IRON AND DHA IN RELATION TO EARLY COGNITIVE DEVELOPMENT

BY

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ABSTRACT

Background: Iron and docosahexaenoic acid (DHA) are important nutrients for brain development; however, it has been shown that when status of both nutrients is low, DHA supplementation may actually impair cognitive outcomes.

Objective: To determine if DHA status interacts with iron deficiency (ID) during pregnancy and influences cognitive function of the offspring. We studied a sample of convenience (n=271, women given a placebo oil or 600 mg/d DHA during the last two trimesters of pregnancy). Maternal and infant nutritional status with respect to iron and DHA were also assessed.

Design: Maternal blood samples were obtained postpartum to assess for iron and DHA status while offspring blood samples were measured through cord blood at delivery and also at 4 months. In the maternal infant pairs, we identified ID by enzyme-linked immunosorbent assay (ELISA) and determined DHA status (erythrocyte weight %) by gas liquid chromatography. ID was analyzed by several markers including serum transferrin receptor (sTfR), serum ferritin (SF) and body iron (BI). DHA status was defined as low or normal by a median split. We measured offspring cognitive performance on the Bayley Scales of Infant Development Index II (BSID-II) Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) at 18 months (n=191). Analysis of variance (ANOVA) was used to examine the relationship between maternal iron

and DHA status with cognitive performance; covariates were taken into account. A priori comparisons of ID-low DHA and ID-normal DHA were conducted. Student's t-tests assuming equal variance and simple correlations were also used.

Results: ID was observed in 18% of the maternal population at delivery, 0% of the infant population at delivery, and 2% of the infant population at 4 months of age based on BI concentration. The only significant correlations seen between individual maternal iron and BSID outcomes were with SF and PDI and first and third Hb measurements and MDI. A significant negative correlation was observed between RBC-phospholipid-DHA (RBC-PL-DHA) and PDI. ANOVAs and ANOVAs with covariates taken into account showed no significant results. A priori comparisons resulted in the observation of MDI scores of (mean ± SD) of 90.1±7.3 and 98.2±9.4, t=2.07, p=0.054 among the mothers with ID, low DHA and ID, normal DHA.

Conclusions: Our population has iron status similar to that of other pregnant American women based on BI. Our results differ from recent findings examining the potential interaction between iron and DHA status on infant cognitive outcomes. Although our sample size of ID women was limited, we detected a trend suggesting that higher DHA status in ID pregnancy may protect against the adverse effects of maternal ID on the BSID-II MDI.

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Chapter 1

INTRODUCTION

Iron and docosahexaenoic acid (DHA) are two nutrients known to be influential in the development of the central nervous system. Iron deficiency (ID) has been associated with negative developmental outcomes, while DHA is a nutrient of interest being studied in relation to improved developmental outcomes.

Research examining both iron and DHA in relation to development is limited, and there are differences in results in the literature. Several attempts have been made to establish an association between maternal iron and DHA status in conjunction with infants' cognitive performances. These studies have not resulted in significant findings and have led researchers to suggest larger scale studies are needed (1). Recently, a negative effect of DHA supplementation on working memory was reported in children with anemia (though not necessarily iron deficiency anemia [IDA]) compared to iron deficient (ID) children without anemia, while ID females had negative outcomes on long term memory (2). Similar findings were reported in the male rat model when repletion of either DHA/EPA or iron was given to rats presenting with a dual deficiency (3).

Statement of Purpose

The purpose of this study is to assess the relationship between maternal DHA and iron status in conjunction with infants' cognitive outcomes at 18 months. The cohort study were children of women who were randomly assigned to DHA during the last half of pregnancy in the KU DHA Outcomes Study (KUDOS).

Research Questions

Primary Research Questions

1. What is the nutritional status, pertaining to iron and DHA, of mother and child?
2. Does maternal iron or DHA status predict Bayley Scales of Infant Development (BSID-II) Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) scores at 18 months of age?

Secondary Research Question

1. Do maternal iron and DHA status interact to influence early developmental outcomes (BSID-II MDI and PDI) of children at 18 months of age?

Chapter 2

LITERATURE REVIEW

Iron and DHA have been studied independently in relation to early developmental outcomes including motor, language and cognitive development. However, the potential relationship between the two in relation to developmental outcomes has been minimally studied. While it might be presumed that these nutrients would both have positive effects on development, recent reports show a negative effect of repletion with DHA when iron stores are low; two recent studies found negative cognitive outcomes in rats and children with deficient iron-stores and supplemented with DHA (1-3).

Iron

ID is the most common micronutrient deficiency in the world (4). The most recent statistic from a World Health Organization (WHO) report on the presence of anemia claims an estimated two billion people worldwide are affected by ID (5). Analysis of worldwide data found children and pregnant women to have the highest risk for anemia, with one in four affected (6). It is estimated that 9-11% of non-pregnant, US women of reproductive age has ID, with IDA prevalent in 2-5% of this same population (7).

ID adversely affects health and development with and without IDA, and therefore IDA and ID are distinguished in discussing the literature (5). I will use the definitions discussed below when referring to ID and anemia. ID is present when iron stores are below the required amount to support normal physiological function of bodily tissues (5). There are various measurements to determine this,

with serum ferritin (SF) being the preferred method for the detection of depleted iron stores (8). However, SF decreases late in pregnancy and is not a preferred measurement during this timeframe (8). SF concentrations of <15 μg/l for females of reproductive age and <10 μg/l for children <6 years of age are defined as levels implying that iron stores are depleted (8-10). However, SF values reflect a less sensitive measurement of iron status during states of inflammation such as pregnancy (10). Serum transferrin receptor (sTfR) is a relatively new addition to testing for ID. An advantage of this measurement is that it does not vary with pregnancy (5, 8). sTfR increases as iron stores are depleted. (8). There is not a universal reference value for sTfR, however, individuals with a plasma or serum value ≥8.4 mg/dL are typically considered ID. Body iron (BI) concentration is an additional, also relatively new measurement of iron status that is determined from SF and sTfR concentrations (11). A BI concentration <0mg/kg indicates the absence of iron stores and is another indicator of ID (11).

Anemia is defined as a hemoglobin concentration less than the threshold necessary to adequately transport oxygen that is due to ID (6). Thresholds are described by age and sex, with a separate threshold for pregnant women compared to non-pregnant women (5). The WHO considers women older than age 15 who are not pregnant to be anemic if their blood hemoglobin concentration is less than 12 g/dL (8). Pregnant women are considered anemic with blood hemoglobin levels less than 11 g/dL (8). Severe anemia is defined as blood hemoglobin levels less than 7 g/dL (8). If the anemia is due to iron deficiency, it is termed IDA.

The prevalence of ID can be attributed to inadequate intake of dietary iron in order to meet needs for growth and development, and IDA occurs when ID is so severe that the body cannot maintain adequate hemoglobin in the blood. Aside from fortified sources and supplementation, dietary iron sources consist of heme iron and non-heme iron. Dietary heme iron is found in some animal foods and is absorbed more efficiently by the body than non-heme iron, a derivative of plant sources (12). Heme iron in meats generally comprises 30-70% of iron content, with 15-35% of the heme iron absorbed by the body (12). It is estimated that less than 10% of non-heme iron is absorbed from plant based foods (12). Iron requirements are ultimately increased in pregnancy due to fetal uptake (13). Iron needs are the lowest in the first trimester due to the absence of menstruation, however, iron needs increase in the second and third trimesters due to increasing fetal absorption of iron (13). Iron requirements are highest in the third trimester of pregnancy, which is also the period of maximal fetal iron uptake (13, 14).

DHA

DHA is a long-chain omega-3 polyunsaturated fatty acid (LC-PUFA) that is transferred from mother to the fetus in utero and to the infant fed human milk. Fetal iron accumulation in the brain and retina is highest in the third trimester, (15), however, maternal intake earlier in pregnancy likely contributes to fetal uptake into other tissues (e.g., adipose tissue) that may provide DHA for the brain and retina in the 3rd trimester. The fetal brain and the placenta are limited in

their capacity to produce LC-PUFAs (1). Thus, it is important that maternal intake is adequate to allow for sufficient fetal uptake.

Dietary intake of DHA is low in many western countries, and therefore a dietary supplement during pregnancy may be needed (16). Smithers et al. states that some researchers suggest a supplement of 200 mg/day of DHA during pregnancy, but they conclude that recommendations for supplement amounts are varied (16). Recommendations have come from expert groups, but there is currently not an official organization with a recommendation for an amount of DHA intake (e.g., the IOM DRIs address omega-3 LC-PUFA only by reference to intake of the precursor, α-linolenic acid).

In 2007, European Commission research project committees, the PeriLip Steering Committee (PERILIP) and the Project Coordinating Committee of the Early Nutrition Programming Project (EARNEST), made recommendations of DHA intake during pregnancy and lactation based on extensive review of scientific evidence as well as through a general consensus workshop with international experts on the matter (17). Their conclusions are similar to that of Smithers et al., and address lactation requirements as well. These groups concluded that the evidence suggests that DHA is the only dietary fatty acid that should increase during pregnancy and lactation in most women. They concluded that arachidonic acid (ARA) is more important postnatally (17). After reviewing the many randomized control trials providing pregnant women with anywhere from 200-1200 mg DHA/d, the European Commission committees concluded 200 mg DHA per day for pregnant and lactating women is appropriate (17).

Preformed-formed dietary DHA intake is best because the plant-based omega-3 fatty acid, α-linolenic acid, is not efficiently converted to DHA (17). Therefore, the committees suggest 200 mg DHA per day through fish oil supplement intake or one to two servings of sea fish per week (17).

Through the consensus report, *Seafood Choices: Balancing Benefits and* Risks (18), the Institute of Medicine recommends that pregnant and lactating women adhere to guidelines for good nutritional intake that include two servings of seafood per week, for a serving size of four ounces raw or three ounces cooked seafood. The highest amounts of DHA are found in fatty fish including Atlantic salmon, Pacific and eastern oysters and rainbow trout (18). Other highly recommended sources include sardines and halibut (19). Government recommendations caution against consumption of swordfish, tilefish and shark and suggest limiting albacore tuna to six ounces per week during pregnancy and lactation due to concerns about methyl mercury (18, 19). One paper recommends consumption of omega-3 fatty acids during pregnancy through intake of fish without high concentration of methylmercury rather than fish oil supplements due to the higher likelihood of the fish oils containing contaminants, (19) however, it has been shown that most fish oils do not contain environmental contaminants. While the highest dietary sources of DHA for children and adults include fish and fish oils, human milk is the primary source of DHA for infants (20, 21). As of 2002, a majority of US infant formulas have been fortified with the addition of LC-PUFA's DHA and arachidonic acid (ARA) (21).

Potential Mechanism

Fatty acids and iron are important for optimal brain growth and function, and associations between the two at a metabolic level provide plausibility for my proposed research questions (4, 22). The brain concentration of both iron and DHA suggests the potential for neuronal interactions. Both nutrients are important for normal myelination. While DHA is a major component of grey matter of the brain, a deficiency in brain DHA accumulation such as occurs in the peroxisomal disorder, Pseudo-Zellweger Syndrome results in inadequate brain myelination (23). Iron is required for the myelination of cerebellar folds in the white matter of the brain, along with that of the spinal cord (24, 25). Delta-6 desaturase, an enzyme that aids in the synthesis of DHA requires iron as a cofactor (1). Iron contains catalase, an enzyme that may reduce peroxidation of LC-PUFA such as DHA in membranes. With respect to oxidative stress, this interaction between adequate iron intake and DHA is possibly beneficial (26). Inflammation can result from oxidative stress, leading to potential memory deficits (27). Finally, both DHA and iron have been shown to influence function of the monoamine neurotransmitter, dopamine (28-30).

