IRON STATUS AND DIETARY AND ANTHROPOMETRIC RISK FACTORS IN US TODDLERS

by

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ABSTRACT

Iron deficiency (ID) is a common problem among US children but diagnosis requires four tests. An equation using only the logarithmic ratio of circulating transferrin receptor to ferritin has been proposed to quantify body iron in adults, however, its application in children is limited. Our objective was to determine the body iron of middle-class US toddlers using this equation and to compare calculated body iron to dietary and anthropometric covariates previously associated with ID. Eighty-six healthy, 18-to 36-month-old toddlers were recruited. Anthropometric, dietary, and hematological data were collected. Multiple regression analyses revealed dietary calcium intake and body mass index negatively influenced body iron, and birth weight, dietary iron intake, and energy intake positively influenced body iron. These predictive variables are similar to previously reported iron status risk factors. Our findings support the use of the body iron equation as a simple measure of iron status in healthy young children.

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ABBREVIATIONS

Iron Deficiency	ID
Iron Deficiency Anemia	IDA
Transferrin Saturation	TS
Free Erythrocyte Protoporphyrin	FEP
Mean Cell Volume	MCV
Total Iron Binding Capacity	TIBC
Zinc Protoporphyrin	ZPP
Hemoglobin	Hgb
Transferrin Receptor	TfR
Body Mass Index	BMI

CHAPTER 1: INTRODUCTION

Summary of Relevant Research

Iron deficiency (ID) and iron deficiency anemia (IDA) are two of the most common nutritional deficiencies among children and women worldwide (1). Recent data from of the National Health and Nutrition Examination Survey (NHANES) IV demonstrated that the prevalence of ID in US toddlers ages 1 to 3 years decreased from 9% to 8% in the last decade. Among toddlers with ID, a disproportionate number were overweight and did not attend day care (2). In 2006, the Pediatric Nutrition Surveillance System (PedNSS) as part of the National Centers for Disease Control and Prevention estimated the national ID prevalence to be 14.0% of children under the age of 5 years (3). Approximately 9.4% and 17.5% of children in Kansas and Missouri, respectively, met the criteria for ID (4).

The effects of ID even at an early age may be widespread and long-lasting. Specifically, infants and children with ID and IDA are thought to have impaired neurodevelopment, decreased language ability, and poorer fine-motor skills compared with their iron-replete counterparts (5-6). These adverse effects may or may not be reversible as a result of the timing and duration of deficiency (7-9).

Dietary habits during infancy and the transition to table foods are known to affect iron status. Compared to formula fed infants, infants fed breast and cows' milk at 8 months of age have poorer iron status at 12 months (10), and infants who are breast fed exclusively over 6 months of age may be at increased risk for IDA (11). In children aged

12 to 24 months, cows' milk, calcium, cheese, and butter intake were negatively associated with iron status (12-15) whereas iron, vitamin C, fish, and meat intake were positively associated with iron status (12, 15).

Traditionally ID and IDA are diagnosed using a battery of laboratory measures including ferritin, transferrin saturation (TS), free erythrocyte protoporphyrin (FEP), and hemoglobin (Hgb) (16). Iron deficiency is diagnosed if two of the first three indicators are abnormal. Iron deficiency anemia is diagnosed if the criterion for ID is met and Hgb is low. Critics cite the limited specificity and sensitivity of these measures along with their tendency to be elevated during chronic disease, inflammation or infection as evidence for the need to find better indicators (18-20).

A more recent development in the detection of subclinical ID that is not subject to these limitations includes measurement of circulating transferrin receptor (TfR) (21-22). A soluble truncated form of cellular TfR, circulating TfR is primarily affected by cellular ID and the subsequent up-regulation of cellular TfR synthesis (23). It is also a marker and valuable measure of erythropoiesis (24). Circulating TfR is directly proportional to the total body mass of cellular TfR (25).

In 1990, Skikne, Flowers, and Cook completed the hallmark study supporting the use of circulating TfR in the detection of subclinical ID (26). During repeated phlebotomies of 14 healthy adult subjects, they observed serum ferritin levels dropped significantly during the initial phase of storage iron depletion while serum TfR and other markers of iron status remained stable. When iron stores were depleted, the subnormal levels of serum ferritin did not change but serum TfR concentration increased

dramatically. In fact between storage iron depletion and the development of anemia, the only significant change in blood marker status occurred in serum TfR concentration. Their results also supported the use of serum TfR and serum ferritn in tandem to establish a serum TfR:ferritin ratio. The ratio has since been logarithmically transformed and has been proposed as part of an equation to predict milligrams iron per kilogram body weight (27-28).

Vazquez Lopez et al. stated that circulating TfR has an advantage over traditional measures in pediatrics because it is sensitive, specific, shows little variability, and its concentration can be determined from small quantities of serum (29). To date several studies have examined the usefulness of circulating TfR, ferritin and the circulating TfR:ferritin ratio in children. As a whole, these studies report circulating TfR is higher in children compared to adults and is not influenced by disease state or gender (30-33).

Justification for Further Investigation

There are several gaps in the current literature regarding circulating TfR, ferritin, and the use of the recent total body iron equation in young populations. For example, studies outside the US have determined circulating TfR concentrations in infants less than 12 months old and young children 3 to 7 years old, but the number of studies examining circulating TfR in US toddlers aged 18 to 36 months is limited. Also, although the total body iron equation has been reported as a suitable measure for quantifying the iron status of adults, quantitative phlebotomy studies have not been done to confirm its use in children and few studies have explored its validity as a measure of childhood ID and

IDA. Specifically, no known studies have compared total body iron to dietary and anthropometric covariates previously associated with ID.

Statement of Purpose

The objectives of this study are to: a) determine the current iron status of children aged 18 to 36 months in the Kansas City metropolitan area using plasma ferritin, plasma TfR, and calculated total body iron, b) identify dietary and/or anthropometric risk factors that relate to total body iron, and c) compare significant dietary and anthropometric risk factors with those previously related to ID as a means of assessing the validity of total body iron in toddlers.

Research Questions

1. What is the current iron status of young children aged 18 to 36 months in the Kansas City metropolitan area using plasma ferritin and plasma TfR to calculate total body iron?
2. What significant correlations exist between hemoglobin, plasma ferritin, plasma TfR, total body iron, and dietary and anthropometric measures in this population?
3. Do relationships between total body iron and dietary and anthropometric measures support the assessment of total body iron as a meaningful measure of iron status in toddlers?

Thesis format

The format of the following chapters is different than a traditional thesis. Chapter 2 will consist of a complete literature review, but rather than separate chapters for methods, results, and discussion, all three will be combined with a shorter introduction in Chapter 3.

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CHAPTER 2: REVIEW OF LITERATURE

The objectives of this study are to: 1) determine total body iron in middle-class US toddlers using plasma transferrin receptor (TfR) and plasma ferritin concentrations and 2) compare total body iron to dietary and anthropometric covariates previously associated with ID. The following review of literature includes the history, classification, prevalence, hematological indicators, clinical presentations, and influential factors of Iron Deficiency (ID) and Iron Deficiency Anemia (IDA). Other causes of anemia including anemia of chronic disease as well as B12 and folate deficiency will not be discussed.

History of Iron Deficiency and Iron Deficiency Anemia

Similar to other nutritional deficiency diseases, ID and IDA were not originally attributed to diet insufficiency. The first modern description of hypochromic anemia was reported by Johann Lange in 1554 (1). Lange described a girl as weak and noted, "her face which in the last years was distinguished by rosiness of cheeks and redness of lips, is sadly paled…the heart trembles with every movement of her body…her stomach loathes food, particularly meat". Termed, *morbus virgineus,* the disease Lange wrote of was thought to be caused by retained menstrual blood. The treatment was to "live with men and copulate" and recovery would occur with conception (2).

Sixty years later, in 1615, the term c*hlorosis* replaced *morbus virgineus* to reflect the greenish tint of skin these patients exhibited. Its reference to a gynecologic disease, however, did not diminish. A successful English physician, T. Sydenham, classified

chlorosis as a hysterical disease coming from the word *hysteron* meaning uterus. Similar to Lange's treatment, Sydenham's followers recommended marriage as the best remedy for this mental disease. It was later noted Sydenham recommended "mineral water impregnated with the Iron Mine" not because he knew iron was an important constituent of blood, but because he thought his patients were weak and iron was a symbol of strength (1).

In later years, physicians strayed from the use of iron as a cure for chlorosis. In 1872, a French clinician named A. Trousseau noted, "The palpitations of the heart, spasms of the stomach, intestine, and uterus must mean chlorosis is derived from a nervous system disorder" (3). Also in 1872, a German physician, R. Virchow, attributed the disease to congenital hypoplasia of the heart and blood vessels. Building upon this idea, English physician A. Clark suggested the disease was aggravated by wearing tight corsets that displaced abdominal organs, obstructed the bowel, and allowed buildup of toxic substances in the blood (1).

It was not until 1895 that another physician related chlorosis to dietary iron (4). R. Stockman discovered patients with chlorosis on average consumed less iron than healthy subjects. He concluded excessive menstrual loss together with habitually low dietary iron intake caused the disease. His theory, however, was not well accepted, because at that time the absence of a substance was not thought to cause disease.

At the end of the nineteenth century, scientific interests shifted yet again and the diagnosis of chlorosis nearly disappeared (4). The idea of anemia and poor iron status, however, was not forgotten and in the late 1920s a scientist by the name of Helen

Mackay made the first definite conclusions about ID and IDA. Mackay scientifically tracked hemoglobin (Hgb) levels in infants and noted that concentrations are high at birth but drop after 6 months of age (5). She also discovered low Hgb is more common and severe in non-breast-fed infants and low birth weight babies. Supplementation with iron salts produced dramatic changes and Mackay documented a 50% reduction in respiratory tract infections, diarrhea, and specific fevers among supplemented infants. Her recommendation that iron should be given to non-breast fed infants from the first months of life stands today.

Classification of Iron Deficiency and Iron Deficiency Anemia

The progression from ID to IDA is commonly described using three stages (6). The first stage, termed iron depletion, represents the time when stored body iron decreases but functional iron status is unchanged. If uncorrected, this stage progresses to iron deficient erythorpoiesis and the continued loss of stored iron impairs the synthesis of functional iron compounds. The final stage, iron deficient anemia, is characterized by Hgb concentrations that fall below the lower limit of normal as a result of the now severe deficit of iron. This last stage is commonly classified as a microcytic hypochromic anemia because of the abnormally small red blood cells that are light in color.

