

**INFLUENCE OF FADS1 AND FADS2 GENOTYPES ON MATERNAL DOCOSAHEXAENOIC
ACID AND INFANT DEVELOPMENTAL STATUS**

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degree of Doctor of Philosophy

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ABSTRACT

FADS1 and FADS2 encode the rate-limiting enzymes responsible for arachidonic acid (ARA) and docosahexaenoic acid (DHA) synthesis. Single nucleotide polymorphisms (SNPs) in FADS1 and FADS2 influence the proportion of blood lipid and breast milk DHA, and breastfeeding confers an IQ-point advantage to children carrying the major allele for a SNP in FADS2. Previous studies have not examined the interaction between FADS genotypes and DHA supplementation, controlled for maternal DHA status to isolate the effect of FADS SNPs on breast milk DHA, or established whether maternal FADS genotypes influence infant cognition. This series of studies aimed to (1) elucidate the effect of DHA supplementation and FADS1 rs174553 and FADS2 rs174575 genotypes on red blood cell (RBC) ARA and DHA in a cohort of pregnant women, (2) determine if SNPs in maternal FADS1 and FADS2 influence the proportion of breast-milk DHA after controlling for the proportion of DHA in maternal RBCs, and (3) determine if toddler performance on the Bayley Scales of Infant Development Mental Development Index (BSID MDI) at 18 months is predicted by either maternal or child genotype in breastfed and formula-fed infants. The study population consisted of a subset of women enrolled in an NICHD-funded Phase-III clinical trial designed to determine the effects of consuming 600 mg/day of DHA throughout gestation on maternal and infant/toddler outcomes. Women provided blood and breast-milk samples the morning after and six weeks following parturition, respectively. Milk- and RBC-DHA were quantified by gas chromatography in comparison with weighed standards. Genomic DNA was extracted from buccal collection brushes, and genotyping performed

with TaqMan SNP Genotyping Assays. MDI was assessed at 18 months of age. FADS1 minor allele homozygotes had a lower proportion of RBC-ARA and DHA than major-allele carriers ($P \leq 0.027$) at enrollment. At delivery, minor allele homozygotes in the placebo group had a lower RBC-DHA than major-allele carriers ($P \leq 0.031$), whereas women in the treatment group had similar RBC-DHA regardless of genotype ($P = 0.941$). Both FADS minor alleles were related to lower ARA among women assigned to the treatment group ($P \leq 0.029$). RBC-ARA was not reduced in major allele homozygotes ($P = 0.899$). The concentration of breast-milk DHA was higher among women assigned to the treatment group than those assigned to the placebo ($P < 0.001$). However, when controlling for RBC-DHA to eliminate the influence of DHA supplementation and dietary intake, FADS2 minor allele homozygotes had a lower proportion of breast-milk DHA than major-allele carriers ($P = 0.033$). MDI was not related to maternal FADS1 or FADS2 genotypes. Finally, breastfed (but not formula-fed) infants carrying two copies of the FADS2 minor allele had a lower MDI at 18 months than major allele carriers ($P = 0.007$). Together, these results suggest that DHA supplementation compensates for the lower proportion RBC-DHA observed among FADS1 minor-allele homozygotes, but exaggerates the supplementation-associated reduction in RBC-ARA among FADS minor-allele carriers. They support the hypothesis that polymorphisms in FADS2 affect DHA in breast milk and confirm the previous observation that the FADS2 rs174575 genotype of the infant moderates the association between breastfeeding and a measure of cognition.

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CHAPTER ONE:

INTRODUCTION

LONG CHAIN POLYUNSATURATED FATTY ACIDS

Polyunsaturated fatty acids (PUFAs) are essential constituents of all biological systems. They serve as critical components of cellular membranes and regulate multiple physiological processes. During critical periods of development, dietary-induced perturbations in PUFA homeostasis and metabolism have been linked to alterations in neurotransmitter systems (1), abnormalities in inflammatory (2) and synaptic (3) signaling, and neurocognitive deficits (4). In addition, a growing body of evidence suggests that the composition of PUFAs in blood and tissue phospholipids is implicated in the pathophysiology of several diseases, including coronary heart disease (5), hepatic steatosis (6), rheumatoid arthritis (7), and psychiatric disorders such as major depression, bipolar disorder, and schizophrenia (8-10).

The effects of PUFAs on the aforementioned conditions are thought to be mediated primarily by long-chain polyunsaturated fatty acids (LC-PUFAs) (11, 12), lipids with at least 20 carbon atoms and 3 double bonds, such as arachidonic acid (ARA; 20:4n-6) and docosahexaenoic acid (DHA; 22:6n-3). Humans are not able to synthesize fatty acids with double bonds located 3 (n-3) or 6 (n-6) carbon atoms from the methyl terminus. Thus, LC-PUFAs must be provided directly by the diet or via their essential dietary precursors, α -linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6).

LC-PUFAs modulate the integrity and fluidity of cell membranes (13), act as second messengers in intracellular signaling pathways (14, 15), regulate gene transcription of proteins involved in lipid metabolism (16-20), and serve as precursors for the synthesis of prostaglandins, thromboxanes, and leukotrienes (21). During

development, an adequate concentration of LC-PUFAs in neuronal cell membranes is essential for efficient neurogenesis (22), neurite outgrowth (23), myelination (24), dendritic maturation (25), and neurotransmission (26).

IMPORTANCE OF DHA AND ARA IN FETAL DEVELOPMENT AND INFANT COGNITION

DHA and ARA are arguably the most important LC-PUFAs in animals. While they serve numerous essential functions throughout the lifespan (27), their effects are most notable during critical periods of fetal growth and in infancy, where they play an indispensable role in the maturation of the visual system and cognitive development (28).