Dopamine is related to many physiological roles in the body, as it is known as the main neurotransmitter associated with reward functions (31). The existence of individual roles of iron and DHA with dopamine is well established. When DHA brain content was decreased through an α-linolenic acid deficient diet in a rodent study, dopamine receptors were altered in a way similar to that of many animal study findings of dopamine receptor alterations in relation to

depression (28). Erikson et al. showed that iron-deficiency in the rat model leads to decreased density among dopamine receptors (29). Another study showed that marginal iron intake during the developmental period of mice, when compared to adequate iron intake, led to not only decreased brain iron concentrations, but also altered biochemical status including disrupted dopamine metabolism (30).

Studies on both DHA and iron suggest potential dopaminergic outcomes when the two nutrients interact (2, 3). One of the recent studies examining combined and individual provision of iron and DHA/EPA on children theorized that these nutrients together produce a lesser inflammatory response than when administered alone and that the negative cognitive outcomes seen in their study with individual provision of DHA/EPA to IDA children may be due to a greater inflammatory response, as this nutrient was supplemented without the other in the case of a dual deficiency (2) However, DHA and EPA are both know to have anti-inflammatory effects, so this seems unlikely. Also, a recent study providing various combinations of iron and DHA/EPA to replete male rats with both ID and DHA deficiency suggested that repletion with a single nutrient exacerbated existing deficits caused by the dual deficiency, affecting the dopaminergic pathway differently than when dual repletion was provided (3).

Cognitive Assessment

The Bayley Scales of Infant Development (BSID) is a common assessment tool for cognitive function and is the primary assessment tool that will be used in this study. Although both are commonly seen in current research,

the BSID-III was published in 2005 and is the most current version of the Bayley Scales, updating the 1993 published BSID-II with the division of the mental developmental index (MDI) measurement into two different cognitive and language analyses as the major revision. The BSID seems to be a consistent measurement about studies examining DHA and cognition, with some studies looking at iron in relation to cognition with this testing method as well. The BSID has the capability of measuring language, motor and cognitive development. It does so by examining a wide variety of tasks including body control, coordination of muscles, sensorimotor development and memory and is generally used on infants and children <42 months of age. (32-35)

Maternal Iron Status on Cognitive Development

Animal studies have examined iron and neurologic relationships more extensively than human studies for the obvious reason that more invasive procedures may be employed in animal models. Iron-restriction in pregnant rats allowed researchers to determine that perinatal ID is associated with decreased size of the hippocampus and neurochemical alterations in the adult rat even if the concentration of brain iron is corrected after birth (36). Although brain-iron concentration recovered to that of the control group, hippocampal size remained 12% smaller in the group of rats made iron deficient prior to birth (36). This implies that neural deficits caused by ID are not fully reversible despite supplementation. This outcome is related to a conclusion from a 2011 review of long-term consequences of ID and behavior in humans. That review concluded

early ID is associated with neurobehavioral impairment regardless of later normal iron status (4).

Although the developmental effects of ID can be tested more readily in rat models, several human studies have attempted to examine both the acute and long-term effects of ID and supplementation (4, 37). A recent study in western China concluded that iron supplementation alone was not responsible for increased developmental outcomes in children (35). This study used the BSID to assess development at three, six, and twelve months of age among offspring of women supplemented with either a multi-micronutrient supplement, folic acid, or folic acid and iron from early in the second trimester until delivery (35). Increases in mental development measured by the BSID were seen among all supplement groups, but only the multi-micronutrient supplement, which included 30 mg of iron, led to significant increases in mental development at twelve months. The increase was not found in women supplemented daily with 60 mg of iron and folic acid (35). The data suggest that a multi-micronutrient supplement that includes iron may be more beneficial for mental development than the provision of iron with only one other nutrient.

Results of human studies examining long-term effects of ID vary, but most literature suggests that there are long-term adverse consequences of ID on neural development and behavior (4). Supplementing pregnant women with 20 mg of iron per day from the middle of gestation until term led to a significant reduction in IDA among mothers in the iron supplemented group, but supplementation did not result in significantly increased intelligence quotients

among offspring at 4 years of age (37). In Nepal, a study supplemented mothers with daily iron and folic acid, iron and zinc, or multiple micronutrients beginning early in pregnancy. The investigators found that iron and folic acid was the only combination to significantly increase intellectual and motor function when children reached seven to nine years of age (38). This suggests that perhaps there is an interactive effect of iron and other nutrients that leads to varying developmental outcomes.

DHA Supplementation on Cognitive Development

The importance of consuming adequate DHA to allow for proper uptake and concentration in the brain and retina is supported by a majority of studies reviewing DHA intake and visual acuity. It is possible that the timing of administration, whether DHA is supplemented during pregnancy or after birth, is most important for early visual outcomes (16, 21, 39, 40). Consistent with other studies examining DHA supplementation, through formula containing LC-PUFA's DHA and ARA, postnatal administration to children results in supported visual development. When varying concentrations of DHA supplemented formula are provided to formula fed infants through one year of age, those receiving any formula with DHA concentration of at least 0.32% show significantly higher visual acuity than those receiving control formula (21). The effects of feeding formula with DHA on visual development remained positive through 39 months of age (40).

When DHA is increased during pregnancy the effect on visual acuity is inconsistent. For example, Innis et al. show higher visual acuity in two-month-old

infants of Canadian mothers who received a daily DHA dose of 400 mg DHA during pregnancy (measured by the Teller Acuity Card Procedure) (39). However, a more recent study from Australia does not support maternal supplementation of an even higher dose of 800 mg DHA per day during pregnancy and increased infant visual acuity (by measurement of visual evoked potential) at four months of age, however these mothers may have been DHA deficient prior to beginning supplementation (16). Both of these studies began DHA supplementation after 16 weeks gestation, facilitating DHA uptake in the third trimester when uptake into the brain is at the highest rate in intrauterine life (15, 16, 39).

The BSID is a frequently used measurement of cognition among researchers (32, 33, 40). Supplementing enteral feeds for pre-term infants until adjusted full-term age did not result in significant increases in cognitive development at 18 months of age, but did suggest a sex-dependent relation as BSID MDI scores in females were increased (32). In another study, DHA supplemented formulas with varying amounts of DHA fed to full-term infants through one year of age led to significant improvements in cognition at 18 months of age (33). Higher cognitive function was also found through 39 months of age when infants are fed a DHA supplemented formula through the first year of life (40). Few studies have gone on to relate motor development stemming from increased neural development, but DHA supplemented formulas throughout the first year of life may also be related to faster development of motor skills, particularly sitting without support (41).

In comparison to infant supplementation with DHA, maternal supplementation during pregnancy has not to date shown a direct effect on developmental outcomes based on the BSID but may be better shown by other neurological measures (34, 42). Offspring of women supplemented with 800 mg of DHA and 100 mg of EPA per day for at least the last half of pregnancy did not have higher BSID scores at 18 months (34). A study examining supplementation to pregnant women with 500 mg DHA and 150 mg EPA per day for a similar time frame looked at neurological development at a later age and found that a higher DHA concentration in cord blood was related to developmental improvements at five and one-half years of age (42). This suggests that developmental assessments other than the BSID or assessments later than 18 months of age may be more sensitive to the effects of DHA supplementation during pregnancy.

Another issue is that DHA status is highly variable among women within and among populations. It is possible that failure to find an effect of supplementation is due to this variability and significant overlap in DHA status between DHA supplemented and not supplemented groups. To illustrate this, several randomized trials have not found an effect of supplementation; however, these same studies show a significant effect of maternal DHA status at delivery on infant development. There are also many observational studies, which show this same finding.

Iron and DHA During Pregnancy and Development

Studies examining the relationship between iron and DHA status are limited. Human studies on the topic are becoming an area of interest. One study

examined the relationship between maternal iron and DHA status with cognitive performance on offspring at six months of age but found no significant relationship (1). However, it is possible that confounding variables played a role as this was an observational study and did not control for factors such as prenatal iron supplementation, DHA supplementation or breastfeeding (1). As well, only 3.2% and 9.5% of mothers in this study had IDA or were anemic, respectively (1).

Two recent studies have examined the effects of DHA repletion in the presence of inadequate iron stores (2, 3). Baumgartner et al. recently completed a study on male rats suggesting a negative effect of DHA/EPA supplementation on those with a dual deficiency of LC-PUFA's and iron, shown by decreased spatial and memory performances (3). The same first author showed a similar effect in children ages six to eleven years (2). All children in the study population were ID, on the basis of one of three markers including SF <20 μg/L, sTfR >8.3 mg/L, or zinc protoporphryin >70 μmol/mol heme (2). Nearly 20% of the children in this population were anemic, though in many cases not due to ID (2). After eight and one-half months of DHA/EPA supplementation (420 mg DHA and 80 mg EPA provided with or without combination of 50 mg iron), the researchers found that working memory was significantly decreased in DHA/EPA supplemented subjects who were anemic at entry to the study (2). The study also found a sex-specific effect of DHA/EPA supplementation on long-term memory of subjects who were ID upon entry, with girls showing a negative response in testing and boys actually performing better (2). These two latter studies imply an

underlying negative interaction resulting from DHA/EPA supplementation in animals or individuals with ID; however, relationships of cognitive function to iron status earlier in development would be of interest.

Conclusion

It is clear that both iron and DHA are important for optimal brain development and that the two are related at the metabolic level. A 2006 review evaluated the connection between iron and DHA in relation to infant's functional outcomes, but concluded the evidence was inadequate due mainly to too few studies of the subject (43). Similar studies to those analyzed in this review of literature led to the suggestion that the high prevalence of ID in pregnancy and potential metabolic interactions between iron and DHA during the perinatal time frame warrant the need for large, long-term studies analyzing iron and LC-PUFA interactions at the metabolic level for nutrient interaction and possible detrimental effects on cognition (43). Since then, two studies have shown a negative interaction between DHA/EPA supplementation and either a deficiency in only iron or in both LC-PUFA's and iron (2, 3). The aim of my study is to examine DHA and iron in relation to early developmental outcomes. The study was undertaken with the hypothesis that DHA supplementation would enhance infant development, however, recent studies suggesting a potential for harm from DHA in ID (2,3). My study took advantage of a cohort of women randomly assigned to DHA supplementation and whose iron and DHA status at delivery were known and whose offspring had been scored on the BSID MDI and PDI.

Chapter 3

METHODS

Overview

Analysis of DHA and iron status, and early cognitive development are secondary or tertiary outcomes of the KU DHA Outcomes Study (KUDOS), a Phase III Clinical Trial initiated in 2006 in Kansas City, KS. The KUDOS study is a two-phase project. The first phase examined the safety and efficacy of administration of 600 mg/day of DHA to pregnant women while the second phase consisted of a postnatal follow-up on visual and cognitive outcomes in infancy and childhood related to DHA administration. Subjects in the control group were provided 3 – 500 mg capsules of placebo oil while those in the treatment group were provided 3 – 500 mg total fatty acid capsules, containing a total of 600 mg DHA per day. Women were instructed to consume the randomized capsules from enrollment until delivery, which was approximately during the last two trimesters of pregnancy. The postnatal follow-up phase of the study did not include an intervention and has been completed through 18 months of age.