Traditionally ID and IDA are diagnosed using a combination of transferrin saturation (TS), free erythrocyte protoporphyrin (FEP), ferritin, and Hgb (7-8). The reference values for young children are listed in Table 1. An individual is iron deficient

if any 2 of the first 3 values are abnormal for age. An individual has IDA if the ID criterion is met plus Hgb is low.

TABLE 1 Reference limits for ID and iron IDA in young children

Indicator	1-2 years	3-5 years
Transferrin Saturation	<10%	<12%
RBC Erythrocyte Protoporphyrin	>1.42 μmol/dL	>1.24 μmol/dL
Ferritin	<10 μg/L	<10 μg/L
Hemoglobin	<11.0 g/dL	<11.1 g/dL

Prevalence of Iron Deficiency and Iron Deficiency Anemia

The World Health Organization estimates all cause-anemia affects approximately 2 billion people worldwide (9). In developing countries high rates are attributed to insufficient dietary intake, infectious disease, other micronutrient deficiencies, or inherited conditions such as thalassaemia.

In the United States, rates are much lower and mostly reflect insufficient dietary iron intake. Results from the third National Health and Nutrition Examination Survey (NHANES III) (1988-1994) found 9% of US toddlers ages 1 to 2 had ID and 3% had IDA (7). The more recent NHANES IV (1999-2002) data revealed the prevalence of ID decreased to 8%; however, IDA could not be measured because of insufficient sample size (10). In 2006, the Pediatric Nutrition Surveillance System (PedNSS) as part of the National Centers for Disease Control and Prevention observed an estimated national ID prevalence of 14.0% in children under the age of 5 years (11) with approximately 9.4%

and 17.5% of children in Kansas and Missouri affected respectively (12). Despite recent decreases in incidence, ID in children remains a national public health issue. The reduction of ID to less than 5% of 1 to 2 year olds and 1% of 3 to 4 year olds is currently a *Healthy People 2010* objective (13).

Hematological Indicators of Iron Deficiency and Iron Deficiency Anemia

Common measures of iron status include: Hgb, ferritin, serum iron, TS, FEP, mean corpuscular (MCV), and total-iron-binding capacity (TIBC). By grouping these indices, an overall picture of iron status is thought to be attained; however, it is well documented that most of these measures are affected by various physiological states and laboratory constraints. These conditions include infection, disease, gender, age, body size, tobacco use, alcohol use, exercise habits, contraceptive pill use, and available laboratory and economic resources (14-15). Also, the sensitivity of each to detecting the range of ID and IDA has been questioned (16). Ferritin, for example, decreases only during storage iron depletion while Hgb decreases much later during functional iron depletion (17). Serum iron and TS are affected by diurnal fluctuations (18), TIBC has low specificity (16), and most indicators aside from ferritin and Hgb require a large sample of blood for analysis.

More recently, circulating transferrin receptor (TfR) has been employed as an iron status marker (19). Uninfluenced by disease state and relatively easy to obtain and analyze, circulating TfR has begun to replace traditional measures. Also because of its ability to provide a more complete picture of iron status between the depletion of ferritin

and Hgb, the combination of Hgb, ferritin, and circulating TfR may be useful. For the purpose of this review, only Hgb, ferritin, and circulating TfR will be discussed in detail.

Hemoglobin is a three-dimensional molecule composed of two globulin chains and four heme groups (20). It is embedded in the red blood cell and is vital for the transport of oxygen and carbon dioxide. Based on this important role, Hgb is the primary functional iron compound in the body. Changes in Hgb concentration occur only in the latest stage of ID denoting the onset of IDA. Blood Hgb is often measured because of its low cost, ease of testing, and usefulness for establishing the presence of IDA. Normal adult levels vary depending on gender; however, this is not the case in children and reference limits are 11.0g/dL and 11.1g/dL for children 1-2 years and 3-5 years respectively (8).

Opposite of Hgb, ferritin is the main iron storage compound in the body (21). The cellular form, synthesized by smooth endoplasmic reticulum, is thought to be concentrated in the liver, spleen, and bone marrow. A small amount may be found and measured in circulating plasma (22-23). Although the cellular form stores more iron, few other differences between the circulating and cellular molecules exist. It is estimated that 1μg/L of plasma ferritin represents approximately 120μg of storage iron per kilogram of body weight in children (21). Ferritin may be quantified in either plasma or serum and levels are commonly measured to assess body iron storage. Ferritin is one of the first hematological markers to change during iron depletion (17) and extremely low concentrations (<12ug/L in adults and <10ug/L in children) are one indicator of ID and IDA. In some populations ferritin is an inaccurate representation of iron status because it

is an acute phase reactant and may be falsely elevated. These conditions include acute and chronic infections, inflammatory diseases, malignancies, liver disorders, smoking, exercise, alcoholism, and contraceptive pill use (14-16).

Circulating TfR has only recently been measured as an indicator of subclinical ID. Discussion of a possible cellular TfR started in the 1960s, but it was not until the 1980s that the structure of the transferrin receptor was described (24). In 1984, Schneider et al. reported cellular TfR a transmembrane glycoprotein that exists as two identical subunits linked by disulfide bonds. (25) When iron-loaded transferrin approaches the cell, transferrin binds to the receptor and receptor-transferrin complex is internalized. Once in the cell, the iron is released from transferrin and the receptor returns back to the cell surface. Approximately 80% of cellular TfR is in the erythroid marrow; however, virtually all cells have cellular TfR (24).

In 1987, a scientist by the name of Y. Kohgo first detected a circulating form of cellular TfR in human serum using an immunoradiometric assay (19). In 1989 an enzyme-linked immunosorbent assay (ELISA) was developed, (26) and in 1990 it was discovered that the circulating TfR is the same as the cellular TfR but truncated and bound to transferrin (27). Either serum or plasma can be used for the measurement of the receptor. The prime determinant of circulating TfR concentration is cellular iron deficiency, and it is well accepted that circulating TfR concentration is inversely related to ferritin in adults (28). Circulating TfR is also affected by erythropoesis in bone marrow (29-30). Circulating TfR has an advantage over other iron indices in that it is not an acute phase reactant and remains stable during inflammation (31). This means that

measurements of circulating TfR in combination with Hgb make it possible to distinguish IDA from anemia of chronic disease.

The hallmark study for supporting the use of circulating TfR as a means of detecting subclinical ID was done by Skikne, Flowers, and Cook in 1990 (17). In this study, serial phlebotomies were performed in 6 male and 8 female volunteers ranging from 24 to 46 years of age. The end point of phlebotomy was considered the point when the individual's serum Hgb levels had fallen 2 g from their baseline mean and stayed that way for at least 3 weeks. A variety of hematological markers, including serum iron, TS, Hgb, TIBC, FEP, MCV, serum ferritin, and serum TfR were evaluated throughout the process. Results indicated serum ferritin concentration fell dramatically until storage depletion whereas all other iron status markers were unchanged. After overt anemia was developed, all markers differed from their baseline value except FEP. However, between the period of iron depletion and anemia, the only significant change in iron status occurred in serum TfR levels. This suggests circulating TfR is the best indicator of iron status between the time of ID and IDA.

Based on the observation that ferritin reflects changes in iron storage while circulating TfR reflects changes in functional iron, Skikne et al. suggested a ratio of circulating TfR:ferritin could provide a log-linear relationship to iron storage (17). The utility of the circulating TfR:ferritin ratio was confirmed when it was shown that the ratio of an iron supplemented group of Jamaican women was significantly different from a group that was not supplemented (28,32).

Two major advantages of using the ratio have been reported. 1) The total number of measures required for diagnosis of ID is far less compared to traditional measures. This creates fewer burdens on participants and requires less laboratory work. 2) Only microliters of blood are needed compared to the milliliters required for the other tests. It should be noted the circulating TfR:ferritin ratio could be susceptible to the fluctuations seen in ferritin during inflammation and some support its use in combination with an inflammatory marker such as C-reactive protein (33).

Since the discovery of circulating TfR, a great number of studies have evaluated its use as an iron status indicator. For the purpose of this literature review, only studies examining circulating TfR in infants and children will be examined. A significant limitation to comparing these studies, however, is the lack of standardization of reference intervals between different assay systems (33-36). Yeung et al. compared three distinct ELISA methods and determined differences among methods were so large comparison of circulating TfR values from different assays was unacceptable (36). More recently, a fully automated circulating TfR immunoturbidimetric assay showed good comparison to two ELISA assays ($r>0.8$) and it was determined results of the three could be compared (37). Because published studies have not necessarily used methods that are comparable, extreme care in interpreting and comparing results is required. For the purpose of this review, the circulating TfR assay method will be documented for each study when possible.

One of the first studies of circulating TfR in children and infants was reported in 1997. Yeung and Zlotkin examined plasma TfR and plasma ferritin concentrations and

the log TfR:ferritin ratio in 485 healthy, middle class urban infants aged 8 to 15 months (38). Plasma TfR was measured using an in-house ELISA assay method. The mean plasma TfR concentration and log TfR:ferritin ratio were 4.4 ± 1.1 mg/L and 6.2 ± 1.1 μg/μg respectively. The authors did not find an association between plasma TfR and Hgb, FEP, or plasma ferritin, which they report could be attributed to the normal Hgb concentrations of the population (12.68 ± 1.48 g/dL). However, other research has shown associations between these iron markers (39-40).

Detailed statistical analysis using circulating TfR and ferritin was defined by Punnonen et al. They examined the use of serum TfR and serum ferritin in the identification of true IDA versus anemia of chronic disease and reported the circulating TfR:ferritin ratio was a good indicator of iron deficiency (AUC^{ROC} 0.98) (41). However, this ratio was not statistically better than circulating TfR alone. Other possibilities of combining circulating TfR and ferritin were evaluated and the logarithmic transformation of the ferritin and the calculation of the circulating TfR:log ferritin ratio provided a better statistical model (AUC^{ROC} 1.00). Based on these results, the ratio of circulating TfR to ferritin is now often termed serum TfR-F index and is calculated using circulating TfR:log ferritin.