DHA is essential for optimal neuronal development of the fetus (29-35). It is the most abundant (n-3) fatty acid in the mammalian brain and typically accounts for 25-33% of the total membrane aminophospholipids [phosphatidylethanolamine (PE) and phosphatidylserine (PS)] in the gray matter and 40-50% of the aminophospholipids in the visual elements of the retina (29-33, 36-39). In humans, the most rapid rates of brain DHA accumulation occur during the last intrauterine trimester and the first 6-10 months after birth (29, 30, 38, 40). This corresponds to the period of time in which brain growth is at peak velocity and suggests that the third trimester fetus and newborn infant are particularly susceptible to developmental deficits when maternal intake of DHA is limited (29, 30, 36). The critical role of DHA in neurogenesis, however, suggests that adverse effects of inadequate DHA in early gestation are also important (36).

Prior to birth, DHA is provided by placental transfer and accumulates in the fetal brain in a manner that is dependent on maternal status (31). Several observational studies in humans have linked higher intrauterine DHA exposure to a number of positive developmental outcomes, such as improved cognitive and visual function in children (29, 30, 41), while animal models provide evidence that early DHA exposure may influence and program dopaminergic (42-45), serotonergic (43, 46), cholinergic (47), and γ -amino butyric acid neurotransmitter systems (48). Similarly, postnatal supplementation has shown benefits on the Brunet-Lezine Scale (49, 50), Bayley Scales of Infant Development (51), and Weschler Primary Preschool Scale of Intelligence (52), and a recent randomized, controlled trial found that postnatal DHA supplementation lowered infant heart rate and increased sustained attention at 4, 6, and 9 months of age (53).

Conversely, a dietary deficiency of (n-3) fatty acids has been shown to decrease brain and retinal DHA, impair neurogenesis, reduce learning ability, alter emotional reactivity, decrease the kinetics of the visual photocycle, and alter gene expression and neurotransmitter metabolism (31, 36, 54). Behaviors observed in nonhuman primates with reduced brain DHA accumulation include altered electroretinogram (ERG) responses and lower visual acuity (55), changes in attention suggestive of slower brain maturation (56), a higher frequency of stereotyped behavior (57), and increased locomotor activity indicative of behavioral reactivity (57).

In contrast to DHA, ARA is considered important for fetal and infant growth (58, 59) and is currently added to US infant formulas with DHA. ARA is found in phospholipids throughout the body and serves as a precursor for eicosanoids pivotal in

numerous immunological and inflammatory pathways (31, 39, 60-62). Eicosanoid metabolism is complex. Although early work considered eicosanoids derived from eicosapentaenoic acid as anti-inflammatory and those derived from ARA as pro-inflammatory, recent advances have demonstrated that ARA-derived lipoxins are important in the resolution of inflammation (63).

A recent study demonstrated that a dietary deficiency of ARA results in reduced growth, reproductive failure, skin and hair changes, and abnormal liver pathology (64). The extent of the developmental effect appears to be related to one's maturational stage and is influenced by the concentration and ratio of DHA to ARA in the tissue (64). Long chain n-3 fatty acids lower ARA by inhibiting the conversion of linoleic acid to ARA and competing for acylation into phospholipids (36). Thus, an appropriate balance of ARA and DHA is important to support normal growth, immune function, and neuronal development (36). When given in combination with DHA supplementation, dietary ARA exhibits limited beneficial effect on brain development and function (51). However, some evidence suggests that low ARA status may be involved in the development of neuromental disorders such as schizophrenia (65).

PLACENTAL TRANSFER OF DHA AND ARA

Intrauterine life constitutes a particularly vulnerable period of brain development, as the fetus is entirely dependent upon the maternal supply of nutrients for growth (30, 31). While the fetus can synthesize some saturated and monounsaturated fatty acids *de novo* from glucose, the DHA and ARA required for fetal development must be provided

by placental transfer (29, 31, 40) because the placenta lacks the $\Delta 5$ and 6 desaturase enzymes required for conversion of essential fatty acids to LC-PUFAs (66), and the fetus has only limited desaturase activity (67). (Please refer to the section entitled “Endogenous Synthesis of DHA and ARA” for a detailed discussion of ARA and DHA synthesis from their dietary precursors, α -linolenic acid and linoleic acid, respectively.)

The transfer of DHA and ARA across the placenta involves a multi-step process of uptake and translocation facilitated by fatty acid binding proteins, such as fatty acid transport protein 4 (FATP-4), fatty acid translocase, and the plasma membrane fatty acid-binding protein (FABPpm) (41, 68). Although the endogenous synthesis of DHA and ARA is likely to be higher in preterm than in term infants (69), the amount of DHA and ARA produced from their dietary precursor is insufficient to match the rate of in utero accretion (70), providing further evidence that placental transfer serves as the primary source of these important LC-PUFAs in fetal development.

The proportions of DHA and ARA differ significantly in maternal and fetal circulation. Specifically, the proportions of DHA and ARA are higher in cord than in maternal plasma phospholipids (71, 72). This phenomenon, referred to as “biomagnification,” gave rise to the hypothesis that DHA and ARA are transferred preferentially across the human placenta to support their accretion in nervous tissue during periods of rapid brain growth (73, 74). Indeed, DHA and ARA accumulate in the fetal brain in a manner that is dependent on maternal status (31), and observational and intervention studies concur that higher dietary intake of DHA and ARA during pregnancy results in an increased maternal-to-fetal transfer of DHA and ARA (31).