The specific aims of the KUDOS study were to: 1) determine whether maternal RBC-phospholipid-DHA (RBC-PL-DHA) can be significantly increased by supplementation, 2) assess the effect of DHA supplementation on duration of gestation, 3) evaluate adverse events in women and infants in the treated and placebo groups, 4) evaluate the effect of maternal DHA supplementation on visual evoked potential acuity in infancy and 5) to evaluate the effect of DHA supplementation on the development of fundamental measures of cognitive

function in infancy. I used this sample to investigate the DHA and iron status of the mother at delivery in relation to early cognitive and motor development of her offspring.

Sample

Subjects of the KUDOS study included 350 enrolled pregnant women between the ages of 16 to 35.99 years. All women were randomized to consume either capsules of DHA or a placebo for consumption during the time from enrollment into the study to delivery, were available to be contacted frequently by telephone and agreed to be available for postnatal study visits.

Women were excluded from the study if serious health conditions existed to negatively influence pregnancy outcomes, including those that may affect the growth and development of the fetus and postnatal newborn as well as the health of the mother. Maternal health risks that put subjects up for exclusion at baseline included but were not limited to cancer, lupus, hepatitis, diabetes mellitus (Type I, Type II, gestational) or HIV/AIDS. Women carrying multiples during pregnancy were excluded due to risk of increased fetal complications. Other baseline measurements that excluded the subject were morbid obesity (BMI ≥ 40) and elevated high blood pressure (systolic ≥ 140). Women were also excluded if they were non-English speaking due to lack of standardization of postnatal tests for multiple cultural groups.

Recruitment and Research Setting

Women between 8 and 20 weeks gestation were primarily recruited in OB clinics at Truman Medical Center (Kansas City, Missouri), St. Luke's Hospital

(Kansas City, Kansas), and the University of Kansas Medical Center (Kansas City, KS) for inclusion into the study. Upon enrollment the subjects were presented with a background, purpose, benefits and risks of the study before officially enrolled. After enrollment, prenatal data was collected at the respective hospitals of study subjects and postnatal data was collected at follow-up visits at the University of Kansas Medical Center. Postnatal visits took place when the infant was 6 weeks, and 4, 6, 9, 10, 12, and 18 months of age.

Ethics

The KUDOS study was approved by the Human Subjects Committee at the University of Kansas Medical Center (HSC#10186). Subjects accepting participation in the study provided written, informed consent (see Appendix A). Welfare of the study subjects was upheld in accordance to the guidelines from the Declaration of Helsinki. Health Insurance Portability and Accountability Act (HIPPA) disclosure information was also provided to subjects. All research team personnel abided by University confidentiality policies, the Privacy Protection for Research Subjects, and remained current in NIH Human Subjects protection and HIPAA certification. Patient confidentiality was maintained throughout the study. Subjects received a random identification number, and this number along with patient initials was used for identifying infants during postnatal follow-up visits. Patient information was stored in a location accessible only by approved study protocol members.

Procedures

This sub-study design was a retrospective cohort study to examine a possible association between iron and DHA in relation to early cognitive development.

Personnel who were trained for consistency completed blood collection and fatty acid analysis. Maternal blood samples were drawn at enrollment and the morning after childbirth. Blood was collected from an antecubital vein in EDTA and placed on ice. Infant blood, collected in the same manner as maternal samples, was obtained from cord venous blood at delivery and from an antecubital vein at 4 months of age. Blood was centrifuged, plasma was removed and the precipitated RBCs were washed three times with EDTA and stored at -80 C until analysis. RBC lipids were extracted and left as organic solvents. Phospholipids (PL) were isolated by thin later chromatography. Individual fatty acid methyl esters were separated by gas chromatography. Peaks from this process were identified by comparison to authentic standards and a weighed standard mixture was used to adjust fatty acids for area/weight to calculate a final percent weight of total fatty acids. RBC-PL-DHA is reported as a percentage of total fatty acids by weight.

Maternal iron status was determined by quantifying hemoglobin (Hb), SF, sTfR and by calculating BI from SF and sTfR. Anemia was defined as Hb less than 11 g/dL in the first and third trimesters while severe anemia was defined as Hb less than 7 g/dL during this same timeframe (8). Those pregnant women with SF levels less than 15 μg/L were classified as ID (8). More sensitive indicators

during this timeframe, including sTfR and BI were used to further investigate iron stores. ID, with respect to these indicators, was considered with sTfR values ≥8.4 μg/mL (test kit value) and negative BI (mg/kg) (11, 44). The following equation was used to calculate BI from the sTfR/SF ratio, also known as the R/F ratio: – [log(R/F ratio) – 2.8229]/0.1207 (45).

We used the same measures to quantify infant iron status with the exception of Hb, which was not measured in infant blood samples. SF values <10 μg/L were used to classify ID in these infants (46). We used the same sTfR and BI concentrations to indicate ID in infants as we used for maternal measurements.

The BSID-II is the primary assessment tool used in this study for cognitive (MDI) and motor (PDI) testing. These BSID subtests were performed at 18 months of age to yield IQ-like scores representing developmental sensorimotor milestones and basic skills representing cognitive development.

The Peabody Picture Vocabulary Test (PPVT) was administered to mothers in the study, but in a few cases, it was given to fathers or grandparents when mothers were not available. This vocabulary assessment test was used in assessing the potential for confounding associations between parental intelligence and cognitive outcomes.

A nutrient database created by Mallory Bratton, MS, RD, as part of her thesis project was the source of information on whether a separate iron supplement was consumed on the advice or recommendation of the woman's physician.

Statistical Analysis

Summary statistics of maternal and infant iron and DHA status are reported as mean ± SD, while minimum and maximum values are also reported. BSID outcome measures are also reported as mean ± SD. Decimals of all mean values are rounded to the nearest tenth. Iron measures are categorized in accordance to the values previously discussed as indicators of low iron status. As study personnel other than the PIs remain blinded to the DHA supplementation, we used a median split of maternal and infant DHA status at delivery to categorize women as having low or normal DHA status. Maternal and newborn iron and DHA values and BSID outcomes were compared by measuring correlation coefficients. T-tests were used to investigate the relationship between low and high maternal iron and DHA status and BSID outcomes. Simple descriptive statistics were used to report the BSID outcomes based on a potential interaction between iron and DHA status.

Analysis of variance (ANOVA) was used to evaluate the interaction between DHA and iron status on the BSID MDI and PDI scores, however, we also did preplanned comparisons between ID women with a DHA status above and below the median in relation to infants BSID MDI and PDI scores. We also used ANOVA controlling for maternal education, income by zip code, and PPVT to evaluate the interaction between DHA and iron status on the BSID MDI scores. Correlation coefficients and t-tests were used to assess other data of interest. All t-tests were two sample t-tests assuming equal variance. Data were analyzed by

IBM SPSS Software 20.0 and *P* values less than 0.05 were considered statistically significant.

Chapter 4

RESULTS

Subject Characteristics

We studied a subset of 271 women who were among the 350 recruited into the KUDOS study for evaluation of DHA supplementation and pregnancy outcomes. The subset of women included those with a blood sample available at delivery. All available data from the narrowed population were used to generate summary statistics, simple correlations and t-tests (n =100 to 271). Of the infants born to these women, we had a valid 18 month BSID MDI for 194 children and a valid PDI for 196 children. A total of 191 pregnancies had maternal iron and DHA status postpartum and BSID scores on the offspring at 18 months. Maternal and infant summary statistics are displayed in Table 1.

TABLE 1. Summary statistics of maternal and infant populations

Characteristic	Mean ± SD	Min – Max
Maternal[1]		
Race (%white / %black / %other)	64/35/1	
Age at enrollment (y)	25.7 ± 4.8	16.06 – 35.97
Education (y)	14.3 ± 2.8	8 – 20
Income by zip code ($)	46,610.5 ± 17,766.9	18,333 – 100,289
Number of living children	0.9 ± 1.0	0 – 5
BMI at enrollment (mg/kg^2)	27.1 ± 26.5	16.53 – 42.56
PPVT	99.6 ± 14.9	67 - 100
Infant[3]		
Gender (%male / %female)	52/48	
Race (%white / %black / %other)	65/29/6	
Birth weight (g)	3,317.6 ± 534.3	1,010 – 4,704
MDI	97.0 ± 11.6	71 – 135
PDI	94.5 ± 9.2	71 – 119

[1]Two hundred seventy-one values for age at enrollment, number of past living children and BMI at enrollment, 270 values for race, 266 values for income by zip code, 231 values for education, 209 values for PPVT. One hundred ninety PPVT exams given to mother, 18 given to father, 1 given to grandmother.
[2]Two hundred seventy-one values were available for birth weight, 270 values for gender, 223 values for race, 196 values for PDI, 194 values for MDI.

Nutritional Status

With the exception of Hb, all maternal iron status markers were drawn at delivery and are referred to as postpartum iron markers. The mean and median values and ranges for maternal and infant measures of iron and DHA status are shown in Table 2. We also included the descriptive analyses of iron and DHA status of the infants for whom blood was obtained at 4 months of age, however these were not evaluated further because there were many missing data.

According to conventional concentrations for iron status biomarkers, 12% (n=29) had ID by sTfR concentration, 29% (n=71) by SF and 18% (n=43) by BI.

Anemia occurred in 5% of the women during the first trimester of pregnancy and in 25% during the third trimester of pregnancy (n=63).

Fifty-one percent (n=86) of the infants had a cord sTfR ≥8.4 mg/L, however, we do not conclude that this many were ID, because there are no standards for sTfR in cord blood. Using SF and BI, which may be more reliable indicators in newborns, one infant had ID according to cord SF while also only one infant had ID according to cord BI, however these were two different subjects. At four months of age, 11% of the infant population had ID according to sTfR. Two infants had ID according to both 4-month SF and 4-month BI. These were the only two infants with ID according to these individual markers.

All maternal DHA values reported and discussed in this study were drawn at delivery. A median split was used to classify those with lower DHA status from those with higher DHA status. Infant DHA values were assessed in cord blood as well as at 4-months of age. As with the maternal population, a median split was used to classify the infant population with lower DHA status from that with a higher DHA status. Summary statistics of maternal and infant iron and DHA status are shown in Table 2, followed by a description of the maternal and infant populations with low iron or DHA status in Table 3.