In 1998 Kling et al. observed the change in plasma ferritin, plasma TfR and erythropoietic activity of 22 infants aged 0 to 7 months consuming iron-fortified formula (42). Plasma TfR was determined by the Quantikine™ IVD™ Human TfR Immunoassay kit (R&D Systems, Minneapolis, MN). Mean plasma TfR levels varied ($p<0.002$) over the first 7 months of life with the lowest plasma TfR levels at 1 month (0.88 ± 0.03 mg/L)

and the highest at 4 months (1.97 ± 0.84 mg/L). Plasma ferritin levels decreased with postnatal age ($p<0.0001$). Plasma TfR levels were positively associated with plasma EPO (z coefficient 3.22; $p<0.001$) but negatively associated with Hgb (z coefficient; -2.60 $p< 0.009$) and plasma ferritin (z coefficient -4.62; $p<0.0001$). The authors speculate that during the first months of life, increases in plasma TfR levels reflect the increased use of iron in the process of erythropoiesis.

In a group of 36 healthy 1 year-old infants with no history of chronic disease, Virtanen et al. found a mean serum TfR concentration of 7.8 mg/L (95% reference interval: 4.5-11.1 μg/L) using the Ramco Laboratories ELISA kit (Ramco Laboratories, Inc, Houston, TX) (39). The serum ferritin geometric mean was 24μg/L (95% reference interval 9-62 μg/L) and the geometric mean serum TfR:ferritin ratio was 316 μg/μg (95% reference interval 94-1059 μg/μg). There was an inverse correlation between serum TfR and log serum ferritin concentration ($r=-0.50$; $p=0.002$) and between serum TfR concentration and MCVs ($r=-0.-0.40$; $p=0.02$). When combined with other age groups in a multiple linear regression model, the best predictors of serum TfR concentration were log serum ferritin ($p<0.0001$) and serum iron ($p=0.004$). This study is a good model for our current study because the same TfR assay was utilized and the lifestyle patterns of the subjects were similar. Fifty percent of the infants were breast fed at least 6 months, 19% for 9 months and 8% for 11 or more months. All but 3 infants were given iron-fortified formula when not exclusively breastfeeding. None of the infants had a serum ferritin of <12μg/L.

Choi et al. measured circulating TfR concentration in a variety of age groups of Korean children to examine changes in circulating TfR over time (43). In total, 849 healthy nonanemic subjects aged 0 to 62 years were examined. Serum TfR was measured using the IDeA^{TM} sTfR kit (Orion Diagnostica, Espoo, Finland). Mean serum TfR concentrations for neonates less than 5 minutes of age, infants aged 4 to 24 months, and young children aged 3 to 7 years were 4.95 ± 1.24, 4.51 ± 1.12, and 3.02 ± 0.76 mg/L respectively. The serum TfR concentrations were significantly higher in infants compared to young children ($p<0.01$). The conclusion was made that circulating TfR levels decrease with age even during the early years of life.

Circulating TfR and log circulating TfR:ferritin was measured in 716 healthy, low to low-middle class Chilean infants aged 4 to 15 month(40). Fourteen percent of the children had abnormal Hgb (<11.0g/dL) and 46.9% had abnormal serum ferritin (<10μg/L). Circulating TfR was measured using an in-house ELISA method. The median circulating TfR was 8.5 mg/L (95% CI 5.9-13.5) and the geometric means for serum ferritin and log circulating TfR:ferritin were 10 μg/L (range 5-22μg/L) and 951 μg/μg (range 398-2356μg/μg) respectively. Significant correlations were observed between circulating TfR, log circulating TfR:ferritin, log serum ferritin and regular laboratory indicators of iron status including Hgb, MCV, FEP, serum iron, and TIBC. As the severity of iron deficiency progressed, the mean circulating TfR concentration increased ($p<0.00001$, ANOVA), however, there was no change in circulating TfR:ferritin ratio. Like Punnonen et al., Olivares et al. observed circulating TfR and circulating TfR:ferritin had better sensitivity and specificity for detecting ID compared to

serum ferritin (AUC^{ROC} 0.75 ± 0.02, 0.72 ± 0.02, and 0.67 ± 0.03 for circulating TfR, circulating TfR:ferritin ratio and serum ferritin respectively). Unlike Punnonen et al., Olivares et al. did not see an increased benefit of using the circulating TfR:ferritin ratio compared to circulating TfR alone.

In 301 healthy children aged 6 months to 18 years, Suominen et al. found no significant gender effect in serum TfR concentrations in infants and children up to 10 years of age using an automated immunoturbidimetric assay (IDeA® sTfR-IT; Orion Diagnostica) (p=0.37) (44). There was an age-related decrease in serum TfR concentration when children between 0.5 to 4 years were compared to those 4 to10 years of age (p<0.001). Age-based reference limits for the assay were derived and set at 1.5-3.3 mg/L for children aged 6 months to 4 years and 1.3–3.0 mg/L for children aged 4 to 10 years.

In a study to evaluate the diagnostic usefulness of circulating TfR in recognizing iron deficiency without anemia, Lopez et al. examined a total of 251 healthy children (45). Two hundred six subjects had normal iron status and 45 had ID (ferritin <10µg/L in children 1 to 5 years and <12µg/L in those 6-10 years old). Serum TfR was measured by the Quantikine™ IVD™-Human sTfR ELISA kit (R&D Systems, Minneapolis, MN). The mean serum TfR of the iron sufficient group (1.93mg/L ± 0.41) was significantly lower than the iron depleted group (2.28mg/L ± 0.5) (p=0.001). The serum TfR:ferritin ratios for the iron sufficient and iron depleted groups were 80 µg/µg ± 36.8 and 567 µg/µg ± 358 (p=0.001) respectively. The serum TfR-F Index for the iron sufficient and iron depleted groups were 1.38 µg/µg ± 0.34 and 4.1 µg/µg ± 2.1 (p=0.001) respectively.

Serum TfR was the only biomarker that showed a significant difference between the two groups of children. The cut off levels for diagnostic purposes were defined as 2.5mg/L for serum TfR, 175 μg/μg for serum TfR:ferritin ratio, and 2 μg/μg for serum TfR:log ferritin.

From this review it is evident several approaches to reporting circulating TfR and ferritin have been utilized as well as different assay methods. Regardless of these differences, there are three consistent findings. 1) Circulating TfR decreases with age. Theories for this occurrence include both increased cellular iron needs during growth and development and increased erythropoesis. 2) Circulating TfR levels are higher in iron depleted individuals compared to iron sufficient individuals. This agrees with the original findings that circulating TfR only increases after iron stores are depleted. 3) There are no gender differences in circulating TfR concentration.

Controversy still exists regarding the correlations between traditional iron status markers, circulating TfR, ferritin, and the various proposed ratios. Some researchers report strong correlations while others report none at all. Also, the difference in reporting circulating TfR:ferritin ratios makes comparing studies difficult.

Acknowledging the need to develop a practical iron surveillance system, Cook et al. proposed a formula to express body iron in milligrams per kilogram body weight (33). Derived from calculated iron stores from serial phlebotomy, the equation is based on a logarithmic circulating TfR:ferritin ratio. Positive calculated values indicate storage iron while negative values indicate tissue iron deficiency. Although the use of this equation is still limited by circulating TfR standardization, the potential for expressing body iron on

the basis of body weight is promising. Perhaps the largest advantage is that expressing body iron based on weight could potentially eliminate the need for absolute reference values that are often inappropriate for target populations.

In 2005, the body iron equation was used to evaluate the iron status of 800 Bolivian children less than the age of 5 (46). The mean body iron stores were 1.71 ± 4.53 mg/kg and 11.8% of the children had tissue iron deficits, consistent with anemia. Specifically, children less than 2 years old had a high prevalence of tissue iron deficit, but a linear increase in body iron with age was observed and the average total body iron of children 4 years old was 3.85 mg/kg.

Clinical Presentations and Consequences f Iron Deficiency and Iron Deficiency Anemia

Commonly known physical symptoms of IDA include fatigue, dizziness, headaches, pallor, brittle nails, angular stomatitis, glossitis, and pica. Decreased aerobic capacity, work capacity, immune function, and school function as well as increased rates of infection and impaired temperature regulation are also well documented. Specifically related to young children, iron status is thought to affect cognition and behavior. The exact relation to iron and brain development is unknown; however, it is thought that inadequate iron during early life may lead to alterations in neurotransmitter metabolism and myelination in the brain (47-48). Controversy still exists regarding the timing and level of iron deficiency required to identify cognitive delay as well as the duration of delay after repletion.

In two well known developmental studies in which iron treatment therapy was implemented, a decrease in Hgb below the conventional cutoff limit for anemia was necessary to significantly affect mental and psychomotor development scores (49). In one of the studies, children with chronic IDA (≥3 months) had significantly lower mental and motor test scores on the Bayley's Scales of Infant Development than those with IDA of shorter duration (50). In the second study, IDA infants maintained closer contact with caregivers during play and showed less pleasure and delight (51). They were also more wary or hesitant, experienced more fatigue, and were less vocal.

In support of these trials, other iron intervention studies show that despite improved iron status, formerly anemic infants may be unable to improve their psychomotor performances to the level of nonanemic controls (49). In the longest trial to date, children 11 to 14 years of age who had severe (Hgb<10.5g/dL) and chronic (≥3 months) IDA during infancy scored lower on measures of overall mental and motor functioning (52). The previously IDA children were also more likely to repeat a grade, be referred for special services, and demonstrate anxiety, depression, social problems, and attention problems. It should be noted other variables such as unidentified nutrient deficiencies, parental intellectual and emotional limitations, or environmental disadvantages both early and late may confound results at such a late age.

Influential Factors Affecting Iron Deficiency and Iron Deficiency Anemia

Late infancy and early childhood are often cited as high risk periods for the development of ID and IDA (53-56). Influential factors to be discussed in this review

include: gestational risks, birth weight and rate of growth during early life, gender, dietary iron intake, height-for-weight status, and preschool/daycare attendance.