ENDOGENOUS SYNTHESIS OF DHA AND ARA

While DHA and ARA can be provided directly by the diet via animal fats, such as fish, fish oils, and specialty egg and dairy products, they are also synthesized endogenously from their essential dietary precursors, linoleic acid (18:2 n-6) and α -linolenic acid (18:3n-3), respectively (31, 36, 37, 39). The conversion pathway consists of a succession of desaturations and elongations in the endoplasmic reticulum and in one terminal cycle of β -oxidation in the peroxisomes (31, 33, 36, 37, 39) (**Figure 1.1**). DHA may also be synthesized through the same pathway from an upstream metabolic precursor abundant in fat fishes and marine products, eicosapentaenoic acid (EPA, 20:5n-3) (37).

Two key enzymes, Δ -5 and Δ -6 desaturase, encoded by FADS1 and FADS2, respectively, are thought to govern the rate of endogenous DHA and ARA synthesis (**Figure 1.1**) (31, 36, 37, 39). Although both Δ -5 and Δ -6 desaturase are expressed in the majority of human tissues, the highest concentrations are found in the liver, brain, heart, and lung (75, 76), and the liver serves as the primary site of conversion.

FADS1 and FADS2 are located in a cluster on chromosome 11 (11q12-13.1) with head-to-head orientation (**Figure 1.2**). The first exons of FADS1 and FADS2 are separated by an 11-kb region, and each contains 12 exons and 11 introns (75-78). Figure 1.2 depicts the position of the exons, hypothetical promoter regions, and hypothetical transcription factor binding sites for the Δ -5 and Δ -6 desaturase genes (79).

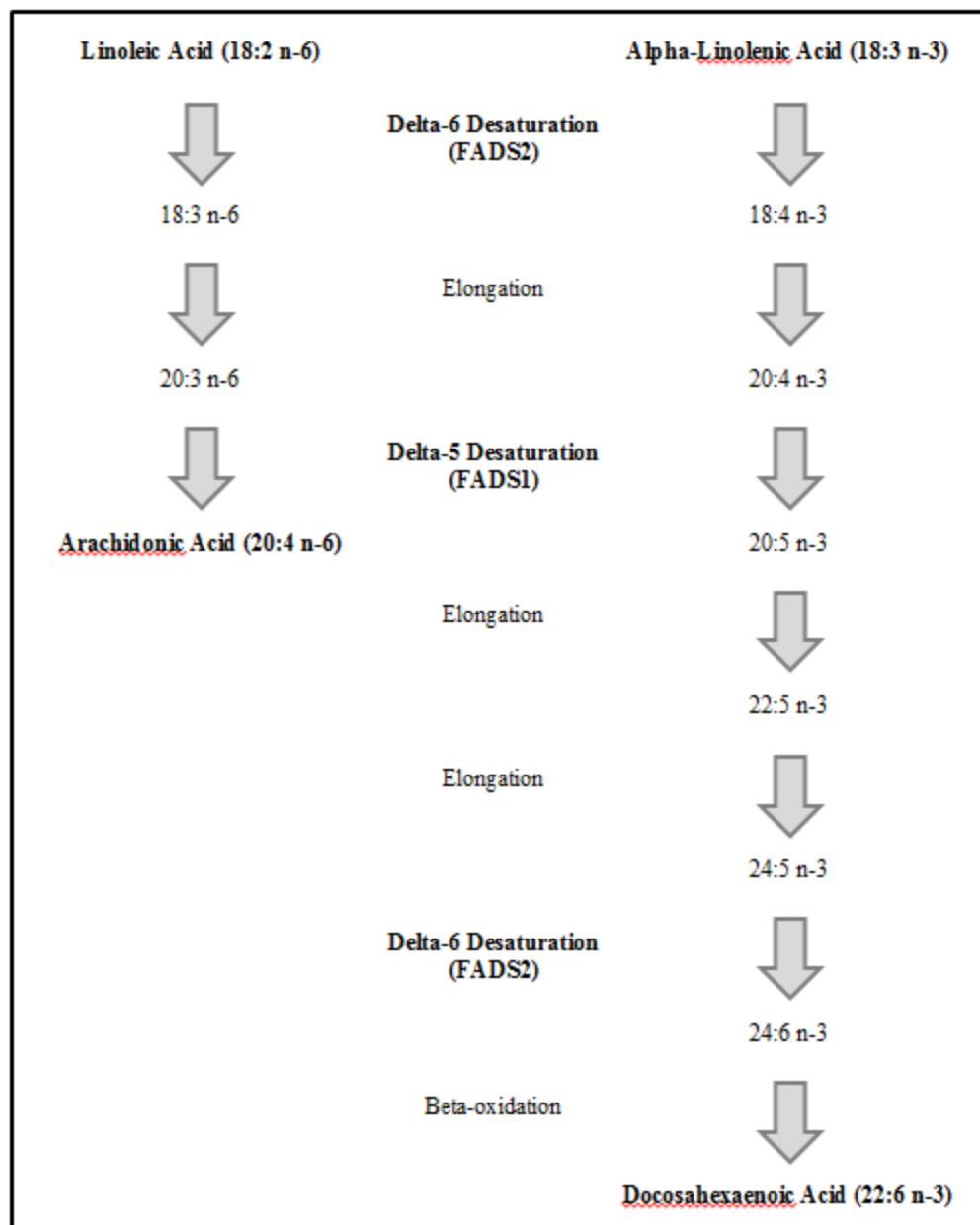


Figure 1.1. Mammalian pathway of endogenous arachidonic acid and docosahexaenoic acid synthesis from essential dietary precursors, linoleic acid and α -linolenic acid.