TABLE 2. Summary statistics of maternal and infant iron and DHA status

Characteristic	Mean ± SD	Median	Min – Max
Maternal iron[1]			
Postpartum sTfR (mg/L)	5.4 ± 2.9	4.67	0.19 – 19.52
Postpartum SF (μg/L)	27.1 ± 27.1	27.13	3.35 – 208.40
Postpartum BI (mg/kg)	4.3 ± 4.0	4.98	-6.85 – 14.95
First trimester Hb (g/dL)	12.6 ± 0.9	12.6	9.9 – 15.4
Third trimester Hb (g/dL)	11.5 ± 1.0	11.5	8.4 – 14.0
Infant iron[2]			
Cord sTfR (mg/L)	8.9 ± 2.8	8.43	0.85 – 19.73
Cord SF (μg/L)	149.0 ± 93.9	127.87	9.53 – 650.31
Cord BI (mg/kg)	8.3 ± 2.7	8.37	-0.03 – 14.86
4-month sTfR (mg/L)	6.5 ± 1.5	6.25	3.19 – 12.83
4-month SF (μg/L)	67.4 ± 44.2	59.00	6.78 – 257.51
4-month BI (mg/kg)	6.3 ± 2.7	6.54	-3.76 – 12.41
Maternal DHA[3]			
RBC-PL-DHA	6.1 ± 2.0	5.47	2.30 – 12.26
Plasma-PL-DHA	4.3 ± 1.6	3.91	1.54 – 9.12
Infant DHA[4]			
Cord RBC-PL-DHA	6.6 ± 1.8	6.49	2.32 – 11.68
Cord plasma-PL-DHA	4.9 ± 1.8	4.54	2.10 – 12.14
4-month RBC-PL-DHA	6.2 ± 1.8	5.87	2.78 – 14.08
4-month plasma-PL-DHA	4.5 ± 1.3	4.29	1.94 – 9.98

[1]Two hundred sixty-five values were available for first trimester Hb, 254 values for third trimester Hb, 243 values for postpartum sTfR, 242 values for postpartum SF and postpartum BI.
[2]One hundred sixty-nine values were available for cord sTfR, 166 values for cord SF, 165 values for cord BI, 115 values for 4-month sTfR, and 100 values for 4-month SF and 4-month BI.
[3]Measured in g/100g Total FA. Two hundred sixty-three values were available for RBC-PL-DHA and plasma-PL-DHA.
[4]Measured in g/100g Total FA. One hundred ninety values were available for cord RBC-PL-DHA, 189 values for cord plasma-PL-DHA, 143 values for 4-month plasma-PL-DHA, 126 values for 4-month RBC-PL-DHA.

TABLE 3. Maternal and infant populations classified with low iron or DHA status

Characteristic	Standard[1]	% Population[2]
Maternal iron marker[3]		
Postpartum sTfR	≥ 8.4 mg/L	12
Postpartum SF	<15 µg/L	29
Postpartum BI	<0 mg/kg	18
First trimester Hb	<11 g/dL	5
Third trimester Hb	<11 g/dL	25
Infant iron marker[4]		
Cord sTfR	≥ 8.4 mg/L	51
Cord SF	<10 µg/L	0
Cord BI	<0 mg/kg	0
4-month sTfR	≥ 8.4 mg/L	11
4-month SF	<10 µg/L	2
4-month BI	<0 mg/kg	2
Maternal DHA marker[5]		
RBC-PL-DHA	5.47	50
Plasma-PL-DHA	3.91	50
Infant DHA marker[6]		
Cord RBC-PL-DHA	6.49	50
Cord plasma-PL-DHA	4.54	50
4-month RBC-PL-DHA	5.87	56
4-month plasma-PL-DHA	4.29	51

[1]Conventional standards used to describe iron status. Maternal sTfR, SF and BI indicate ID. Maternal Hb indicates anemia. Infant iron standards indicate ID. DHA status represented in g/100g Total FA and based on median split.
[2]Percent of population with values < standard used for iron status and ≤ standard used for DHA status.
[3]Two hundred sixty-five values were available for Hb first trimester, 254 values for Hb third trimester, 243 values for postpartum sTfR, 242 values for postpartum SF and postpartum BI.
[4]One hundred sixty-nine values were available for cord sTfR, 166 values for cord SF, 165 values for cord BI, 115 values for 4-month sTfR, and 100 values for 4 month SF and 4-month BI.
[5]Two hundred sixty-three values were available for RBC-PL-DHA and plasma-PL-DHA.
[6]One hundred ninety values were available for cord RBC-PL-DHA, 189 values for cord plasma-PL-DHA, 143 values for 4-month plasma-PL-DHA, 126 values for 4-month RBC-PL-DHA.

Individual Maternal Iron and DHA Markers in Relation to BSID Scores

Correlations between the BSID outcomes and maternal iron and DHA status were run to view potential relationships. The relationships between all maternal iron status markers assessed, as well as those of all maternal DHA markers assessed, and respective infant cognitive outcomes are shown in Table 4. The only significant correlations seen between maternal iron markers and MDI scores were hemoglobin measurements in the first and third trimester, which were both positive correlations. Postpartum SF and RBC-PL-DHA were significantly negatively correlated with PDI scores.

TABLE 4. Correlations between maternal iron and DHA markers and BSID outcomes

	MDI	PDI
	r	*r*
Postpartum sTfR	-0.107	0.031
Postpartum SF	0.043	-0.162*
Postpartum BI	0.079	-0.101
First trimester Hb	0.167*	-0.048
Third trimester Hb	0.165*	-0.029
RBC-PL-DHA	0.081	-0.157*
Plasma-PL-DHA	0.109	-0.086

*Correlation is significant at $p<0.05$

In further analyses, BSID outcomes of respective high and low maternal iron and DHA status were examined. When comparing the MDI or PDI scores of mothers with high iron status and mothers with low iron status, no significant

difference was found based on postpartum sTfR. When comparing the MDI or PDI scores of mothers with high iron status and mothers with low iron status, no significant difference was found based on postpartum BI. When comparing the MDI or PDI scores of mothers with high RBC-PL-DHA concentrations and mothers with low RBC-PL-DHA concentrations, no significant difference was found. These data are shown in Table 5. Conventional markers used to classify high and low maternal iron and DHA status for this analysis were those reported in Table 3.

TABLE 5. Individual relationships between low and normal maternal iron and DHA status and BSID outcomes[1]

	MDI			PDI		
	Mean 1 ± SD	Mean 2 ± SD	*P*	Mean 1 ± SD	Mean 2 ± SD	*P*
Postpartum sTfR[2]	94.4 ± 9.2	97.3 ± 11.4	0.285	97.7 ± 9.8	94.2 ± 9.2	0.116
Postpartum BI[3]	95.3 ± 10.7	97.3 ± 11.4	0.374	97.2 ± 8.3	94.0 ± 9.4	0.093
RBC-PL-DHA[4]	96.7 ± 11.7	97.3 ± 11.5	0.753	95.4 ± 9.5	93.9 ± 9.0	0.256

[1]Mean 1 representing mean for ID and low DHA groups, Mean 2 representing mean for normal iron and DHA groups.
[2]MDI: Mean 1 n=19, Mean 2 n=170. PDI: Mean 1 n=19, Mean 2 n=172.
[3]MDI: Mean 1 n=29, Mean 2 n=160. PDI: Mean 1 n=29, Mean 2 n=162.
[4]MDI: Mean 1 n=85, Mean 2 n=106. PDI: Mean 1 n=86, Mean 2 n=107.

Potential Influence Between Maternal Iron and DHA Status and Infant Outcomes

To evaluate the association with maternal iron and DHA status on early developmental outcomes of the child population at 18 months of age, we used the 191 mother infant dyads with a maternal postpartum DHA blood sample and usable BSID scores in the final descriptive statistics and ANOVA model.

Mother infant dyads were grouped according to maternal postpartum iron and DHA status. Four groupings were created for nutrient status: ID with RBC-PL-DHA less than or equal to the median; ID with RBC-PL-DHA above the median; normal iron with RBC-PL-DHA less than or equal to the median; normal iron with RBC-PL-DHA above the median. ID was defined as either a sTFR ≥8.4 mg/L or a BI<0 BSID MDI (n=188) and PDI (n=190) outcomes were compared by iron and DHA status.

Among all mother infant dyads with a low postpartum iron status, no significant difference in BSID outcomes was observed depending on DHA status. Results for BSID outcomes categorized by postpartum iron and DHA groupings are shown in Table 6.

TABLE 6. BSID outcomes categorized by postpartum iron and DHA[1] groupings

	MDI[2]		PDI[3]	
	Mean ± SD	n	Mean ± SD	n
sTfR[4], DHA groupings				
Iron Deficient, low DHA	90.1 ± 7.3	9	101.7 ± 8.9	9
Iron Deficient, normal DHA	98.2 ± 9.4	10	94.1 ± 9.6	10
Normal iron, low DHA	97.4 ± 11.1	74	94.6 ± 9.4	75
Normal iron, normal DHA	97.2 ± 11.8	95	93.9 ± 9.1	96
BI[5], DHA groupings				
Iron Deficient, low DHA	93.3 ± 10.1	16	99.2 ± 9.7	16
Iron Deficient, normal DHA	97.8 ± 11.1	13	94.7 ± 5.8	13
Normal iron, low DHA	97.4 ± 11.1	67	94.4 ± 9.3	68
Normal iron, normal DHA	97.2 ± 11.7	92	93.8 ± 9.5	93

[1]DHA categorized into low DHA and normal DHA groups based on original study population median value of 5.47 g/100g Total FA; low as RBC-PL DHA ≤ median, normal as RBC-PL-DHA > median
[2]One hundred eighty-eight values available for MDI
[3]One hundred ninety values available for PDI
[4]sTfR categorized into ID and normal iron groups with ID as postpartum sTfR ≥ 8.4 mg/L and normal iron as postpartum sTfR <8.4 mg/L
[5]BI categorized into ID and normal iron groups with ID as postpartum BI <0 mg/kg and normal as postpartum BI >0 mg/kg

To compare the BSID MDI and PDI in relation to the maternal iron and RBC-PL-DHA status, MDI and PDI were entered into two separate two-way ANOVAs by iron status (ID or normal) and DHA status (below or above the median) as between subject factors. No significant outcomes were seen among any of the four ANOVAs ran. The MDI analysis did not yield any significant factors when ID was defined by sTfR: Iron Group, $F(1,184) = 1.317$, $p = .253$; DHA Group, $F(1,184) = 2.055$, $p = .153$; Iron x DHA, $F(1,184) = 2.338$, $p = .128$. The PDI analysis did not yield any significant factors when ID was defined by

sTfR. Iron Group, F (1, 186) = 2.668, p = .104; DHA Group, F (1, 186) = 3.428, p = .066; Iron x DHA, F (1, 186) = 2.377, p = .125. The MDI analysis did not yield any significant factors when ID was defined by BI: Iron Group, F (1, 184) = .613, p = .435; DHA Group, F (1, 184) = .863, p = .354; Iron x DHA, F (1, 184) = 1.075, p = .301. The PDI analysis did not yield any significant factors when ID was defined by BI: Iron Group, F (1, 186) = 2.257, p = .135; DHA Group, F (1, 186) = 1.872, p = .173; Iron x DHA, F (1, 186) = 1.060, p = .304.

While MDI and PDI were significantly correlated, no other significant correlations were observed in conjunction for PDI. On the other hand, several variables correlated significantly with MDI including maternal education (r = 0.268, p < 0.01), income categorized by zip code (r = 0.262, p < 0.01) and maternal PPVT scores (r = 0.326, p < 0.01). A significant negative correlation was also observed between MDI and number of living children (r = -0.253, p < 0.01).