Most infants are born with sufficient iron stores to support infant growth and development until approximately 6 months of age (56-58). However, several high-risk gestational conditions including gestational diabetes (GDM), intrauterine growth restriction (IUGR), and maternal binge drinking have been documented to decrease infant iron status at birth and increase ID and IDA risk. Sixty-five percent of infants born to mothers with GDM in a 1990 study had abnormal serum iron profiles characterized by decreased ferritin and TIBC and increased FEP (59). Abnormally higher Hgb concentrations were observed in IUGR infants (16.4 g/dL) compared to appropriate weight for gestational age (AGA) infants (15.7 g/dL) ($p<0.001$) in 1999 (60). Both studies cited intrauterine hypoxia and compensatory Hgb synthesis as possible mechanisms. In 2007 Carter et al. reported maternal binge drinking was associated with a significantly higher incidence of IDA at 12 months of age and proposed prenatal alcohol exposure may disrupt transport of iron across the placenta (61).

Even if the child is born without a high-risk gestational condition, other factors such as birth weight and growth rate early in life may affect iron status because of the important role iron plays in growth. Wharf et al. observed birth weight had positive effect on ferritin ($p<0.001$) at 4 months of age (62) and both Persson et al. and Sherriff et al. noted a positive association between birth weight and ferritin levels at 12 months of age (63-64). Regarding growth during the first year, Michaelsen et al. reported a negative association between knee-to-heel growth velocity and serum ferritin ($p=0.006$)

at 12 months of age (65). Sherriff et al. reported rapid growth between 8 and 12 months of age was associated with depleted ferritin levels ($r=-0.154$; $p=0.004$), but not between 12 and 18 months ($r=-0.07$; $p=0.168$). Using serum TfR as an indicator of iron status, Thorsdottir et al. saw a positive association between serum TfR and linear growth ($r^2=0.14$; $p=0.04$) (54).

Gender differences among infants in relation to iron markers have been described by Domellof et al (58). After adjustment for birth weight and postnatal weight gain, at 4, 6, and 9 months, boys had lower Hgb ($p=0.013$), MCV ($p<0.001$), and ferritin ($p<0.001$) and higher zinc protoporphyrin (ZPP) ($p=0.007$) and plasma TfR ($p<0.001$) compared to girls (58). The gender difference in MCV and ZPP remained evident at 9 months of age even after controlling for iron supplementation and growth variables. The authors speculated that these results were due to possible hormone-mediated or genetic differences in iron metabolism between young males and females. Other studies, however, show no difference in circulating TfR concentration between sexes in children (38-39,44).

Dietary iron intake during infancy is important after 4 to 6 months of age when fetal stores are depleted (66). A recent US study observed that young children exclusively breastfed for 6 months were at increased risk for ID (67) and a UK study found infants consuming iron-fortified formula were better protected from ID and IDA at 8 and 12 months of age compared to those consuming cows' or breast milk (68). As young children transition to the family diet pattern, the need to monitor their dietary intake for insufficiency becomes even more important (69). Foods and nutrients found to

be inversely associated with iron status during these transition years include cheese, butter, calcium, tea, and cows' milk (54,65, 70-72). Vitamin C, iron, fish, and meat intakes have been positively associated with iron status (54,71). In a study to evaluate the nutritional quality of childrens' diets in the second year of life, Picciano et al. found only 40% of 12-month-olds and 20% of 18-month-olds met daily iron intake requirements (73). However, the 1989 Recommended Dietary Allowance (RDA) for iron was used to assess the mean iron intake in this population. The current practice to assess the adequacy of population nutrient intake is to use the Estimated Average Requirement.

In a very recent national analysis of ID risk in the United States, both daycare attendance and weight-for-height status were independently associated with ID in young children after controlling for race/ethnicity and interview language (10). Specifically, 20.3% of overweight children between the ages of 1 and 3 years compared to 7.1% of normal weight children were iron deficient. Also, 5.2% of children attending daycare versus 10% of those not attending daycare were iron deficient. The authors stated prolonged bottle-feeding and the subsequent link to increased juice intake during childhood were cited as possible causes for the association between overweight and ID. Higher quality diets at daycare that protect against poorer family nutritional practices were cited as possible mechanisms behind the association between daycare attendance and ID association.

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CHAPTER 3: IRON STATUS AND DIETARY AND ANTHROPOMETRIC RISK FACTORS IN US TODDLERS

This work will be submitted for publication in the American Journal of Clinical Nutrition

Abstract

Iron deficiency (ID) is a common problem among US children but diagnosis requires four tests. An equation using only the logarithmic ratio of circulating transferrin receptor to ferritin has been proposed to quantify body iron in adults, however, its application in children is limited. Our objective was to determine the body iron of middle-class US toddlers using this equation and to compare calculated body iron to dietary and anthropometric covariates previously associated with ID. Eighty-six healthy, 18-to 36-month-old toddlers were recruited. Anthropometric, dietary, and hematological data were collected. Multiple regression analyses revealed dietary calcium intake and body mass index negatively influenced body iron, and birth weight, dietary iron intake, and energy intake positively influenced body iron. These predictive variables are similar to previously reported iron status risk factors. Our findings support the use of the body iron equation as a simple measure of iron status in healthy young children.

Introduction

Iron deficiency (ID) and iron deficiency anemia (IDA) are common problems among children in the US and iron is the most common nutrient deficiency worldwide (1). Recent data from the National Health and Nutrition Examination Survey (NHANES) IV demonstrated that the prevalence of ID in US toddlers ages 1 to 3 years decreased from 9% to 8% in the last decade (2). However, this prevalence is still far from the national goal to reduce ID to less than 5% of 1 to 2 year olds and 1% of 3 to 4 year olds by the year 2010 (3).

The consequences of ID and IDA can be severe. Several studies indicate infants with chronic IDA have impaired neurodevelopment, decreased language ability, and poor fine-motor skills (4-6). Furthermore, even after treatment, previously IDA children may be more likely to repeat a grade, be referred for special services, or demonstrate anxiety, depression or attention problems (7). The exact relation to iron and brain development is still unknown; however, it is thought that inadequate iron during early life may lead to alterations in neurotransmitter metabolism and myelination in the brain (5-6).

Dietary habits and rapid growth during infancy and early childhood are known to affect iron status. Compared to formula fed infants, infants fed breast and cows' milk at 8 months of age have poorer iron status at 12 months (8). It is also thought that exclusively breast fed infants over 6 months of age may be at increased risk for IDA (9). In children aged 12 to 24 months, cows' milk, cheese, and butter intakes negatively affected iron status (10-13) possibly because of the role dietary calcium plays in inhibiting iron absorption. However, iron, vitamin C, fish, and meat intakes positively

influenced iron status (11,13). Rapid growth during the first year of life is also thought to decrease iron status and negative associations have been found between growth velocity and serum ferritin at 12 months of age (14)

Traditionally ID and IDA are diagnosed using a battery of measures including ferritin, transferrin saturation, free erythrocyte protoporphyrin and hemoglobin (Hgb). Critics cite the limited specificity and sensitivity of these measures along with their tendency to be elevated during inflammation as evidence for the need to find better indicators (15-18). A newer marker, circulating transferrin receptor (TfR), has been accepted for early ID detection (19-20). Recently, an equation using circulating TfR and ferritin to quantify total body iron as a measure of milligrams iron per kilogram body weight has been proposed in adults, however, reports of its use in children are limited (16,21-22).

The purpose of this study was to determine the iron status of healthy US toddlers using Hgb, plasma TfR, plasma ferritin and the calculated total body iron equation, and to identify associations between iron status and dietary and anthropometric variables in this population.

Subjects and Methods

Study population

Caregivers of subjects were recruited using a targeted phone directory or through word of mouth among families who had previously participated in infant nutrition research at the University of Kansas Medical Center. Eighty-six subjects were recruited

between the months of July 2006 and March 2007. At the first visit, subject gender, race, weight, height (Appendix A), 24-hour dietary recall (Appendix B) and venous blood sample were obtained before they were randomized to a milk-based drink that contained different levels of nutrients. To be eligible for the parent study, subjects had to be 18 to 36 months old and of normal weight, be daily consumers of milk or a milk-based beverage, and live with their parent(s). Subjects were ineligible if they were receiving breast milk at time of randomization, were from a multiple birth, had an intolerance to cows' milk, were diagnosed with an underlying disease, or had an active infection 0 to 7 days prior to randomization. Written informed consent was obtained from the caregiver before participation in the study (Appendix C). The University of Kansas Medical Center Human Subjects Committee approved the study protocol (HSC#10466).

Study design

A total of three visits were required for the parent study. At each visit, dietary recalls were acquired by a registered dietitian who had been trained to use the 3-pass method. Results from the three separate recalls were averaged to improve reliability. For the purpose of this study, only blood drawn at enrollment was used to measure Hgb, ferritin, and plasma TfR unless insufficient amounts were obtained, in which case blood from the third visit was used. Measured height and weight also from enrollment were used to calculate Body Mass Index (BMI). A supplemental questionnaire was approved by the Human Subjects Committee and mailed to study families in November 2007 (Appendix D). Information regarding the mother's use of supplements before, during, and after pregnancy as well as the subject's birth weight, duration of breast milk and/or

formula feeding, age at first food, and use of supplements was obtained using this questionnaire.

Laboratory methods

Whole blood was collected in 6 mL sodium-heparin tubes (BD Vacutainer, Franklin Lakes, NJ) and centrifuged at 3000 g for 10 minutes at 4ºC. Plasma was stored in 1 mL microcentrifudge tubes at -80ºC. Both whole blood and plasma samples were sent to LabOne laboratories (now Quest Diagnostics, Madison, NJ) daily and analysis of Hgb and ferritin was completed at a local Kansas City site using automated analysis.

Batches of approximately 32 samples were analyzed in duplicate for plasma TfR with an enzyme immunoassay kit (Ramco Laboratories, Inc, Houston, TX) (Appendix E). If intra-assay variability was higher than 8.5%, samples were re-run. The final mean intra-assay variability was 4.3 ± 3.1%. Total body iron was calculated using Cook's logarithmic equation of circulating TfR:ferritin (16).