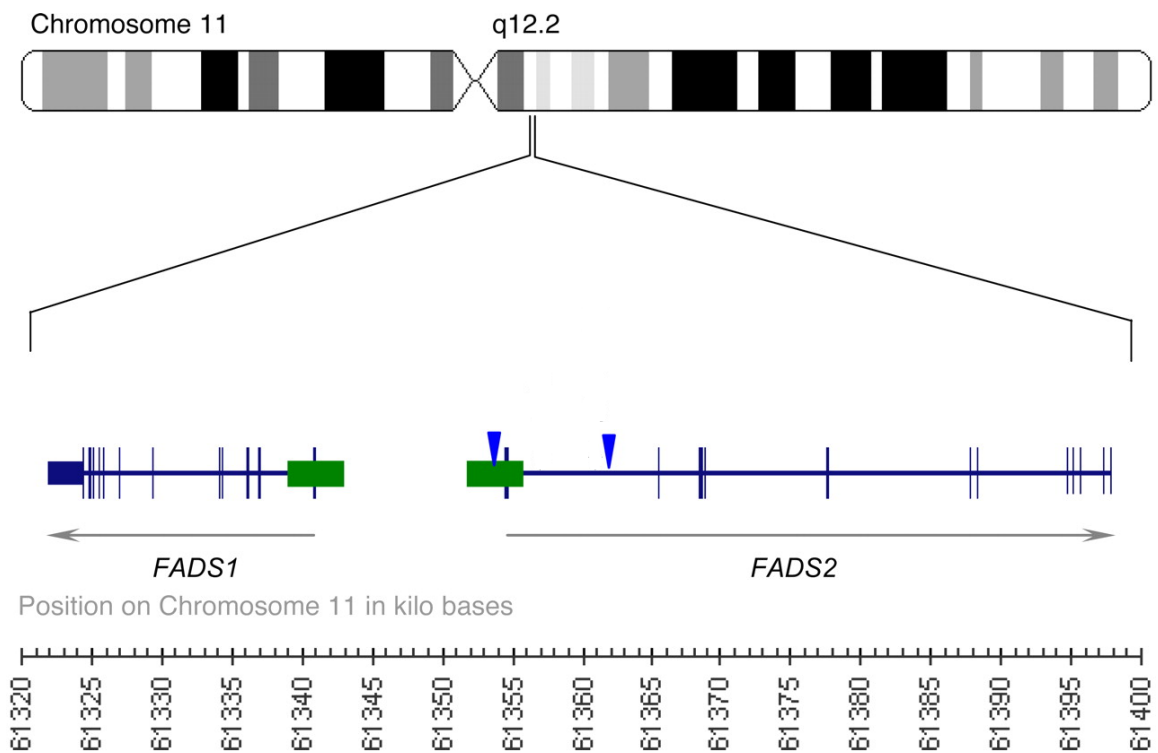


Figure 1.2. The FADS1 and FADS2 gene structure on chromosome 11q12.2. The figure depicts the position of the exons (vertical blue lines), hypothetical promoter regions (green rectangles), and hypothetical transcription factor binding sites (blue arrowheads) (79). Adapted from Caspi *et al* (80).

IMPORTANCE OF THE FATTY ACID DESATURASE GENES: FADS1 AND FADS2

It is well established that single nucleotide polymorphisms (SNPs) in the FADS1 and FADS2 gene cluster influence fatty acid composition in adult populations, with minor allele carriers having lower product to precursor ratios and reduced proportions of ARA and DHA in plasma and red blood cell (RBC) phospholipids (81-87). For example, Koletzko *et al.* explored the relation between 17 SNPs in the FADS gene cluster and the composition of RBC fatty acids in more than 4000 pregnant women participating in the Avon Longitudinal Study of Parents and Children (82). Independent of dietary effects, FADS minor alleles were consistently positively associated with precursor fatty acids and negatively associated with LC-PUFAs and product:substrate ratios of n-6 and n-3 pathways (82). Similarly, Xie and Innis found that minor allele homozygotes of rs174553 (G/G), rs99780 (T/T), and rs174583 (T/T) have a lower proportion of ARA, but higher linolenic acid in plasma phospholipids and erythrocyte ethanolamine phosphoglyceride and decreased n-6 and n-3 fatty acid product to precursor ratios at 16 and 36 weeks of gestation (85). Together, these results demonstrate that FADS1 and FADS2 genotypes influence the proportions of DHA and ARA in maternal phospholipids and may affect the supply of DHA to the growing fetus.

Previous studies have also demonstrated that SNPs in FADS1 and FADS2 influence the proportion of breast-milk DHA (82, 83, 85). After birth, human milk and supplemented formulas serve as the primary source of DHA and ARA. Several observational studies in humans have linked breastfeeding to positive developmental outcomes (88-91), and breast-fed infants have a greater proportion of erythrocyte- and

cortical-DHA relative to those fed with unsupplemented formulas (92). While breastfeeding is often correlated with a more favorable socioeconomic environment, a recent randomized, controlled trial found that postnatal DHA supplementation in infant formula lowers infant heart rate and increases sustained attention, independent of environmental factors (53). This suggests a significant dose-response relationship exists between infant cognition and postnatal, dietary exposure to DHA.

Interestingly, one study observed that the proportion of DHA in plasma phospholipids increases with dietary intake, irrespective of the genotype, while DHA proportions in milk increase only in FADS major-allele carriers (83). Caspi *et al.* found that breastfeeding confers a 6.4 to 7.0-IQ-point advantage only among children carrying the major allele for a SNP in FADS2 (80). This suggests that genetic variations in FADS may confer particular benefits of breastfeeding among some children.