As a result of the significant correlations observed with MDI, two-way ANOVAs by iron status (ID or normal) and DHA status (below or above the median) as between subject factors where we controlled for maternal education, income categorized by zip code, and maternal PPVT scores were performed. The results from the ANOVAs controlling for covariates were also nonsignificant. The MDI analysis controlling for the three mentioned covariates did not yield any significant factors when ID was defined by sTfR: Iron Group, F (1, 175) = .989, p = .321; DHA Group, F (1, 175) = .269, p = .605; Iron X DHA, F (1, 175) = 1.610, p = .206. The MDI analysis controlling for the three mentioned covariates did not yield any significant factors when ID was defined by BI: Iron Group, F (1, 175) =

0.22, p = .882; DHA Group, F (1, 175) = 0.85, p = .770; Iron x DHA, F (1, 175) = 1.726, p = .191. As nothing other than MDI showed a strong correlation in relation to PDI, the idea that there is no need for an ANOVA controlling for covariates with PDI outcomes is supported.

Because one of our questions was if DHA could actually be harmful to cognitive development in conjunction with ID, we compared MDI and PDI performance at 18 months for children of women with ID and lower DHA compared to ID and higher DHA. There was a nonsignificant trend toward higher child MDI when DHA status was higher in ID mothers, as measured by sTfR (p=0.054). Figure 1 shows the effects observed between iron and DHA status on the MDI. Figure 2 shows the effects observed between iron and DHA status on the PDI.

FIGURE 1. Iron by DHA effect on MDI (means of groupings represented)

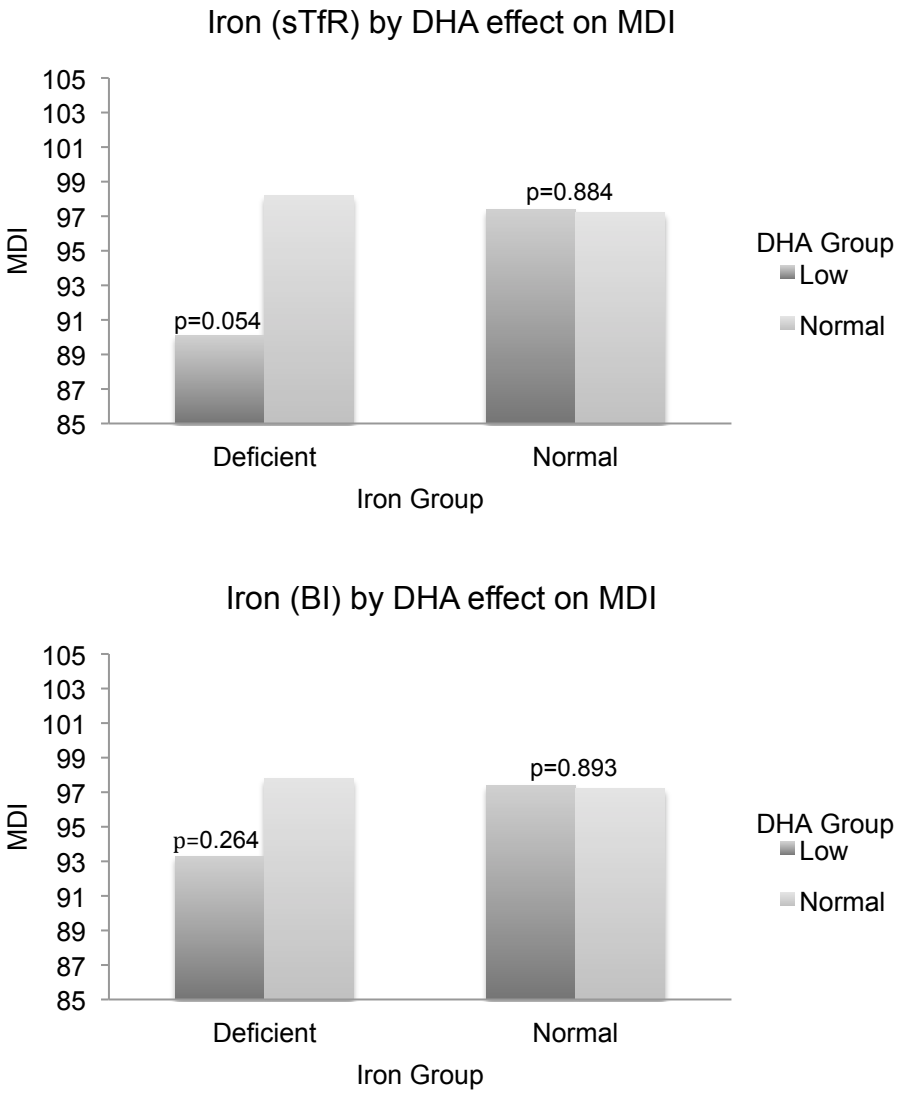
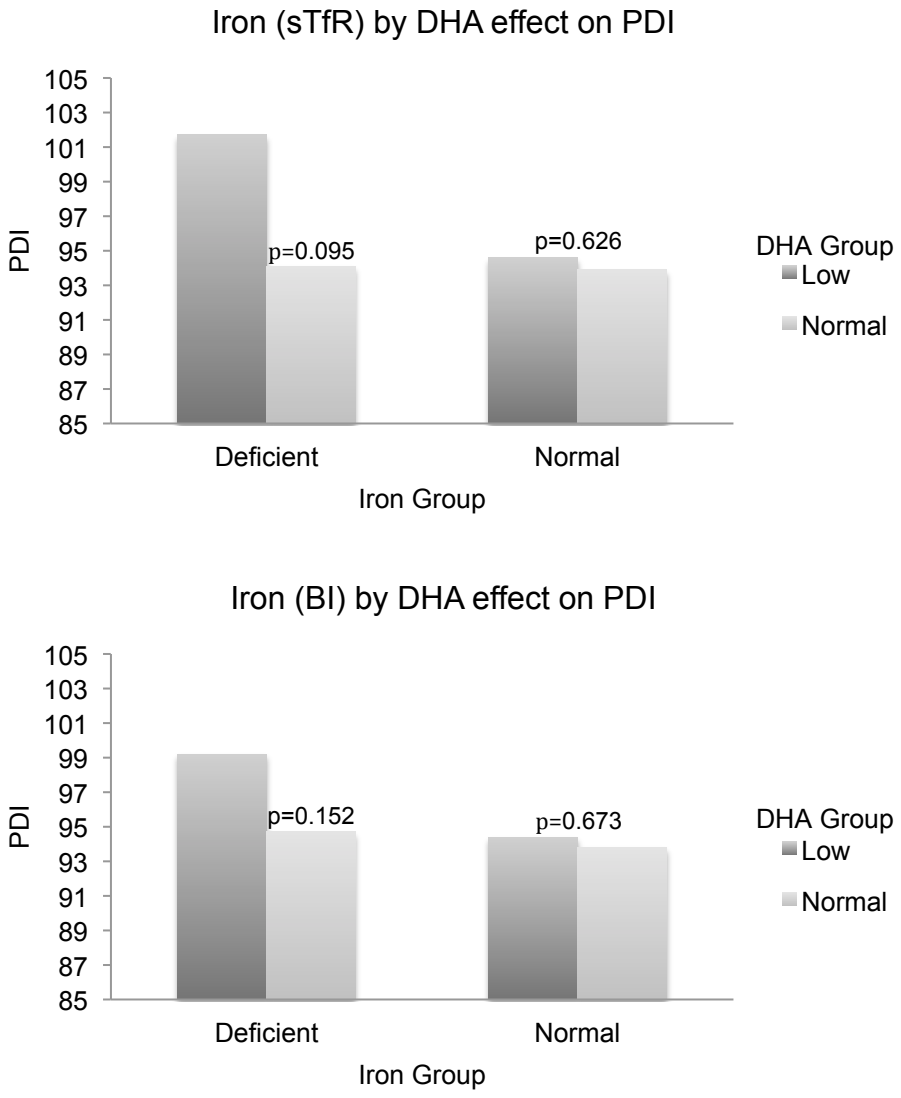


FIGURE 2. Iron by DHA effect on PDI (means of groupings represented)



Association Between DHA and Iron Status

Maternal RBC-PL-DHA was significantly correlated with postpartum BI (r = 0.265, $p < 0.01$) and SF (r = 0.261, $p < 0.01$) and also with sTfR (r = -0.191, $p <$ 0.01). When maternal BI of women with RBC-PL-DHA status below the median was compared to that of women above the median there was a difference in body iron ($p < 0.01$). The mean BI (mg/kg) for those with DHA below the median was 3.3 ± 4.0 while that of women with a DHA status above the median was 5.3 ± 4.0.

Physician Recommended Iron Supplementation

Fifty of the 191 women whose offspring had available BSID scores were prescribed iron by their physician. At delivery 14 of these had a ID based on a negative BI and/or elevated sTfR. Of these 14 women, eight reported intake of the iron supplement recommended by their physician. The remaining 36 women prescribed iron by their physician had normal iron status at delivery by BI and sTfR. All reported taking their recommended supplement. Of the 191 mother infant pairs studied, 33 women had ID by postpartum BI and/or postpartum sTfRr.

Chapter 5

DISCUSSION

Iron Status

Through measurement of BI as the representative marker of iron status, the presence of ID of pregnant women examined in this study appears to representative of the pregnant women of the US population. A 2011 report by Mei et al. using BI as the representative marker examined the 1999-2006 National Health and Nutrition Examination Survey and claims to be the first study to assess iron status in a representative population of US pregnant women (47). The latest measurement of iron status of pregnant women in the NHANES study was taken in the third trimester (47). Using these markers in comparison to our markers at delivery, mean BI was observed to be the same in our group compared to that of the NHANES study (4.3 mg/kg) (47). Mean SF of the two respective groups was similar (27.1 µg/L vs. 26.3 ug/L) but sTfR was higher in our cohort (5.4 mg/L vs. 3.45 mg/L) than in NHANES (47). As well, the mean Hb in our population during the third trimester was lower (11.5 g/dL) than that of the NHANES study (12.6 g/dL) (47).

Pregnant women from the NHANES study had an 18% incidence of ID by BI <0 mg/kg (47). Low BI was more prevalent in Mexican American and non-Hispanic black pregnant women during the second and third trimesters of their pregnancy, as well as in those with a parity ≥ 2 (47). Among the pregnant women in our study population, the presence of ID by BI was 18%. A BI less than 0 was

significantly more likely to occur in mothers with parity ≥ 2 ($P < 0.01$). We did not find a relationship between maternal race and BI in our sample.

Compared to pregnant women who have been studied in other parts of the world, US pregnant women appear to have less ID (10). A study in Belgium found the prevalence of ID to be 40% based on SF. However, it was interesting that this same group of pregnant women had a lower prevalence of ID based on sTfR (7% compared to 12% in our population) and a lower prevalence of anemia based on Hb (21% compared to 25% in our population (10). Only the median BI was reported in the Belgian women (3.6 mg/kg), but it was lower than the median BI of those in our population (4.3 mg/kg). Iron assessment occurred during the 3rd trimester of pregnancy in the Belgian population while our assessments were made within hours after delivery (10). Nearly two-thirds of the women in the Belgian population claimed to have taken supplements containing iron during pregnancy (actual amounts of iron intake not specified), however it should be noted that 40% of these women were still considered ID during the third trimester based on SF < 15 ug/L (10). Rioux et al. studied maternal DHA and iron status and found the prevalence of ID in the Canadian women they studied to be 34.9%, based on a SF < 10 ug/L. This is much higher than in our population, which had a prevalence of 17% using this cut-off. (1).