Statistical analysis

Of the original 86 subjects enrolled, dietary intake data were available for 85 (99%) subjects. Hemoglobin, plasma ferritin, and plasma TfR concentrations were available for 79 (92%), 70 (81%), and 83 (97%) subjects respectively. Seventy-one caretakers (83%) returned the supplemental questionnaire. Sixty-nine subjects (80%) had all three hematological indicators and fifty-seven subjects (66%) had all data available.

Means, standard deviations, and ranges were calculated for descriptive, dietary, and hematological data. The geometric means and exponentiated standard deviations were computed for plasma ferritin and TfR:ferritin. Logarithmic transformation of

ferritin was used to create a normal distribution, and Pearson's Product Moment correlations were used for simple correlation analysis. Student *t* tests were used to compare means and one-way ANOVA was used to compare differences between total body iron status groups. When the results of the ANOVA were significant, identification of significant differences between groups was based on Newman-Keuls post hoc test.

Multiple linear regression analysis was used to evaluate associations between total body iron and anthropometric and dietary intake markers. Previously identified covariates considered for the models included calcium, iron and energy intakes, gender, race, birth weight, age, BMI, childhood vitamin and maternal prenatal supplement use, duration of breast milk and/or formula feeding, and age at first food. Because dietary iron and energy intake were highly correlated ($r=.480$; $p<0.01$), separate regression models to examine total body iron were constructed that included either iron intake or energy intake. All statistical procedures were done using Microsoft Office Excel 2007® for Windows (Microsoft Corp., Seattle, WA) and SAS® version 9.2 (SAS Institute Inc., Cary, NC). Significance was determined by alpha less than 0.05 and all p values were based on two-sided tests.

Results

All available data (n=69 to 86) were used to generate summary statistics and simple correlations. Only the 57 subjects with a complete set of data were used in the final multivariate regression models. Summary statistics of the population are shown in Table 2. Birth weight, duration of breast and/or formula feeding, age at first food, and

duration of child's vitamin and/or mother's prenatal supplementation use were self reported. No subject was >85th percentile age for BMI. Ninety-one percent of the mothers who completed the mailed questionnaire reported they consumed a prenatal vitamin during their pregnancy.

TABLE 2 Summary statistics of study population[1]

Characteristic	Mean	SD	Min – Max
Sex			
male , *n=45*			
female, *n=41*			
Age (mo)	25.9	5.9	18.0 - 35.2
Birth weight (g)	3323	407	2041 – 4354
Breast fed (mo)	7.0	6.4	0 – 26
Formula fed (mo)	7.1	4.6	0 – 13
Age at 1st food (mo)	5.6	1.8	3 – 11
Weight (kg)	13.0	1.8	8.7 - 17.7
BMI	16.0	1.0	14.0 - 18.6
Energy intake (kcal/d)	1184	278	666 – 1771
Iron intake (mg/d)	8.9	3.6	3.2 - 20.8
Calcium intake (mg/d)	781	300	291 – 1772
Mother's PNV intake (mo)	18.6	16.9	0 – 64

[1] Eighty-six values were available for age, weight, and BMI, 85 for energy, calcium, and dietary intake, 71 for breast and formula fed and for age at first food, and 70 for mother's PNV intake

Summary statistics of iron status markers are shown in Table 3. Iron deficiency and IDA could not be diagnosed according to conventional standards because transferrin saturation and free erythrocyte protoporphyrin markers were not available. However, the prevalence of low iron storage based on plasma ferritin alone (ferritin <10μg/L) was 7% (5 of 70), and the prevalence of abnormal Hgb levels associated with IDA (Hgb

<11.0g/dL) was 4% (3 of 79). Approximately 7% (5 of 69) of subjects had negative body iron using the total body iron equation. No age or gender differences were seen in any of the iron status markers, thus, male and females of all ages were grouped together for analysis. Hemoglobin and log plasma ferritin were not correlated (r=0.085), though, both Hgb and log plasma ferritin were negatively correlated with plasma TfR (r= -0.306; p=0.01 and r= -0.363; p=0.01 respectively).

TABLE 3 Summary statistics of iron status markers[1]

Characteristic	Mean	SD	Min – Max
Hgb (g/dL)[2]	12.25	0.8	10.6 - 14.4
Ferritin (μg/L) [3]	22.22	14.3	3 – 66
TfR(mg/L)[2]	6.22	2.0	2.01 - 14.93
TfR/ferritin (μg/μg) [3]	260.21	714.0	65.27 - 4975.37
TfR/log ferritin (μg/μg)[2]	5.07	4.2	2.04 - 31.28
Total Body Iron (mg/kg)[2]	3.38	2.8	-7.24 - 8.35

[1] Seventy-nine values were available for Hgb, 70 for ferritin, 83 for TfR, and 69 for TfR/ferritin, TfR/log ferritin and total body iron
[2] mean, SD
[3] geometric mean; exponentiated SD

Dietary intake

No subjects consumed less than the Estimated Average Requirement for iron (3 mg/d). However, plasma ferritin and total body iron were lower in toddlers who consumed less than the Recommended Dietary Allowance (RDA) for iron (7 mg/d) compared to those who consumed the RDA (ferritin; p=0.002, total body iron; p=0.008) (Figures 1 and 2).

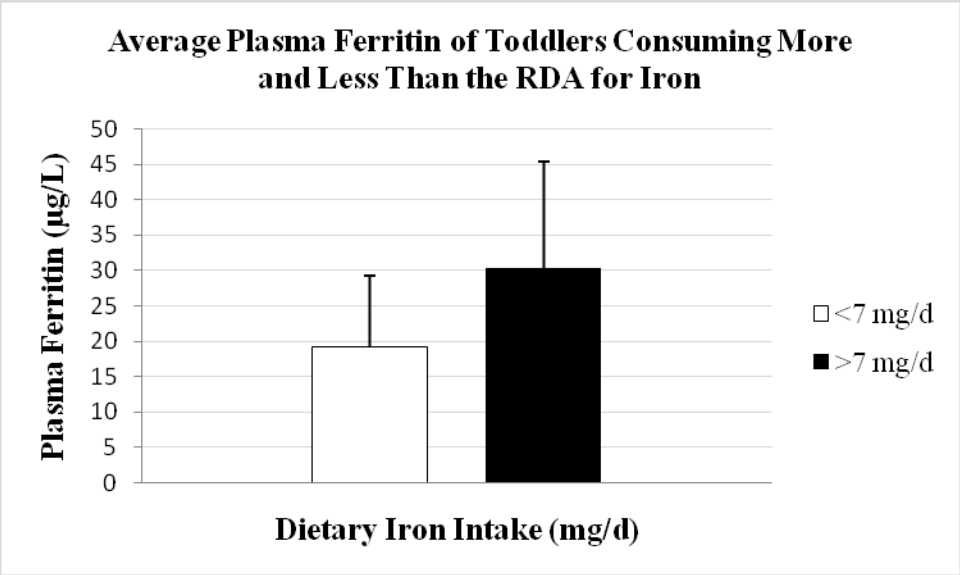

FIGURE 1 Mean (±SD) plasma ferritin concentration of toddlers consuming more or less than the RDA for iron (p=0.002)

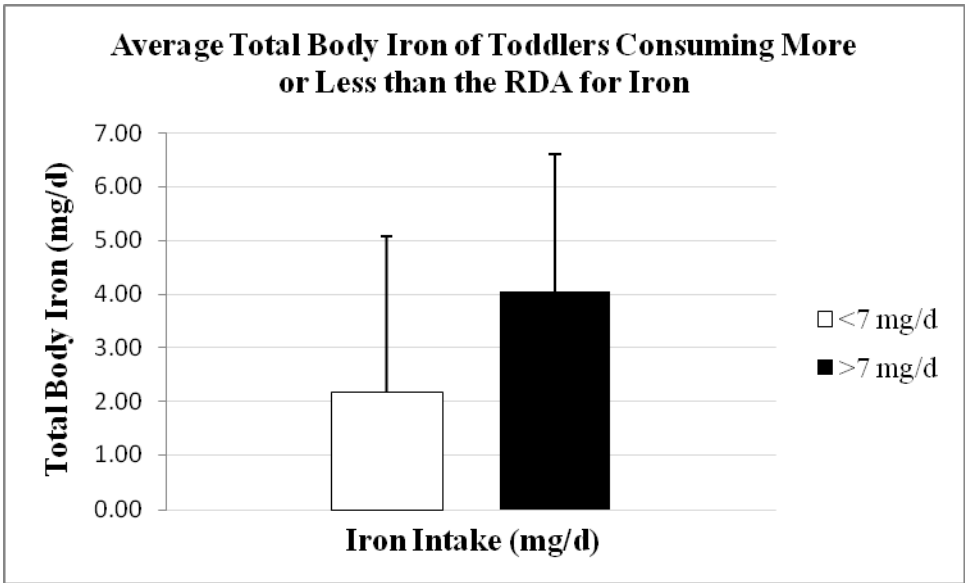

FIGURE 2 Mean (±SD) total body iron concentration of toddlers consuming more or less than the RDA for iron (p=0.008)

When total body iron was divided into quartiles, dietary iron intake was significantly different between the 1st and 2nd and between the 3rd and 4th quartiles (Figure 3).

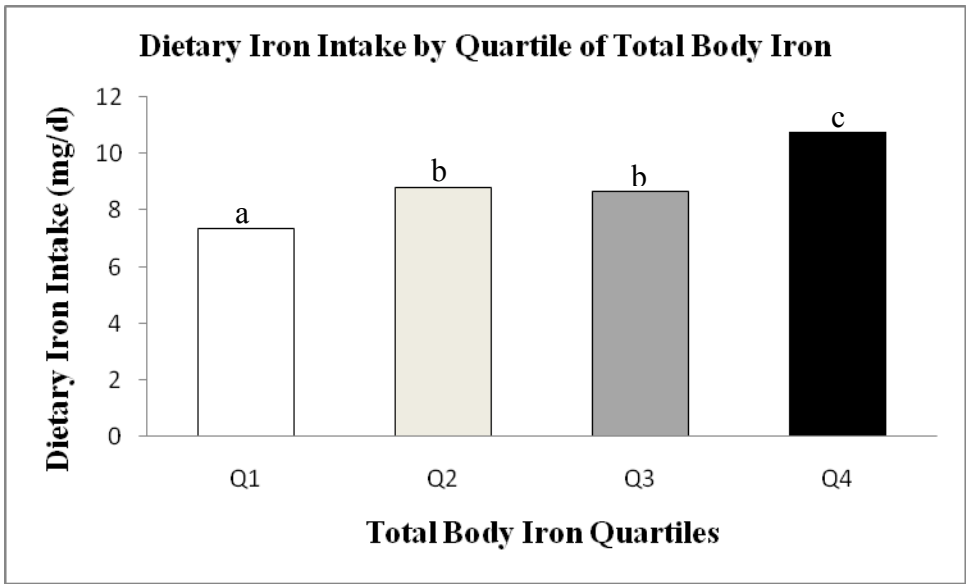

FIGURE 3 Total body iron quartiles based on dietary iron intake. Columns bearing different letters within a group are significantly different (p=0.0377) based on one-way ANOVA model. Mean (±SE) dietary iron intake for the quartiles were: Q1: 7.32 ± 0.81, Q2: 8.80 ± 0.65, Q3: 8.66 ± 0.86, Q4: 10.75 ± 0.96.