Similar to most known polymorphisms, the frequency of FADS minor alleles differs according to race, and new reports indicate that SNPs in the FADS gene cluster may contribute to health disparities between populations of European and African descent (93, 94). For example, some recent studies have linked FADS minor alleles to an increased incidence of asthma, allergic rhinitis, and atopic eczema in pediatric populations (95-97), and others have demonstrated that an association may exist between FADS alleles, intelligence (80, 98), and attention-deficit/hyperactivity disorder (99). Thus, it is important to account for the influence of FADS polymorphisms on DHA and ARA status among studies examining racial differences in LC-PUFA status.

PURPOSE OF DISSERTATION

Continued research regarding the influence of FADS SNPs on maternal LC-PUFA status and outcomes in pediatric populations is warranted. The goal of the present project was to elucidate the influence of maternal FADS SNPs on DHA and ARA status in breast milk and maternal phospholipids and determine whether maternal or infant polymorphisms are predictive of a measure of early developmental status.

CHAPTER TWO:

DOCOSAHEXAENOIC ACID (DHA) SUPPLEMENTATION DIFFERENTIALLY MODULATES ARACHIDONIC ACID AND DHA STATUS ACROSS FADS GENOTYPES IN PREGNANCY

ABSTRACT

FADS1 and FADS2 encode the rate-limiting enzymes responsible for arachidonic acid (ARA) and docosahexaenoic acid (DHA) synthesis. FADS1 and FADS2 influence the proportions of ARA and/or DHA in plasma and red blood cell (RBC) phospholipids, but previous studies have not examined the interaction between FADS genotypes and DHA supplementation. This study aimed to elucidate the effect of DHA supplementation and FADS1 and FADS2 genotypes on RBC-ARA and DHA in a cohort of pregnant women. Women enrolled in a trial designed to determine the effects of consuming 600 mg/day of DHA throughout gestation on maternal and infant/toddler outcomes provided blood at enrollment and the morning following parturition. RBC-ARA and DHA were quantified by gas chromatography. Genomic DNA was extracted from buccal collection brushes and genotyping performed with TaqMan SNP Genotyping Assays. FADS1 minor allele homozygotes had a lower proportion of RBC-ARA and DHA than major-allele carriers ($P \leq 0.027$) at enrollment. At delivery, minor allele homozygotes in the placebo group had a lower RBC-DHA than major-allele carriers ($P \leq 0.031$), whereas women in the treatment group had similar RBC-DHA regardless of genotype ($P = 0.941$). Both FADS minor alleles were related to lower ARA among women assigned to the treatment group ($P \leq 0.029$), RBC-ARA was not reduced in major allele homozygotes ($P = 0.899$). DHA supplementation appears to compensate for the lower proportion RBC-DHA observed among FADS1 minor-allele homozygotes, but exaggerates the supplementation-associated reduction in RBC-ARA among FADS minor-allele carriers.

INTRODUCTION

The long chain polyunsaturated fatty acids, docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are important constituents of neural tissue and play an indispensable role in cognitive and visual development (28). While ARA and DHA can be provided directly by the diet via animal fats, they are also synthesized endogenously from essential dietary precursors, linoleic acid (18:2 n-6) and α -linolenic acid (18:3n-3), respectively. The conversion pathway consists of a succession of desaturations and elongations, and two key enzymes, Δ -5 and Δ -6 desaturase (encoded by FADS1 and FADS2, respectively) are thought to govern their rate of synthesis (**Figure 1.1**). FADS1 and 2 are located in a cluster on chromosome 11 (11q12-13.1) with head-to-head orientation. Both Δ -5 and Δ -6 desaturase are expressed in the majority of human tissues, but the highest concentrations are found in the liver, brain, heart, and lung (75, 76).

It is well established that single nucleotide polymorphisms (SNPs) in FADS1 and 2 influence fatty acid composition in adult populations, with minor allele carriers having lower product to precursor ratios and reduced proportions of ARA and DHA in plasma and red blood cell (RBC) phospholipids (81-86). The frequency of FADS minor alleles differs according to race and may contribute to health disparities between populations of European and African descent (93, 94). The impact of FADS genetic variants on LC-PUFA metabolism, specifically ARA levels, appears to be more pronounced in African Americans due to the larger proportion of individuals carrying the genotype associated with increased FADS1 enzymatic conversion of dihomo-gamma-linolenic acid to ARA

(93, 94). It is thought that these genetic differences may account for the observation that multifactorial diseases of chronic inflammation tend to disproportionately affect African Americans in industrialized settings (94).

Recent studies have linked FADS minor alleles to an increased incidence of asthma, allergic rhinitis, and atopic eczema in pediatric populations (95-97). Others have demonstrated that an association may exist between FADS alleles, intelligence (80, 98), and attention-deficit/hyperactivity disorder (99). To our knowledge, no studies have been conducted to examine the interaction between FADS genotypes and DHA supplementation, and it is not known if supplementation is able to compensate for the observed reduction in DHA status among minor allele carriers. This study aimed to determine if DHA supplementation modulates RBC-ARA and DHA across FADS1 rs174553 and FADS2 rs174575 genotypes in a cohort of pregnant women.