Infant iron status at delivery and during infancy does not seem to be as readily measured as maternal status. However, Rioux et al. found that 6.3% of infants were anemic at 6 months of age based on Hb stores (1). Our study did not measure Hb in the infant population, but it was observed that at 4 months of

age the prevalence of ID was 2% based on BI and 11% based on sTfR concentrations.

Relevant Markers of Iron Status

The WHO has questioned the use of SF concentration to determine iron status in pregnancy because pregnancy and parturition are considered to be inflammatory states. Accordingly, WHO advises against its use as an iron status indicator during pregnancy (8). Interestingly, inflammation increases SF, yet SF concentration in our population suggested a much higher incidence of ID (29%) than did sTfR or BI, 12% and 18%, respectively.

Even when SF is measured during pregnancy, the SF cutoff used to differentiate ID varies. While SF <15 µg/L is the suggested cutoff for ID for females of reproductive age (8-10) and this value has been used during pregnancy, other references have used SF <12 µg/L and SF <10 µg/L during pregnancy (1, 9, 10, 48). If we used an SF concentration <12 µg/L, as our cut-off, ID in our population decreased from 29% (n=71) to 21% (n=52). As noted above, only 17% of women in our study had ID using SF<10 as the cut-off, the value used by Rioux et al (1).

As well, studies use different cut-offs for SF signifying ID in the infant population. The WHO uses a cut-off of <12 ug/L in children under 5 while some researchers suggest <10 µg/L (8, 9). We used SF <10 µg/L, a more conservative number, but the number of ID individuals would not have been increased at delivery or 4 months of age had we used a cut-off of <12 µg/L.

The most selective marker of ID for pregnant women used in this study appears to be sTfR, however, 51% of the cord blood samples had an sTfR concentration ≥8.4 mg/L. We do not think elevated sTfR in cord blood is a reliable indicator of ID. The mean sTfR of our infant cord blood samples (8.9 mg/L) was similar to that reported by Sweet et al. (8.4 mg/L). These authors speculated that the elevated sTfR in cord blood plasma most likely reflected high fetal requirements for erythropoiesis (49). Based on SF and BI concentrations, only two children in our study had ID at birth; these children did not have ID based on both markers as one had ID based on SF and the other had ID based on BI.

In terms of a maternal marker for ID during pregnancy, sTfR is used throughout the literature, however a standard value has not yet been identified. An sTfR >4.4 is the smallest concentration that has been mentioned in publications to indicate ID, however, the authors chose the value because they lacked a standard and because the manufacture specified range for sTfR ended at this value (47). Vandevijvere et al. used sTfR >8.5 mg/L to specify ID in their population of Belgian women, and chose this value based on this cut-off in a previous study in Swedish pregnant women (48). We used ≥8.4 mg/L in our study, because that is the cutoff used for our ELISA, however, the percentage of women in our study classified with ID would remain 12% had we used a cut-off of >8.5 mg/L. One fewer women would have been considered ID. Interestingly, she was considered ID by status was categorized in terms of BI. The subject also had a lower RBC-PL-DHA value, so she would have been lost from the ID, low DHA

group, leaving only 8 women with ID and lower DHA. The lower and higher DHA groups are already small and the effect of DHA on the MDI in ID women is not significant. However, it would be much easier to determine if the apparent protective effect of DHA on child MDI is real in a cohort that includes more women with postpartum ID.

BI was proposed as an indicator of iron status in 2003 and is not yet a validated marker of iron status during pregnancy (45). While it did not appear to be as selective of a marker of ID in our population as did sTfR, it was much more selective than SF, which is still seen commonly used despite suggestions against its use during pregnancy. BI is becoming a more common assessment tool during pregnancy (10, 47).

Hb appears to be a consistent measurement taken to view iron status during pregnancy, however it is not often the primary marker of focus in the literature. This appears to be so as Hb may be lower throughout the progression of pregnancy due to the increased expansion of plasma volume (8). It is often primarily used as a marker of iron-deficiency anemia, especially within the first two trimesters of pregnancy, but due to increased plasma volume its use as a determinant for iron status as pregnancy progresses is questioned and is therefore not a principal assessment marker (10, 48).

DHA Status

We differentiated DHA status of mother and infant based on a median split and used the respective values to separate those with lower DHA status from those with higher DHA status. Although not analyzed in relation to iron or

cognitive outcomes, mean and median values of plasma-PL-DHA were reported in addition to RBC values used in further analysis throughout our study.

The RBC and plasma DHA levels of our maternal sample at delivery were higher than those reported by Rioux et al (1). Mean RBC-PL-DHA values were higher among our population of pregnant women at 6.1 ± 2.0 g/100g total FA in comparison to 4.3 ± 1.98 g/100g total FA (1). Mean plasma-DHA values were also higher among our population at 4.3 ± 1.6 g/100g total FA in comparison to 2.1 ± 0.5 g/100g total FA (1). The main differences between the methodologies of the two studies are that Rioux et al. studied a population of pregnant Canadian women, took blood measurements between 28 and 32 weeks, and did not provide a DHA supplement (1), whereas approximately half of the women in our study were provided 600 mg/day of DHA for over half of pregnancy. The nutritional intake of Canadian women, blood draws prior to delivery, and lack of supplementing with DHA are all possible factors to attribute the higher DHA status of our population.

In a study supplementing pregnant women with 400 mg DHA or placebo, Novak et al. measured maternal PL-DHA at 36 weeks gestation and infant PL-DHA through cord blood at delivery (50). Novak et al. reported DHA values through analysis of the choline PL (PC) and ethanolamine PL (PE) (50). In comparison, the RBC-PL-DHA values of our population included all PL in the RBC membrane. Given that there are approximately equal amounts of PC and PE in RBC PL, and these two PL classes account for approximately two-thirds of RBC PL, I averaged the results from PC and PE in the population studied by

Novak et al. Maternal RBC-PL-DHA values of our population were 6.1 g/100g total FA compared to 5.34 g/100 g total FA in their study (50). Cord RBC-PL-DHA values of our population were also higher at 6.6 g/100g total FA compared to an average of PC and PE of 5.79 g/100g total FA (50).

Whereas our study analyzed plasma-PL-DHA and RBC-PL-DHA in all subjects, plasma-PL-DHA content was analyzed among only 17 of the mother infant dyads of the initial 97 in Novak's study (50). Plasma values of our maternal and infant populations were lower than those reported among the maternal and infant populations of Novak et al. Mean plasma-PL-DHA of our maternal population at delivery (4.3 g/100g total FA) was lower than that reported by Novak et al. (6.45 g/100g total FA) (50). The case was the same for infant cord measurements at a mean of 4.9 g/100g total FA in our study and 7.77 g/100g total FA reported by Novak et al. (50). It is well known that RBC-PL-DHA is higher than plasma-PL-DHA in groups that have been studied previously, so it is not clear why the values found by Novak et al. were so high for plasma. There may have been a problem with their analysis since they also were unable to analyze DHA in most of their plasma samples (80 of 97).

Association Between Iron and Cognitive Outcomes

When we considered all women regardless of iron status, we did not find a significant relationship between the BSID scores of children and the iron status of their mothers when assessed by sTfR or BI, which we consider the most relevant markers of iron status in this study and as a result of the reviewed literature. We did however find a significant relationship between iron status and MDI scores

with Hb concentrations in the first and third trimester (p<0.05). This was not weighed heavily due to the criticism of the fluctuation of hemoglobin and poor reflection of iron status during pregnancy, due to in part to increased plasma volume (8, 10, 48). As well, hemoglobin during pregnancy is not a primary marker of assessment in the literature (10, 48). Postpartum SF was the only iron status marker significantly correlated with PDI scores (p<0.05). This outcome was not weighed heavily either, as SF has been criticized as a poorly sensitive marker of ID during pregnancy, although still seen in the literature (8). Few studies have examined maternal iron status or iron supplementation alone in relation to cognitive outcomes of offspring.

Our results are somewhat similar to a study assessing maternal iron supplementation and child outcomes (37). In the study by Zhou et al., a randomized trial of prenatal iron supplementation did not show a significant effect of supplementation compared to placebo on IQ scores of children at 4 years of age (37). However, it should be noted that this study used SF < 12 μg/L as the primary ID indicator, while also assessing IDA by way of hemoglobin and SF which could not be done in our study (37). While we did see a significant negative correlation between maternal SF and offspring PDI, when a t-test was run differentiating the PDI scores based on ID according to SF, a significant difference in PDI scores was not seen with our cutoff of SF <15 μg/L. When adjusted to SF <12 μg/L for comparison to the results of Zhou et al., a significant difference was still not observed in the mean PDI scores of our population.

Although the children were assessed at an older age, BSID scores at 18 months do correlate modestly with IQ scores later in childhood (37).

Association Between DHA and Cognitive Outcomes

We found that the BSID MDI in children did not differ by the DHA status of their mothers but that BSID PDI in children was significantly negatively correlated ($p < 0.05$) with maternal RBC-PL-DHA, while this difference was not observed with maternal plasma-PL-DHA. Current research has failed to find significant relationships between maternal DHA supplementation and infant cognition. To the best of our knowledge, our study is the first to identify a significant negative relationship between lower maternal DHA status and a lower outcome on the BSID PDI in children. This is suggestive of a negative effect of maternal DHA status on PDI outcomes of offspring. However, when a t-test was run on the PDI scores based on a median split differentiating RBC-PL-DHA, a significant difference was not observed. With the exception of our observation in relation to PDI scores and low RBC-PL-DHA, our results are consisted with the current research in finding no other relationships with DHA status and cognitive outcomes, especially on the MDI.

Although we assessed DHA status based on a median split rather than viewing the supplemented and placebo groups separately in our population, half of the women were supplemented with DHA and we expect that supplemented women constitute the majority of the higher DHA group. The study is still masked to all but the study Principal Investigators so we need not attempt to identify who received a supplement and who did not. Makrides et al. randomized

a large number of Austrailian pregnant women (n=2,399) to received either 800 mg DHA per day or placebo from <21 weeks gestation until delivery and the BSID-III was assessed in the offspring at 18 months (n=726) (34). Because the BSID-III contains more testing divisions than the BSID-II, direct comparison of our BSID results to those of Makrides et al. is not possible (34). However, no significant differences in BSID outcomes were observed between the supplemented and supplemented groups, leading to the conclusion that maternal DHA supplementation does not improve infant performance on the BSID at 18 months of age (34). That said, the BSID may not be the ideal indicator to evaluate early cognitive function. More sensitive measures, and measures targeted to the effects of DHA on brain function have been proposed. In addition, it has been proposed that the effects of DHA on cognitive function may not fully emerge until later in childhood (1).

Potential Interaction Between Iron and DHA and Relationship to Cognitive Outcomes

In 2006, a review by Rioux et al. suggested that large, long-term studies on pregnant women were in need to better understand individual and dual roles of iron and DHA on respective infant cognitive outcomes (43). Since then, the same primary author has attempted to study the relationship between the two nutrients with infant cognition (1). Our study appears to be the only one to examine both maternal iron and DHA status in relation to infant cognition other than Rioux et al. These investigators did not combine DHA and iron status in analysis with BSID outcomes. While Rioux et al. did not supplement pregnant

women, outcomes on the BSID-II at 6 months of age were not associated with maternal iron or DHA status between 28 and 32 weeks gestation (1). As noted, the iron status of our maternal population is difficult to compare to that of Rioux et al. because we have a larger percentage of women with ID based on Hb and a smaller percentage of women with ID based on SF. Rioux et al. did not measure sTfR or BI, which may be better indicators for iron status during pregnancy. Also previously noted, the maternal DHA status was lower than that in our population, and in fact all could be considered to have low DHA status because they were not supplemented with DHA.