Results from the first multiple regression model are shown in Table 4. Iron intake and six other independent variables including calcium intake, race, birth weight, BMI, childhood vitamin use, and age at first food were used. None of the independent variables were correlated with each other. Using this model, 17.7% of the variance in total body iron was explained, and dietary iron intake approached significance as a predictor of total body iron status (B=0.2002; p=0.0556).

TABLE 4 First multiple regression analysis for total body iron (mg/kg)[1]

Independent variable[2]	Parameter Estimate	Standard Error	*P* value
Intercept	3.867	3.690	—
Calcium intake (mg/d)	-0.005	0.001	0.0006
Iron intake (mg/d)	0.2002	0.102	0.0556
Birth weight (g)	0.0019	0.000	0.0377

[1] Adj. $R^2 = 0.1771$
[2] When added to this model, none of the other variables (race, BMI, childhood vitamin usage, and age at first food) were not significant and are not shown

Eighty-nine percent of subjects consumed more than the Adequate Intake (AI) for calcium (500 mg/d) and the mean intake was 781 mg/d indicating the overall group was at low risk for inadequate calcium intake. Dietary calcium was negatively associated with formula feeding duration (r= -0.295; p<0.01), log plasma ferritin (r= -0.224; p=0.05) and total body iron (r= -0.244; p=0.05).

In the second multiple regression model, dietary energy intake was combined with calcium intake, race, birth weight, BMI, childhood vitamin and maternal prenatal supplement use, duration of formula feeding, and age at first food to predict total body iron status (Table 5). Using this model, the explained variance in total body iron was 22.3%. Dietary calcium was inversely correlated with total body iron (*B*= -0.006; p=0.0006) in this model (Table 5) as well as in the first model (*B*= -0.005; p=0.0006) (Table 4).

TABLE 5 Multiple regression analysis model 2 for total body iron (mg/kg)[1]

Independent variable[2]	Parameter Estimate	Standard Error	*P* value
Intercept	1.581	3.902	—
Calcium intake (mg/d)	-0.006	0.002	0.0006
Body Mass Index	-0.262	0.129	0.0478
Energy intake (kcal/d)	0.004	0.002	0.0157

[1] Adj. R^2 = 0.223
[2] When added to this model, none of the other variables (race, birth weight, childhood vitamin usage, prenatal vitamin usage, duration of formula feeding, and age at first food) were significant and are not shown.

Using Pearson's Product Moment correlations, the reported duration of formula feeding was positively correlated with log plasma ferritin (r=0.296; p=0.02) and total body iron (r=0.288; p=0.02) but not to plasma TfR status. However, these relationships were not significant in the multivariate models. No correlations were found between breast feeding and any iron status markers.

Anthropometric measures

Child birth weight was positively associated with Hgb (r=0.263; p=0.02) and negatively associated with plasma TfR (r= -0.266; p=0.02). Height was positively correlated with energy intake (r=0.230; p=0.05) and vitamin usage (r=0.354; p<0.01), but it was not significantly correlated with any iron status markers. Weight at time of enrollment was positively correlated with Hgb (r=0.280; p>0.01) but no other iron marker or dietary factor. In the first multiple regression model (Table 4), child birth weight was significantly correlated with total body iron (*B*=0.0019; r=0.0377). In the second multiple regression model (Table 5), BMI was a significant predictor of total body iron (*B*=-0.262; p=0.0478).

Discussion

ID and IDA remain common public health problems in United States and the current method of identifying ID is subject to a variety of inconsistencies. The evaluation of circulating TfR, ferritin, and ratios of circulating TfR:ferritin and circulating TfR:log ferritin have been proposed as sensitive and efficient measures of early ID even in children (20, 23-24). More recently, Cook et al. developed a quantitative equation of total body iron using log circulating TfR:ferritin to estimate iron status on the basis of body weight rather than absolute values (16). Using Cook's equation, positive values indicate storage iron while negative values indicate tissue iron deficiency. The mean body iron stores in healthy US male and female adults were reported to be 9.89 ± 2.82 mg/kg and 4.87 ± 4.14 mg/kg respectively (16). In a group of mildly anemic Bolivian children aged 1 to 4 years, total body iron was 1.72 mg/kg (22).

The negative correlations observed between Hgb and plasma TfR, and between plasma ferritin and plasma TfR are also consistent with previous findings (25). These associations may be largely explained by examining the effect these indicators have on one another at the cellular level. In an individual with adequate iron status, both Hgb and ferritin concentrations are within normal ranges. In the cell, adequate ferritin stores saturate iron binding proteins, preventing cellular TfR synthesis and subsequently lowering circulating TfR (26). However, during iron deficiency, ferritin stores are depleted and the iron binding proteins are free to promote cellular TfR synthesis. The increase in cellular TfR increases circulating TfR.

Our results for circulating TfR and ferritin concentrations in toddlers are similar to previous studies that used similar methods. Virtanen et al. reported serum TfR and serum ferritin concentrations of 7.8 mg/L and 24 μg/L in 12-month-old infants (24) while we report concentrations of 6.22 mg/L and 22.22 μg/L for plasma TfR and plasma ferritin respectively. Both Virtanen and our results support the observation that circulating TfR concentrations are higher in children compared to adults (27). The lower circulating TfR concentration among our toddlers compared to Virtanens' infants may also indicate even slightly older children have better iron status. Differences in the amount of iron required for growth and erythropoesis during infancy have been cited possible causes (28) though other lifestyle habits including dietary intake and living environment may certainly have an effect. Our results of no gender specific differences in circulating transferrin receptor are also similar to previous findings (24,27,29-30).

As young children transition from iron-fortified baby foods and infant formula to the family dietary pattern, dietary iron intake decreases (31), and it is thought that this may lead to a decrease in iron status. Our results support this hypothesis. Specifically, children consuming less than 7 mg iron per day had poorer iron status than those consuming the current RDA. Also, the significant relationship between dietary iron intake and total body iron status in the ANOVA and in one multiple regression model support the role of iron intake iron status.

Other studies have documented negative associations between cows' milk and calcium consumption and iron status markers (11-13). Our results support this observation and indicate that more than any other factor identified in the study, dietary

calcium best predicted iron status. Specifically, total body iron decreased 0.6 mg/kg for every 100 mg increase in dietary calcium intake. This relationship is significant because it provides evidence that an excessive intake of foods containing high quantities of calcium may not be beneficial for iron status.

The increase in explained variance using the energy intake multiple regression model versus the dietary iron intake model is an interesting concept that deserves further exploration. One explanation may be that children consuming more calories are somehow consuming iron from different sources compared to children with lower energy intakes. Heme iron, for example, is known to be better absorbed and utilized but is found in meat products that have a greater relative energy density compared to non-heme iron sources such as vegetables. A second explanation may be that the nutrient analysis system used to compute dietary data may be more sensitive to energy intake compared to iron intake. If, for instance, a child consumed iron-fortified foods such as orange juice, cereal, or specialty products, the computer system may more accurately estimate calories compared to iron. Future studies that examine the association between the source of dietary iron and energy intake, and child iron status may help answer this question.

Unlike previous studies, our multivariate analyses did not indicate that formula fed children have better iron status compared to those fed breast or cows' milk. Nonetheless, significant simple correlations were found between formula feeding duration and total body iron. There may be two possible reasons for this discrepancy. 1) By eliminating subjects who did not have complete data sets, the analytical power to detect differences may have diminished. It is possible with a larger sample size regression

models would support significant correlations. 2) The age range of our subjects (18 to 36 months) is beyond the usual weaning period. It is possible the dietary habits in early childhood outweighed iron intake during infancy so that any effect of formula or breast feeding was lost.

Higher birth weight has been shown to have an iron-protective effect in early life presumably because less catch-up growth is needed (10-11,14). Our results that children with higher reported birth weights had higher Hgb, lower plasma TfR, and better total body iron status are consistent with this observation. This finding is noteworthy because it suggests child birth weight, which is influenced by a variety of gestational situations, continues to influence iron status at 18 to 36 months of age. Future studies to examine possible reasons for low birth weight, such as gestational age at birth and maternal substance use, in relation to long term childhood iron status may help better predict which children are at highest risk.

The significant negative relationship between child BMI and body iron is consistent with previous findings that overweight children may be at higher risk for ID and IDA (2). It may be possible that even though overweight children consume an excess of calories, their overall quality of diet and iron intake is worse compared to normal weight children. This increased risk of poor iron status adds to the growing list of risk factors associated with childhood weight for length.

The iron status of our population using the total body iron equation was higher than previously reported for young, anemic Bolivian children (22) but lower than US adults (16). This supports the assumption that total body iron is lower among individuals

with functional iron deficits and that children use relatively higher amounts of iron for growth and development and thus have less iron storage. Our finding that total body iron was highly correlated with previously identified iron status risk factors including calcium intake, iron intake, BMI, and birth weight strengthens the argument for its use as an iron status indicator. It shows, even without traditional measures of iron status, similar trends using only the total body iron equation can be seen. However, inconsistencies among commercial circulating TfR kits and the subsequent disparities of reported circulating TfR concentrations limit the use of this equation.