SUBJECTS AND METHODS

SUBJECTS

The study population consisted of a subset of women enrolled in an NICHD-funded Phase-III clinical trial (NCT00266825), designed to determine the effects of consuming 600 mg/day of DHA throughout gestation on maternal and infant/toddler outcomes. A total of 350 women were enrolled in the trial. Those who provided both blood and DNA samples were included in the current analysis (**Figure 2.1**). Women were eligible for enrollment if they were English-speaking, between 16 to 35.99 years of

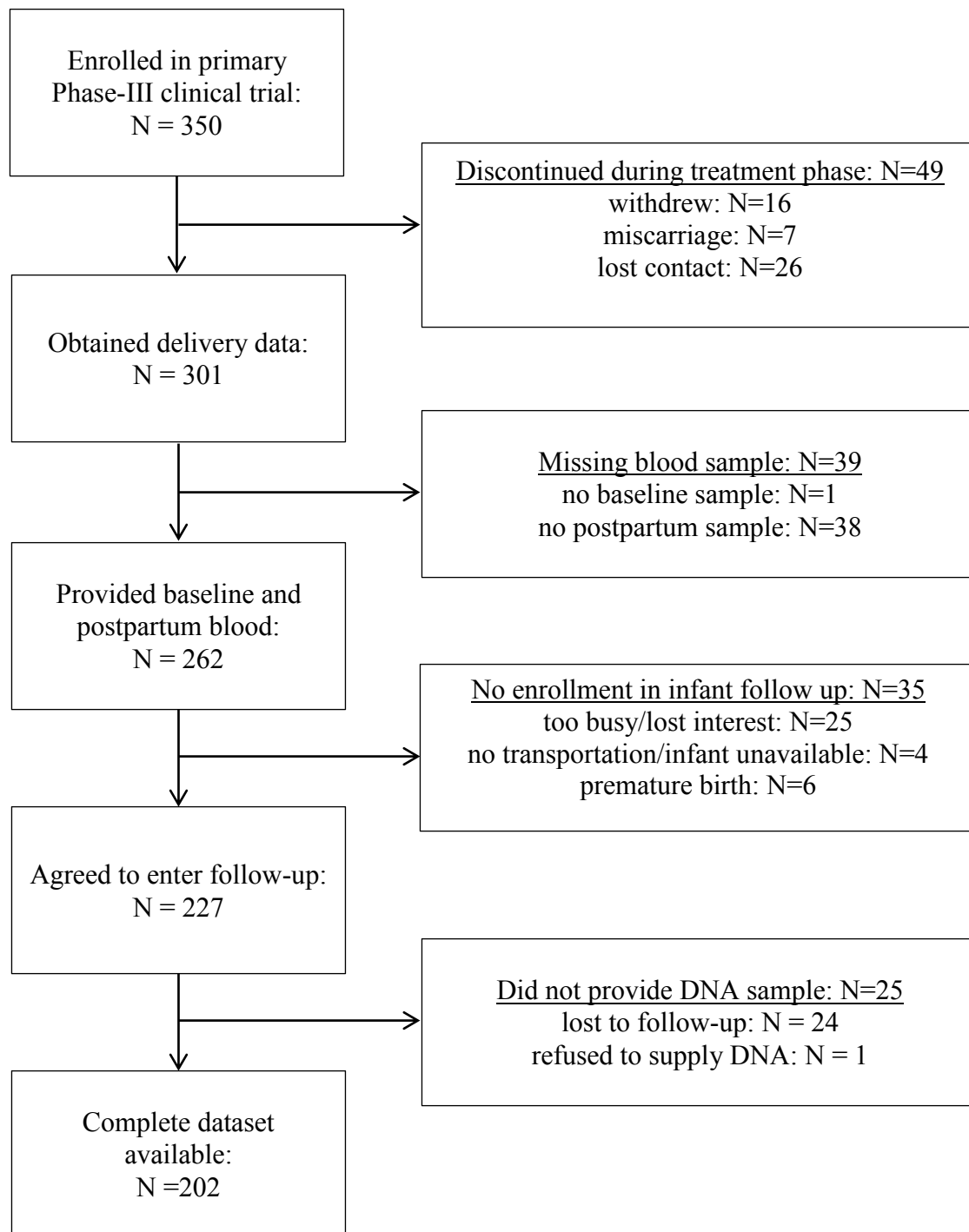


Figure 2.1. Consort flow diagram depicting subjects included in the current analysis.

age, and in their 8th to 20th week of gestation. Subjects were excluded if they were expecting multiple infants or had any serious health condition likely to affect the growth and development of their fetus or the postnatal growth and development of their newborn infants. This included, but was not limited to, subjects with cancer, lupus, hepatitis, HIV/AIDS, and those with pre-pregnancy or gestational diabetes mellitus at enrollment. As morbid obesity and elevated blood pressure present a high risk for co-morbid conditions independent of and including obstetric complications, women were also excluded if they had a baseline BMI ≥ 40 or systolic blood pressure ≥ 140 mm Hg. Subject characteristics, including maternal age, race, and education, were obtained via questionnaire at enrollment (**Table 2.1**). The research protocol and informed consent forms adhered to the Declaration of Helsinki (including the October 1996 amendment) and were approved by the Institutional Review Board/ethics committee at the participating institution, the University of Kansas Medical Center (HSC #10186).