In examining children of women who were ID at delivery (sTfR ≥8.4 mg/L), we saw a nonsignificant trend toward higher BSID MDI in women with a DHA status above the mean. Because there were so few ID women in each DHA group (9 and 10 in the lower and higher DHA groups, respectively), the results suggest a type II error. Offspring of the group of women with ID and low DHA status had an 8-point lower mean MDI scores compared to women with ID but higher DHA status. This was observed even in comparison to those women with normal iron status, regardless of DHA status. This may suggest a protective effect of DHA on cognitive outcomes when maternal iron status is low. It is likely that a significant protective effect of DHA was missed due to the low sample size of mothers with ID.

At least one study has attempted to establish a relationship between supplementation with iron and DHA to establish a relationship between the nutritional status of children regarding the two nutrients and their cognitive

outcomes at an older age. Although these children were from a different country and were between the ages of 6-11 years, ultimately a different outcome in the potential relationship between the two nutrients and cognition was found between our studies (2). Baumgartner et al. observed potential associations between child supplementation with iron or DHA/EPA, or the combined nutrients, in a population of ID and n-3 FA-deficient children, and found that DHA/EPA supplementation in these children had a negative outcome on cognition in children with anemia and girls with ID (2). This study prompted us to evaluate the effect of higher DHA status in conjunction with ID in pregnancy in relation to child cognitive function. As noted above, rather than seeing any suggestion of harm, our data support a benefit of DHA supplementation to women who are ID during pregnancy.

Correlations with BSID Outcomes

MDI was significantly correlated with maternal education, income and the PPVT score, a means of evaluating parent IQ. A negative relationship was observed between parity and the MDI. It was thought that these factors could be related to the outcomes seen on the MDI in comparison to maternal iron and DHA status. Outcomes of ANOVAs run with maternal education, income and PPVT as covariates to the MDI showed the relationships to still be nonsignificant.

Association Between DHA and Iron Status

Our results showed that maternal DHA status might have an effect on maternal iron status. Higher RBC-PL-DHA status was significantly related to higher BI concentration in the mothers of our study population, although this

difference was not observed when DHA was analyzed in accordance to sTfR status, perhaps because more women had ID by BI than by sTfR. Because women with a higher DHA should represent predominantly women who received DHA supplements, this finding suggests that DHA might actually improve iron status. So far we do not have a plausible mechanism to suggest, however a similar result was observed in baboons supplemented with different amounts of DHA and ARA compared to placebo with neither DHA nor ARA. While a potential interaction between the two indicators is suggested here, we note that BI is not yet a validated marker of iron status during pregnancy. To our knowledge, the relationship between BI and DHA has not been observed in other studies examining the two nutrients in accordance with one another.

Physician Recommended Iron Supplementation

Not all mothers with a postpartum ID based on sTfR or BI were advised to take iron during pregnancy. Of the women who were prescribed additional iron that were included among the 191 mother infant dyads studied here, 50 women were advised to take an iron supplement. At delivery, 14 of the 50 had ID. The remaining 36 women did not, but this may be because they were given an iron supplement.

Limitations

Although this data collection from previous research has been improved over time, limitations still exist in this project. Due to delivery at outlying hospitals, cord bloods were not obtained for all pregnancies for which we have maternal blood at delivery. Also, mother infant dyads who had blood drawn but did not complete the BSID appointment are excluded, therefore limiting the sample size of this study. Another limitation to this study is a lack of previous work in pregnant women to clarify the best biomarkers for ID and a lack of consistency in the literature, which limits our ability to compare the results of our study with those of others.

Chapter 6

SUMMARY

Our sample population appears to have similar iron status to that of US women. However, inconsistency among use of appropriate iron markers made it difficult to compare the iron status of our population to that of other countries. The DHA status of our maternal population was also relatively similar to that reported in the literature for US women when we looked at ID and DHA status prior to supplementation.

Outcomes between individual maternal iron and DHA status on infant cognition do not seem to differ significantly from the limited findings available for review, however, we found a potential association between maternal ID and DHA status in relation to infant cognition. We found a potentially positive and protective effect of DHA when maternal iron status is low. We observed a significantly negative correlation with maternal RBC-PL-DHA status and offspring PDI outcomes, however when analyzed based on the median split of our population, a difference in the means was not significant.

Future research is needed to assess proper iron status markers during pregnancy on mother and child, while validation of BI as an assessment tool during this timeframe should be considered. Further studies including a larger number of women with ID, while also paying close attention to DHA status, are needed to assess the relationship between iron and DHA status on respective infant cognitive outcomes.

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APPENDIX A

CONSENT FORM FOR KU DHA OUTCOMES STUDY (KUDOS)

CONSENT FORM
The Effects of DHA on Pregnancy and Infant Outcome
(Kansas University DHA Outcomes Study or KUDOS)

Sponsor: NIH (1R01 HD047315)

INTRODUCTION

As a pregnant woman who is between 8 and 20 weeks of gestation, you are being invited to enroll in a research study of a nutrient (DHA) that is a component of normal brain and important for brain development. The centers involved in the study are the University of Kansas Medical Center in Kansas City, Kansas, St. Luke's Hospital in Kansas City, Missouri, and Truman Medical Center in Kansas City, Missouri. If you decide to enroll in this study, your baby will participate in research procedures at the University of Kansas Medical Center. Dr. Susan Carlson is the main investigator for this study. A total of 350 pregnant women will be enrolled in this study between October 2005 and January 2010.

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

BACKGROUND

Docosahexaenoic acid (DHA) is a fat that is found in very large amounts in the brain. DHA is important for how my baby sees and learns. Breast milk and, since 2002, US formulas contain DHA. Many studies have shown that DHA in the diet helps the baby's vision, attention, and ability to learn. In this way, DHA is considered an important nutrient for babies after they are born.

DHA may also be important before babies are born. Four studies found that women's DHA during pregnancy was related to higher infant/child function. These studies are called observational studies, meaning that the women's normal DHA status was studied in relation to development of the baby/child. There is only one study that gave women DHA during pregnancy and measured development of their babies/children. That study showed higher IQ at 4 years of age in children whose mothers took fish oil capsules during the last 6 months of pregnancy. (Fish oil contains a lot of DHA). However, because women in the study also consumed DHA while they were breastfeeding they provided more DHA to their babies after they were born. Therefore, the study does not prove that giving DHA before babies are born will help their development. There are no studies that have varied DHA intake only during pregnancy. You and your child are being asked to participate in such an experimental study.

PURPOSE

The purpose of this study is to determine if a dietary supplement of DHA during pregnancy will help babies be born at the right time and help their development. If you decide to be in the study, you will have a 50-50 chance of receiving capsules with the supplement of DHA or ordinary food oil, which does not contain any DHA.

PROCEDURES

If you choose to enroll yourself and your infant in this study, the investigators will record some information from your medical record about your pregnancy and medical history. They will also ask you a few questions about foods that you usually eat. You will have a blood sample collected from a vein in your arm. One-half teaspoon of blood will be drawn. The blood will be used to measure DHA in your blood as well as other nutrients. You will be asked to provide a current address and phone number where you can be contacted. .

During pregnancy: You will be randomly assigned (like flipping a coin) to capsules with DHA-oil or ordinary food oil (which does not contain any DHA). The DHA-oil is the same oil that is used in US infant formulas and has been fed safely to millions of infants.

You will be given enough capsules each month to take 3 capsules each day and you agree to try to consume all 3 capsules. If you consume all 3 capsules, you will consume 600 mg of DHA. The capsules are relatively small and you should find them easier to swallow than many nutrient supplements. They are orange-flavored, so if you burp (common in pregnancy and in the first week of taking any nutrient supplement), the taste should not be unpleasant. You do not need to take the capsules at any specific time as they are a nutrient and not a drug. However, you should decide upon a regular time to take them so that taking the capsules will become a habit and you won't forget. For example, you might wish to take them just before you go to bed or when you have your first beverage of the day.

Neither you nor the investigators will know which capsules you have been assigned to. On the day you enroll for the study, we will send you home with your first bottle of capsules. About 30 days later (early enough so that you do not run out of capsule), you will receive another bottle of capsules in the mail. AT THAT TIME, YOU AGREE TO PLACE THE FIRST BOTTLE WITH ANY REMAINING CAPSULES IN THE ENVELOPE AND DROP IT INTO THE MAIL.

This process will be repeated each month until your baby is born and you will continue to take 3 capsules per day until your baby is born. Each time you receive a new bottle, you will mail back the bottle that you have been using and that day will open and begin using the new bottle.

The investigators will contact you by phone at least once per month. They will ask about capsule intake and they will ask how you are doing. Maintaining contact with our study personnel on a monthly basis is very important.

IF YOUR PHONE NUMBER OR ADDRESS CHANGES AT ANY TIME DURING THE STUDY, YOU WILL LET THE INVESTIGATORS KNOW BY CALLING 913-588-3781 AND LEAVING A MESSAGE.

Delivery: After you are admitted to the hospital to deliver, you should telephone study personnel or ask the person at admitting to telephone them. You will be given a cell phone number today to call. Once you deliver your baby, the investigators will visit you in the hospital to collect data about your delivery and your baby's health. A sample of your baby's cord blood will be collected after delivery by nurses at the hospital and given to the investigators. A nurse will also draw a small blood sample (one-half teaspoon) from you while you are in the hospital. The blood samples will be used to measure DHA and other nutrients. The investigators will visit you, and give you an appointment for your baby's first follow-up visit at KUMC.

Visit 1 (6 weeks of age): The investigators will measure how your baby sees using a test that involves placing 3 electrodes directly on your baby's head. The process involves cleaning the area then placing a small amount of paste similar to toothpaste on the head. The electrodes are placed on top of the paste. The electrodes will be used to record your baby's brain waves while he/she is looking at pictures. Your child's weight, height and head circumference will be measured again and you will be asked questions about what your baby eats. If you are breastfeeding your baby, you will be asked to provide a teaspoon of breast milk to the investigator. The sample will be frozen and analyzed for fats that are found in the capsules. The visit should last about 40 minutes. You should arrive on time and allow that amount of time for the visit.

Visit 2 (4 months of age): The investigators will measure how your baby sees using the same test as before and another vision test. Your baby will wear a pair of plastic glasses during the second test. In another test, your child will be given an object to look at several times. The investigator will measure how long he/she looks at the object and how quickly he/she stops looking at the object. Your child will be video recorded during the test. Your baby's heart rate will be measured during the test. Your baby's height, weight and head circumference will be measured and you will be asked about what food your baby eats. Your baby will have a blood sample collected by either heel stick or drawn from a vein. If it is necessary to use a heel stick, the investigator may use a cream or spray that will numb the area before obtaining the sample. One-half teaspoon of blood will be drawn. The blood will be used to measure DHA and other nutrients. You should let the investigator know if your baby has been sick or not acting well since his/her last visit. The visit will take 60-90 minutes.