In conclusion, the iron status of our population using Hgb, ferritin, and circulating TfR indices was better than national averages. Dietary and anthropometric risk factors previously associated with iron deficiency including dietary calcium and iron intakes as well as BMI and birth weight were significant predictors of total body iron. Our findings support the use of the total body iron equation as a simple measure of iron status in healthy young children.

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Appendix A

Subject Data Collection Forms

Mead Johnson Nutritionals
Project 3375-1

Source Document for eCRF: Enroll
Visit: V0

Patient number: 112600 ___ ___ ___

Visit Date: [redacted] (Leave this field blank)
MO DA YEAR

Initials: ___ ___ ___

Birthdate: __ __/__ __/__ __ __ __
MO DA YEAR

Gender: 1☐ Male 2☐ Female

Race: 1☐ White 2☐ Black or African American
3☐ Asian 4☐ Native American/Alaskan Native
5☐ Native Hawaiian or Pacific Islander
6☐ More than one 7☐ Unknown or not reported

Ethnicity: 1☐ Hispanic or Latino 2☐ Not Hispanic or Latino

Randomization Code: ___ ___ ___ (Last 3 digits of the 6-digit participant number found on the randomization schedule inside the randomization envelope)

Product Code: ___ ___ ___ (Last 3 digits of the 7-digit regimen number found on the randomization schedule inside the randomization envelope)

Study Personnel Signature: _____ Date:_____

Mead Johnson Nutritionals
Project 3375-1

Source Document for eCRF: A Inclusion Criteria
Visit: V1

Patient number: 112600 ___ ___ ___ Initials: ___ ___ ___ Birthdate: __ __/__ __/__ __ __ __
MO DA YEAR

Visit Date: [redacted] (Leave this field blank)
MO DA YEAR

Label	Yes/No	Criteria for Participant Inclusion
IN_A	1☐ Yes 0☐ No	A. Participant is 18 to 36 months of age at randomization (36 months is up to and including their 3rd birthday)?
IN_B	1☐ Yes 0☐ No	B. Current weight for length is within the 10th to 90th (inclusive) percentiles as plotted on the CDC Weight-for-Length percentiles growth charts (birth to 36 months – boys and girls)?
IN_C	1☐ Yes 0☐ No	C. Parent/caregiver reports that participant consumes milk or a milk-based beverage on a daily basis?
IN_D	1☐ Yes 0☐ No	D. Participant lives at home with parent(s) or other family members?
IN_E	1☐ Yes 0☐ No	E. Signed informed consent obtained?
IN_F	1☐ Yes 0☐ No	F. Signed authorization obtained to use and/or disclose Protected Health Information?
IN_G	1☐ Yes 0☐ No	G. Was whole blood sample of at least 2.0 ml drawn? **If no, participant should not be enrolled in the study.**

Date obtained Informed Consent: __ __/__ __/__ __ __ __
MO DA YEAR

Date obtained Protected Health Information: __ __/__ __/__ __ __ __
MO DA YEAR

All above questions must be answered YES for the participant to be randomized.

Study Personnel Signature: _____ Date:_____

Mead Johnson Nutritionals
Project 3375-1

Source Document for eCRF: B Exclusion Criteria
Visit: V1

Patient number: 112600 ___ ___ ___ Initials: ___ ___ ___ Birthdate: __ __/__ __/__ __ __ __
MO DA YEAR

Visit Date: [redacted] (Leave this field blank)
MO DA YEAR

Label	Yes/No	Criteria for Participant Exclusion
EX_A	1☐ Yes 0☐ No	A. Participant receiving breast milk?
EX_B	1☐ Yes 0☐ No	B. Participant has an active infection? An active infection is defined as an infection, (bacterial, viral, fungal or parasitic) that was diagnosed 0-7 days prior to randomization.
EX_C	1☐ Yes 0☐ No	C. Participant with a history of underlying disease, chronic disease, or congenital malformation which in the opinion of the Investigator, is likely to interfere with the evaluation of the participant?
EX_D	1☐ Yes 0☐ No	D. Known or suspected intolerance to cow's milk protein?
EX_E	1☐ Yes 0☐ No	E. Participant has been diagnosed with diabetes?
EX_F	1☐ Yes 0☐ No	F. Participant is from a multiple birth and the sibling(s) are still living?
EX_G	1☐ Yes 0☐ No	G. Participant has a sibling that participated in this study?

All above questions must be answered NO for the participant to be randomized.

Study Personnel Signature: _____ Date:_____

(Electronic Signature also required in TAO)

Mead Johnson Nutritionals
Project 3375-1

Source Document for eCRF: C Anthropometrics
Visit: V1

Patient number: 112600 ___ ___ ___ Initials: ___ ___ ___ Birthdate: __ __/__ __/__ __ __ __
MO DA YEAR

Visit Date: __ __/__ __/__ __ __ __
MO DA YEAR

Weight: _____ 1☐ LB 2☐ KG

Length/Height: _____ 1☐ IN 2☐ CM

(Lengthboard recommended for 18 to 24 months of age.
Stadiometer recommended for 24 to 36 months of age.)

Appendix B

24 Hour Dietary Recall Form

24-Hour Dietary Recall Form

Visit:_____

Random #_____ Date of Intake:_____ DOB:_____ EDC:_____

Time	Food/Beverage	Ingredients/Preparation	Amount

Intake: Typical More than Usual Less than Usual Why? _____

_____

Recall: Reliable Unable to recall meals? Unreliable for other reasons? Why? _____

_____

Vitamin/Mineral/Supplement Use? _____

Home / Daycare / Babysitter Number of people responsible for feeding _____

Interviewer Initials: _____

Appendix C

Parent Study Consent Form

CONSENT FORM

Intake of A Toddler Drink With Different Amounts of Docosahexaenoic Acid

Protocol #

Sponsor: Mead Johnson & Company

INTRODUCTION

As a parent of a child between 18 and 36 months of age, you are being asked if you will permit your child to participate in a study of a milk-based drink that contains a nutrient called docosahexaenoic acid (DHA). The study will be conducted at the University of Kansas Medical Center by Susan Carlson, Ph.D. and other members of her study team. Ninety children will be enrolled in the study.

You do not have to allow your child to participate in this research study. It is important that before you make a decision for your child to participate, you read the rest of this form. You should ask as many questions as you need to understand what will happen if your chlld participates in the study.

BACKGROUND

Docosahexaenoic acid (DHA) is recognized to be important for infant development. DHA is found in all breast milk and has been added to US formulas since 2002. DHA accumulates rapidly in the brain until children are at least 2 years of age. After infants are weaned from breast milk or formula, their DHA decreases unless they consume quite a lot of foods with DHA, particularly some kinds of fish. Chicken, eggs and some other animal foods contain DHA as well. Toddlers studied in other countries have lower blood levels of DHA than infants fed breast milk or DHA-containing formulas. There are no data for DHA intake or blood levels of US toddlers.

PURPOSE

The purpose of the study is to determine how a milk-based drink containing constant amounts of nutrients but different amounts of DHA will affect the blood level of DHA and some of those other nutrients in young children between 18 and 36 months of age.

PROCEDURES

If you choose to enroll your child in this study after hearing about how the study will be conducted, and what you and your child will need to do, you will be given an appointment to bring your child to the University of Kansas Medical Center. The investigators will work with you to find a time of day that is a good one for his/her appointment.

Visit 1 Your child will be weighed and measured at his first appointment at KUMC. A blood sample (1/2 teaspoon) will be taken by a person trained to take blood from young children. The blood sample will be used to measure the level of DHA and some nutrients in blood. The investigators will also review the birth and medical history and any medications/immunizations that your child has received within the 7 days before Visit 1. You will be given forms on which to fill out the amount of the milk product that your child consumes and instructed how to fill out the forms. You will be asked what your child has

eaten in the past 24 hours and questioned about your child's general health. If based on the above procedures, your child meets the requirements of the study, he/she will be assigned by chance (like the flip of a coin) to receive a milk-drinkwith one of three amounts of DHA: a marketed product with no DHA or one of two experimental drinks with different amounts of DHA. You will be sent home with the 3 cases of the milk drink your child is assigned to and you will be asked to provide him/her with one serving per day for the next 2 months. This visit will take approximately 1 hour.

Visit 2 One month after your child enters the study, your will bring your child back to KUMC and he/she will be weighed. You will be asked what your child has eaten in the past 24 hours and about your child's general health. You should let the investigator know if your child has been sick or not acting well since his/her last visit. The investigators will collect the forms you have filled out about your child's milk consumption during the past month. You will be sent home with 3 more cases of the milk your child is assigned to and asked to continue providing one serving per day for the next 2 months. The visit will last approximately 30 minutes.

Visit 3 Two months after your child enters the study, your child will be weighed. You will be asked what he/she has eaten in the past 24 hours and questions about his/her general health. You should let the investigator know if your child has been sick or not acting well since his/her last visit as illness can influence the amount of nutrients in blood. A blood sample (1/2 teaspoon) will be taken from your child by a person trained to take blood from young children. The blood sample will be used to measure the level of DHA and other nutrients in blood. The investigators will collect the forms you have filled out about your child's milk consumption during the past month. You will be asked to complete a product assessment form. You will return any unused full cans of study product to the investigator. The visit will last approximately 1 hour.

RISKS

Some redness, soreness, or bruising may occur at the site of blood sampling. There is also a very slight risk of infection from blood sampling. Toddlers who cannot drink cows' milk should not enroll in this study. Even toddlers who are able to drink milk may not tolerate the drink, however, this is considered to be very unlikely.

NEW FINDINGS STATEMENT

You will be informed if any significant new findings develop during the course of the study that may affect your willingness to allow your child to participate in this study.
You may request to know what nutrients were in your child's assigned drink when the study is complete.

BENEFITS

Your child is not expected to benefit from participating in this study. It is hoped that additional information gained in this research study may be useful in the growth and development of toddlers.

ALTERNATIVES

Your child does not have to participate in this research study. If you wish to increase DHA in your child's diet, you may do so by serving him/her more foods with DHA. There

are also supplements such as fish oil that contain DHA. At least one company has a marketed toddler drink that contains DHA and it may be purchased in the Kansas City area.

COSTS
You will not incur any costs because of your child's participation.