SUPPLEMENTATION

Women assigned to the treatment group received capsules of a marine algae oil source of DHA (DHASCO, Martek Biosciences, Columbia, MD) (200 mg DHA/capsule), while those in the control group received capsules containing half soybean and half corn oil (Martek Biosciences, Columbia, MD). All subjects were asked to consume three 500 mg capsules daily throughout gestation. While the soybean and corn oil combination did not contain DHA, each capsule provided 20 mg of α -linolenic acid. Thus, the consumption of 3 control capsules could theoretically result in the synthesis of

Table 2.1: Subset and population characteristics¹

	Subset (N = 205)	Population (N = 299)	P-Value
Mean Age at Enrollment (years \pm SD)	26.3 \pm 4.7	25.4 \pm 4.8	NS
Education at Enrollment (years \pm SD)	14.3 \pm 2.8	13.8 \pm 2.8	NS
Maternal Race			NS
Caucasian (%)	68.66	59.9	
African American (%)	29.85	39.1	
Other (%)	1.49	1.0	
Average Weekly Capsule Intake (# \pm SD)	17.3 \pm 4.1	16.2 \pm 5.0	0.013

¹Values were analyzed using Student's *t*-test; NS, not significant

approximately 8 mg of DHA (100). As this value is far below that provided by the treatment capsules (600 mg DHA/day), the potential conversion of α -linolenic acid to DHA was not considered a limitation of the study design.

A monthly supply of capsules was mailed directly to subjects. A self-addressed, stamped envelope was also provided to return any remaining capsules from the previous month. The Investigational Pharmacy recorded the number of remaining capsules, and the weekly and overall capsule intake of each subject was calculated at the end of the treatment phase.

ANALYSIS OF RED BLOOD CELL DHA AND ARA

Women provided blood samples at enrollment and the morning following parturition. Blood samples were collected by venipuncture into 2 mL K₂EDTA tubes (BD Vacutainer, Franklin Lakes, NJ). Plasma and RBC were separated by centrifugation (3000×g, 10 minutes; 4°C), frozen, and stored under nitrogen at –80°C until analysis. Lipids were isolated according to a modification of the Folch protocol (101), and RBC lipids were fractionated (102) by thin-layer chromatography. RBC phospholipids were transmethyated with boron trifluoride-methanol (103), and the resulting fatty acid methyl esters (FAME) were separated using a Varian 3900 gas chromatograph with an SP-2560 capillary column (100 m, Sigma Aldrich) and a Star 6.41 Chromatography Workstation for peak integration and analysis as previously reported (104). Injector and detector temperatures were programmed at 260° C. The temperature program for the 41-minute column run was: 140° C, 5 minutes; 4° C increase/minute to 240° C; 240° C, 11 minutes.

Individual peaks were identified by comparison with a qualitative standard (PUFA No. 1 Marine Source 100 mg; PUFA No. 2 Animal Source 100 mg; Sigma Aldrich) and a weighed standard mixture (Supelco 37 Component FAME mix, Sigma Aldrich) was employed to determine a final weight percent of total fatty acids.

GENOTYPING

FADS1 rs174553 and FADS2 rs174575 SNPs were selected among those previously studied because of their relatively common minor allele frequencies (33% and 24%, respectively) and observed association with blood lipid and breast milk DHA (82, 83, 85). Genomic DNA was extracted from buccal collection brushes using the Gentra Puregene Buccal Cell Kit (QIAGEN, Hilden, Germany), and genotyping was performed with made-to-order TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) using real-time polymerase chain reaction (PCR). Five-microliter total reactions were prepared according to manufacturer instructions, and individual genotypes were determined with StepOne Software (Version 2.0; Applied Biosystems).

STATISTICAL ANALYSIS

The present study group was compared to the original cohort using Student's *t*-test. One-way ANOVA was used to compare RBC-DHA and ARA across maternal FADS genotypes in samples collected at enrollment and in postpartum samples from women assigned to the placebo group. When indicated, Fisher's Least Significant

Difference (LSD) was used to conduct pairwise comparisons. To control for the effect of variable adherence to the prescribed DHA supplementation regimen in the treatment group, ANCOVA was used to compare RBC-DHA and ARA across maternal FADS genotypes in postpartum samples from women assigned to the treatment group, with average weekly capsule intake serving as the covariate. Although the frequency of FADS minor alleles differs between individuals of European and African descent (93, 94), race was not included as a covariate in the present analyses. This would have introduced multicollinearity into the model and dramatically reduced our power to observe differences in RBC-DHA and ARA across maternal genotypes. Before conducting each ANCOVA, preliminary analyses were performed to evaluate the homogeneity-of-regression (slopes) assumption. When the ANCOVA was significant, follow-up tests using contrast coefficients (L' Matrix) were conducted to evaluate pairwise differences among genotypes. Model assumptions were examined using the Kolmogorov-Smirnov test, Shapiro-Wilk test, and Levene's Test of Equality of Error Variances. All data were analyzed with SPSS Statistics 17.0 software (SPSS, Chicago, IL), and P -values ≤ 0.05 were considered significant.

RESULTS

Compared to the population from which this study originates, the subset of women included in the current analysis consumed, on average, a greater number of capsules per week ($P = 0.013$) (**Table 2.1**). No differences in maternal race, age, and education at enrollment were noted (**Table 1**). The observed genotypic and minor allele

frequencies for each SNP are provided in **Table 2.2**. The normality and homogeneity of variance assumptions were satisfied, and the preliminary analysis evaluating the homogeneity-of-regression (slopes) assumption indicated that the relationship between average weekly capsule intake and postpartum RBC-DHA and ARA did not differ significantly as a function of genotype ($P = 0.421$ and 0.519 for FADS1 vs, DHA and ARA, respectively; $P = 0.449$ and 0.827 for FADS2 vs, DHA and ARA, respectively.)

At enrollment, FADS1 rs174553 genotype significantly influenced both RBC-DHA ($P = 0.035$) and ARA ($P = 0.002$) (**Table 2.3**). Specifically, minor allele homozygotes had a lower proportion of RBC-DHA than major allele homozygotes and heterozygotes ($P = 0.010$ and 0.027 , respectively), and minor-allele carriers had a lower proportion of RBC-ARA than major allele homozygotes ($P = 0.009$ and 0.003 for A/G and G/G, respectively). FADS2 rs174575 genotype was unrelated to RBC-DHA ($P = 0.164$) or ARA ($P = 0.300$) at enrollment (**Table 3**).