Visit 3 (6 months of age): The investigators will measure how your baby sees using the test that requires him/her to wear a pair of plastic glasses. In another test, he/she will be given an object to look at several times (just like at 4 months of age). The investigator will measure how long he/she looks at the object and how quickly he/she stops looking at the object. Your child will be video recorded during the test. Your baby's heart rate will be measured during the test. Your baby's height, weight and head circumference will be measured. You will be asked questions about what your baby eats. You should let the investigator know if your baby has been sick or not acting well since his/her last visit. The visit should take 40 -60 minutes.

Visit 4 (9 months of age): Your baby will have both tests that measure how he/she sees. In another test, your child will be given an object to look at several times (just like at 4 and 6 months of age). The investigator will measure how long he/she looks at the object and how quickly he/she stops looking at the object and your baby's heart rate will be measured during the test. Your child will be video recorded during the test. Your baby's height, weight and head circumference will be measured. You will be asked questions about what your baby eats. You should let the investigator know if your baby has been sick or not acting well since his/her last visit. The visit should take about 40-60 minutes.

Visit 5 (10 months of age): During this visit your baby will be placed on your lap in front of a small table. A test will be completed with a small toy, foam block and 2 cloths that will be placed in front of your child. You will also take a short language test. The small toy will be given to your child to keep. In another test, your baby will be asked to take turns with the researcher building fun toys. After your baby has played for a moment with the pieces, the researcher will show him or her how to build the toy. Then, your baby will be given a turn to put the toy together. Your baby's turn will happen either immediately or after 10-minutes of play with other things. Your child will be video recorded during the tests. You should let the investigator know if your baby has been sick or not acting well since his/her last visit. You will be asked questions about what your baby eats. The entire 10-month visit should last 65 - 70 minutes.

Visit 6 (12 months of age): The investigators will measure how your baby sees using both vision tests. Your child will be video recorded while playing with an interesting toy and the investigator will use the recording to measure some aspects of attention. Your child's height, weight and head circumference will be measured. You will be asked questions about what your baby eats. You should let the investigator know if your child has been sick or not acting well since his/her last visit. The visit should take about 2 hours. It is important that your child be rested before the testing at this visit. If for some reason your baby cannot finish the tests that day – this may happen if he/she is unusually fussy or tired – you will be asked to return to finish the remaining tests within 7 days.

Visit 7 (18 months of age): The investigators will measure how your child sees using the test that he/she had while wearing plastic glasses. Your child will be video recorded while playing with an interesting toy and the investigator will use the recording to measure some aspects of attention. Your child will also be given a standardized test to measure mental and physical development. Your child's height, weight and head circumference will be measured. You will be asked questions about what your baby eats. You will be asked questions about the words your child uses and understands. You should let the investigator know if your child has been sick or not acting well since his/her last visit. The visit should take about 2 hours. It is important that your child be rested before the testing at this visit. If for some reason your child cannot finish the tests that day – this may happen if he/she is unusually fussy or tired – you will be asked to return to finish the remaining tests within 7 days.

RISKS
Some redness, soreness, or bruising may occur at the site of blood sampling. There is also a very slight risk of infection.

You may experience burping from the capsules and find this unpleasant

There are no known risks of consuming the amount of DHA you will be provided if you receive the DHA. Even if you forget to take your capsules for one or two days, there is no known risk of deciding to "catch up" on the third day. The amount is smaller than pregnant women in many countries eat every day. Nevertheless, you could develop a problem that has not been observed before.

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 participate or to allow your child to participate in this study.

BENEFITS
You and your child may or may not benefit from participating in this study**.** If you receive the supplement, it may help your baby to be born at the right time and your baby's/child's development**.** If you will not get the supplement, your baby and you will not be getting any of those benefits. It is also possible that all infants/children will get some benefit from being followed closely with developmental testing. It is hoped that additional information gained in this research study may be useful in understanding if DHA can help your baby be born at the right time and help your baby's vision, attention, and learning as he or she grows. You will receive a video recording of your infant doing the 4, 6, and 9 month looking test when the 12 month visit is complete.

ALTERNATIVES
You do not have to participate in this study to be able to take DHA supplements while you are pregnant. You may purchase capsules containing DHA at local stores without a prescription (for example, Osco, Costco, Wal-Mart). There are also several brands of prenatal supplements with DHA available by prescription or over the counter. The prenatal capsules typically contain 200 mg of DHA each and are marketed to take one capsule/day as a DHA supplement.

COSTS
Capsules containing either DHA or food oil will be provided to you at no cost while you are participating in this study. You will not incur any costs because of your or your child's participation

PAYMENT TO SUBJECTS
If study investigators are able to communicate with you each month you will be given 2 bonus gift cards to either Wal-Mart or Target of $25 each. The first gift card will be given to you half way through your treatment phase if communication is maintained at least one time each month during the first half of your treatment. The second gift card will be

given at delivery if communication maintained at least one time each month during the second half of your treatment.

Additionally, if the study investigators are called after you are admitted for delivery you will be given your choice of a bonus gift card worth $50 from either Wal-Mart or Target. You may make the call yourself or have someone else call for you. Study personnel will give you the gift card when they come to the hospital after your baby is born.

Once your baby is born, you will receive a check for $50 after your baby completes each of the following visits: 6 weeks, 4 months, 6 months, 9 months, and 10 months. You will receive a check for $100 after your child completes each of the following visits: 12 and 18 months. The reimbursements are to cover the costs of transportation and to partially compensate you for your time required to participate in the study**.**

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.

IN THE EVENT OF INJURY

In the event you experience any serious health problem (hospitalization, life-threatening illness, or death) for any reason during your pregnancy, you should immediately seek treatment or help in the way you normally would as if you were not in a study. You should let Susan Carlson, Ph.D. know about any of these problems as soon as possible by calling her office (913-588-5359) or the study office (913-588-3781). A message may be left at both numbers. Dr. Carlson may also be reached at home (816-960-1805).

INSTITUTIONAL DISCLAIMER STATEMENT

If you believe you have been injured as a result of participating in research at Kansas University Medical Center (KUMC), you should contact the Director, Human Research Protection Program, Mail Stop #1032, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160. Compensation to persons who are injured as a result of participating in research at KUMC may be available, under certain conditions, as determined by state law or the Kansas Tort Claims Act.

Truman Medical Center (TMC) will provide medical attention to you if you suffer any injury or harm as a direct result of participating in this research project. TMC, your study doctor, and the sponsor of this study will decide, at their discretion, who should pay for the medical care. TMC will provide treatment to you in the event of any medical emergency while present at TMC, whatever the cause. Moreover, you will have the benefit of the coverage of any existing healthy insurance you own. Participation in this research study does not take the place of routine physical examinations or clinic visits to your person physician. If you believe you have been injured as a result of participating in this study you are encouraged to contact the study investigator, Dr. Susan Carlson, at her work number, 913-588-5359.

The University of Missouri-Kansas City appreciates the participation of people who help

it carry out its function of developing knowledge through research. Although it is not the University's policy to compensate or provide medical treatment for persons who participate in studies, if you think you have been injured as a result of participating in this study, please call the investigator, Dr. Susan Carlson at 913-588-5359 (work) or Sheila Anderman, IRB administrator of UMKC's Adult Health Sciences Institutional Review Board at 816-235-6150

CONFIDENTIALITY AND PRIVACY AUTHORIZATION

Names of subjects or information identifying subjects will not be released without written permission unless required by law. Videotapes of your baby when he/she is looking at pictures and playing with toys will be used only by the investigators and their students and to make a videotape copy for you. The videotapes will be secured under lock and key like all other information that could be linked directly to your child. The videotape of your child will not be shown without specific permission from you and even then would not identify your child by name. Efforts will be made to keep you and 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 you and/or your baby will be removed.

The privacy of you and 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 participate in this study, you will be asked to give permission for researchers to use and disclose your and your baby's health information that is relevant to the study.

To perform this study, researchers will collect health information about me and my child from his/her and my medical records and from the study activities that are listed in the Procedures section of this consent form. My and my baby's study-related health information will be used at KU Medical Center by Dr. Carlson, members of the research team, Truman Medical Center, St. Luke's Hospital and the KU Hospital Medical Record Department. The KUMC Research Institute as well as officials at Truman Medical Center and St. Luke's Hospital that oversee research, including the KUMC Human Subjects Committee, the IRB that governs Truman Medical Center and St. Luke's Hospital and other committees and offices that review and monitor research studies, may also see my and my baby's study-related health information.

Dr. Carlson and her team may share information about me and my baby with representatives of Martek Biosciences, the monitoring company who verifies study data, the laboratory that processes study lab samples, other business partners who help with 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). Martek Biosciences (Columbia, MD) donated the capsules for this study that is otherwise supported by the National Institute of Child Health and Human Development.

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

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

If I enroll in the study, the investigators cannot tell me what capsule I was assigned to until the study ends. This may be after I have stopped taking the capsules.

QUESTIONS

I have read the information in this form. Dr. Carlson or her associates have answered my question(s) to my sati,action. I know if I have any more questions after signing this I may contact Dr. Carlson or one of her associates at (913) 588-5359. If I have any questions about my or my child's rights as a research subject, I 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

My and my child's participation in this study is voluntary and the choice to not 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 my child receives now or in the future at the University of Kansas Medical center. The entire study may be discontinued for any reason without my consent by the investigator conducting the study, by the sponsor of the study, or the FDA. My child's participation can be discontinued by the investigator or by the sponsor if it is felt to be in my child's best interest or if I do not follow the study requirements. If I choose to withdraw before my child is 18 months of age, I may be asked to answer questions about the study on the telephone.

If I want to cancel permission to use my or my child's health information, I should send a written request to Dr. Carlson. The mailing address is Susan Carlson, Ph.D., Dept. of Dietetics and Nutrition, MS 4013, 4019 Delp, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. If I cancel permission to use my child's health information, the research team will stop collecting any additional information about me and my child.

Should the study be terminated prior to the completion of my pregnancy, neither the investigator nor the University of Kansas Medical Center will be under any obligation to provide me with DHA capsules used in the study.

CONSENT

Dr. Carlson or her associates have given me 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, I give my permission for my and my child's health information to be used and disclosed for the purposes of this research study. If I choose not to sign this form, my child and I will not be able to participate in the study.

I voluntarily consent to my and my child's participation in this research study. I have read the information in this form and have had an opportunity to ask questions and have them answered. ***I will be given a copy of the signed form to keep for my records.***

_____
Type/Print Subject's Name

_____ _____ _____
Signature of Subject Time Date

_____
Type/Print Name of Person Obtaining Consent

_____ _____
Signature of Person Obtaining Consent Date

_____
Type/Print Name of Principal Investigator

_____ _____
Signature of Principle Investigator Date

May the investigators contact you after the study is over to ask if you interested in continuing your child's participation? If you agree to be contacted, the investigators would explain any new study to you later and you would have the chance to decide if you wanted to participate at that time (please circle your response).

Yes

No

_____
Type/Print Subject's Name

_____ _____ _____
Signature of Subject Time Date

_____
Type/Print Name of Person Obtaining Consent

_____ _____
Signature of Person Obtaining Consent Date

_____
Type/Print Name of Principal Investigator

_____ _____
Signature of Principle Investigator Date