PAYMENT TO SUBJECTS
You will receive a check for $50 at each visit to the University of Kansas Medical Center to cover the costs of transportation and to partially compensate you for your time required to participate in the study. If you do not have transportation to come for the visit, you may ask the investigators to pay for a cab to and from the appointment and you will be given the $50 check, however, the investigators will have to deduct the cost of the cab from your next check. There will be 3 regularly scheduled visits over the course of 2 months.

Your name, address, social security number, and the title of this study will be given to the KUMC Research Institute. This is done so that the Research Institute can write a check for study payments. Payments are taxable income.

DISCLOSURE OF FINANCIAL INTERESTS
The principal investigator has been paid as a consultant and for program presentation on DHA for Mead Johnson Nutritionals (the sponsor). The University of Kansas Medical Center Conflict of Interest Committee monitors this research project to make it less likely that these financial interests inappropriately influence how the study is conducted. However, you should make your own decision about whether these financial interests affect your decision to participate. If you have any questions about this financial relationship, you may discuss them with the investigator or with the Research Compliance division at 913-588-5492.

INSTITUTIONAL DISCLAIMER STATEMENT
Although the University of Kansas Medical Center does not provide free medical treatment or other forms of compensation to persons injured as a result of participating in research, such compensation may be provided under the terms of the Kansas Tort Claims Act. If you believe your child has been injured as a result of participating in research, you should contact the Office of Legal Counsel, University of Kansas Medical Center, Kansas City, KS 66160-7101. You do not give up any rights by signing this form. In the event you believe that your child has suffered any physical injury as the result of his/her participation in the research program, you may contact Dr. Susan Carlson, 913-588-5359, who can review the matter with you and provide further information on how to proceed.

CONFIDENTIALITY AND PRIVACY AUTHORIZATION
Names of subjects or information identifying subjects will not be released without written permission unless required by law. Study data will be shared with the sponsor, but you will not be identified. Efforts will be made to keep your child's personal information confidential. Researchers cannot guarantee absolute confidentiality. If the results of this

study are published or presented in public, information that identifies your baby will be removed.

The privacy of your child's health information is protected by a federal law known as the Health Insurance Portability and Accountability Act (HIPAA). If you choose to allow your child to participate in this study, you will be asked to give permission for researchers to use and disclose your baby's health information that is relevant to the study.

Your baby's study-related health information will be used at KU Medical Center by Dr. Carlson, members of the research team, the KU Hospital Medical Record Department, the KUMC Research Institute and officials at KUMC that oversee research, including the KUMC Human Subjects Committee, and other committees and offices that review and monitor research studies.

Dr. Carlson and her group may share information about you and your baby with representatives of Mead Johnson (the sponsor of the study), the U.S. Food and Drug Administration (FDA), and U.S. agencies that govern human research (if and when regulatory compliance issues arise). Your child's information may be shared in order to analyze and confirm the results of the study.

Some of the persons or groups that receive your baby's study information may not be required to comply with HIPAA privacy laws. You and your child's information may lose its federal protection if those persons or groups disclose it.

Permission granted on this date to use and disclose your child's health information remains in effect indefinitely. By signing this form you give permission for the use and disclosure of your child's information for purposes of the study at any time in the future.

QUESTIONS

You have read the information in this form. Dr. Carlson or her associates have answered your question(s) to your satisfaction. You know if you have any more questions after signing this you may contact Dr. Carlson or one of her associates at (913) 588-5359. If you have any questions about your child's rights as a research subject, you may call (913) 588-1240 or write the Human Subjects Committee, University of Kansas Medical Center, 3901 Rainbow Blvd. MSN 1032, Kansas City, KS 66160.

SUBJECT RIGHTS AND WITHDRAWAL FROM THE STUDY

Your permission to allow your child's participation in this study is voluntary and the choice not to participate or to quit at any time can be made without penalty or loss of benefits. Not participating or quitting will have no effect upon the medical care of treatment your child receives now or in the future at the University of Kansas Medical center. The entire study may be discontinued for any reason without your consent by the investigator conducting the study or by the sponsor of the study.

You have a right to change your mind about allowing the research team to have access to your child's health information. To cancel your permission you must send a written request to Dr. Carlson at the University of Kansas Medical Center, Dept. of Dietetics and Nutrition, Mail Stop 4013, 3901 Rainbow Boulevard, Kansas City, KS 66160. If you

cancel permission to use your child's health information, he/she will be withdrawn from the study and the researchers will stop collecting information about him/her. The researchers and the sponsor may continue to use and share information that was gathered before your cancellation.

CONSENT

Dr. Carlson or her associates have given you information about this research study. They have explained what will be done and how long it will take. They explained the inconvenience, discomfort and risks that may be experienced during this study.

By signing this form, you give your permission for your child to participate in 3 visits at KUMC. If you choose not to sign this form, your child will not be able to participate in the study.

You voluntarily consent to allow yourchild to participate in this research study. You have read the information in this form and have had an opportunity to ask questions and have them answered. ***You will be given a copy of the signed form to keep for my records.***

_____

Type/Print Name of Subject's Parent or Guardian

_____ _____ _____

Signature of Subject's Parent or Guardian Time Date

_____

Type/Print Name of Person Obtaining Consent

_____ _____

Signature of Person Obtaining Consent Date

_____

Type/Print Name of Witness

_____ _____

Signature of Witness Date

Appendix D

Prenatal Iron and Infant Feeding Questionnaire

Infant & Toddler Nutrition Research Clinic

KU Dietetics & Nutrition
Mailstop 4013
3901 Rainbow Boulevard
Kansas City, Kansas 66160-7200

Dear Parents,

You are receiving this letter because your child participated in a clinical research trial of a DHA study beverage when he/she was a toddler. Although the study is completed, we are still investigating several key nutrients using your child's blood sample(s).

One of the nutrients we are examining is iron. Previous research has shown iron is critical for healthy development and a young child's iron status may be linked to both prenatal and postnatal care. If you are willing, we would like to ask a few questions on the following page about the pregnancy of your toddler as well as your toddler's dietary intake as an infant.

The completion of the questionnaire is completely voluntary and you are not required to return it if you feel uncomfortable providing this information. However, any information received from this survey will be very helpful in understanding the iron status of children who participated in the study.

Thank you for your support of infant research at KU. We appreciate the time and dedication you have shown to helping us understand the health needs of children. And as always, you may call at anytime if you have any questions or concerns.

Thank you and happy holidays,

Elizabeth Kerling, RD

Elizabeth Kerling, RD
Research Assistant and Masters Candidate
Phone: 913-945-6649
Fax: 913-588-5309
Email: ekerling@kumc.edu

Toddler Nutrition Questionnaire

Date:

1. **Please circle yes or no to indicate if the following supplements were consumed during the pregnancy of your study toddler. If taken, please provide the approximate date these supplements were started and stopped (month and year will be fine) . Also, please indicate how frequently they were taken.**

   Prenatal vitamin:

   Yes No Start Date:_____ Stop Date:_____ Frequency: _____

   Iron supplement:

   Yes No Start Date:_____ Stop Date:_____ Frequency: _____

   Other: (Please state what it was)

   _____ Start Date:_____ Stop Date: _____
   Frequency: _____

   _____ Start Date: _____ Stop Date:_____
   Frequency: _____

2. **Please circle if your toddler was breast or formula fed as an infant (or both) . For each, please provide the approximate start and stop date (month and year will be fine) .**

   Breast:

   Yes No Start Date:_____ Stop Date: _____

   Formula:

   Yes No Start Date:_____ Stop Date:_____ Formula Name: _____

3. **Has your child ever consumed any vitamins, minerals, or supplements on a regular basis? If yes, please provide the name and approximate dates as well as duration and frequency.**

   Yes No Name:_____ Start Date:_____ Stop Date:_____
   Frequency:_____

4. **When was food first introduced to your study toddler? (age in months)**

5. **How much did your son or daughter weigh at birth?**

Appendix E

Procedure for Circulating Transferrin Receptor Analysis

Procedure taken from insert of Enzyme Immunoassay for quantifying Human Transferrin Receptors in Serum or Plasma (Catalog #TFR-94)

Ramco Laboratories, Inc
4507 Mt. Vernon
Houston, TX 77006

1. Remove plasma samples from -80ºC freezer and allow to thaw

2. Remove the necessary strips from the packet and place in Microwell Frame (any unused strips must be returned to and resealed in the ziplock foil packet, returned to 2-8ºC and used in 6 weeks)

3. Prepare a 1:100 dilution of each patient sample and Control by dispensing 1ml of Sample Dilutent into a test tube and adding 10µl of patient serum or plasma or Control. Mix thoroughly. DO NOT dilute standards

4. Pipette 50µl of each diluted patient sample and control, in duplicate, into individual wells

5. Pipette 150µl of HRP-conjugate into all individual wells containing samples, controls, and standards

6. Seal the microwell strips with the self-adhesive plastic film and place on a rotating table and mix for 10 minutes at 190nm or rotate by hand, pressing the frame firmly against the counter to avoid sloshing, to ensure mixing of the HRP-conjugate with the samples, controls, and standards. Allow reaction to proceed for an additional 2 hours (no rotation) at room temperature upon completion of mixing

7. Prepare the substrate solution: calculate the amount of substrate solution needed by multiplying 0.2ml by the number of wells in the assay run plus 0.5-1ml dead volume. Mix equal volumes of TMB substrate solution A and TMB substrate solution B just prior to addition of the microwells. This should be used within 30 minutes of preparation.

8. After the 2 hours incubation, remove plastic film, invert the plate, and dump the contents of the microwell strips. Tap the plate dry on absorbent pad or paper towels. Wash the wells with the prepared washing solution (wash solution 5X in 250ml graduated cylinder QS to 250ml) using a plate washing device or by using the wash bottle to flood the wells and then invert and dump the contents of the wells. The

wells should be washed a total of 3 times, tapping dry between each wash. After the final wash, make sure the wells are completely empty

9. Pipette 200µl of substrate solution into each well containing samples, controls, and standards, and incubate in the dark at room temperature for 30 minutes. A blue color should develop in those wells containing TfR

10. Stop the color reaction by pipetting 50µl of acid stop solution into the wells and mix briefly to remove any air bobbles

11. Read the absorbance of each well at 450nm using a microplate reader. It is recommended that the reader be zeroed using blanks prepared with 200µl of substrate solution and 50µl acid stop solution