At delivery, minor allele homozygotes of FADS1 in the placebo group had a lower proportion of RBC-DHA than major-allele carriers ($P = 0.005$ and 0.031 for A/A and A/G, respectively), whereas women in the treatment group had similar RBC-DHA regardless of genotype ($P = 0.941$) (**Figure 2.2A**). In contrast, FADS1 genotype did not influence RBC-ARA in the placebo group ($P = 0.215$), but was related to lower ARA in those assigned to the treatment group ($P = 0.001$) (**Figure 2.2B**). Specifically, heterozygotes and minor allele homozygotes had a lower proportion of RBC-ARA than major allele homozygotes ($P = 0.008$ and 0.001 , respectively). In this case, minor allele homozygotes also had a lower proportion of RBC-ARA than heterozygotes ($P = 0.044$).

Table 2.2: Maternal FADS1 rs174553 and FADS2 rs174575 genotypic frequencies¹

Gene	SNP	M/m Alleles	Genotype (Number of Subjects)		MAF	
FADS1	rs174553	A/G	A/A (109)	A/G (79)	G/G (17)	0.28
FADS2	rs174575	C/G	C/C (110)	C/G (79)	G/G (16)	0.27

¹ SNP, single nucleotide polymorphism; M/m, major/minor alleles; MAF, minor allele frequency

Table 2.3: Mean (\pm SE) RBC-ARA and DHA across FADS genotypes at enrollment¹

	FADS1 rs174553 [*]			FADS2 rs174575		
	A/A	A/G	G/G	C/C	C/G	G/G
RBC-DHA (%)	4.47 \pm 0.10 ^a	4.37 \pm 0.15 ^a	3.67 \pm 0.29 ^b	4.41 \pm 0.11	4.41 \pm 0.13	3.83 \pm 0.26
RBC-ARA (%)	15.31 \pm 0.21 ^a	14.53 \pm 0.22 ^b	13.76 \pm 0.30 ^b	15.05 \pm 0.20	14.78 \pm 0.23	14.25 \pm 0.34

¹ARA, arachidonic acid; DHA, docosahexaenoic acid; RBC, red blood cell

^{*}Columns bearing different letters are significantly different ($P < 0.05$; ANOVA, Fisher's Least Significant Difference).

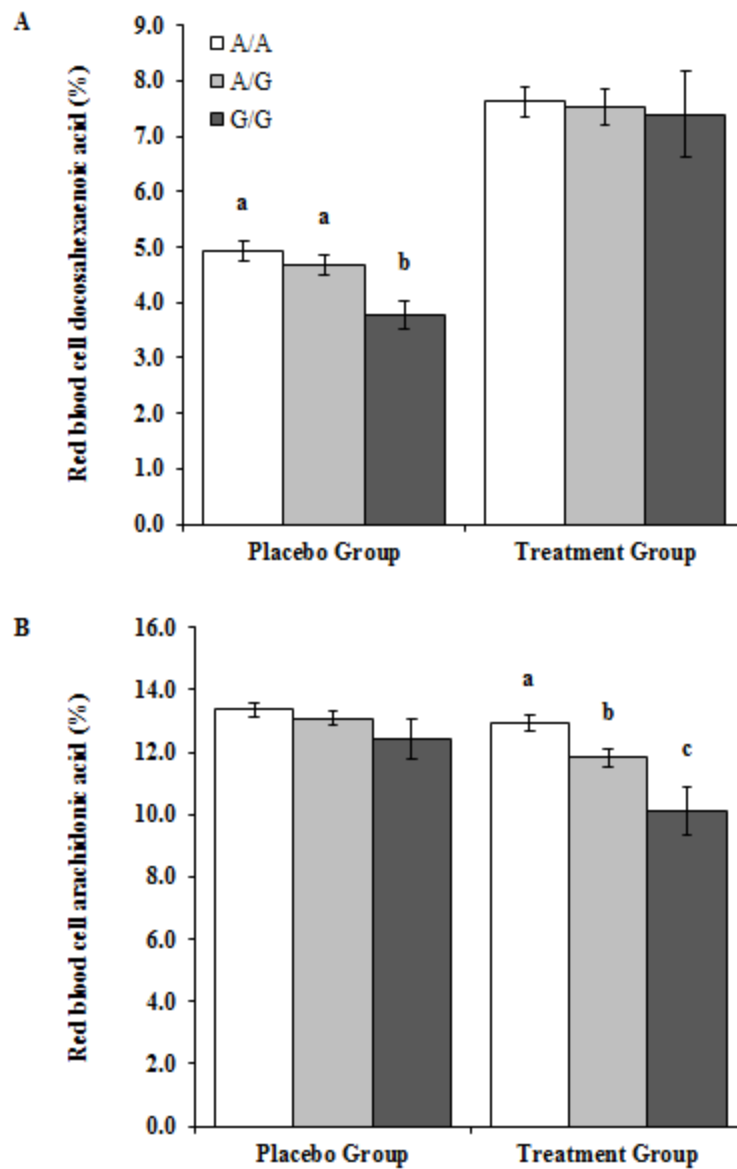


Figure 2.2. Proportion of (A) docosahexaenoic acid and (B) arachidonic acid in RBC phospholipids across FADS1 rs174553 genotypes at delivery. Columns bearing different letters are significantly different ($P < 0.05$; ANOVA/ANCOVA, Fisher's Least Significant Difference). Bars represent means \pm SE.

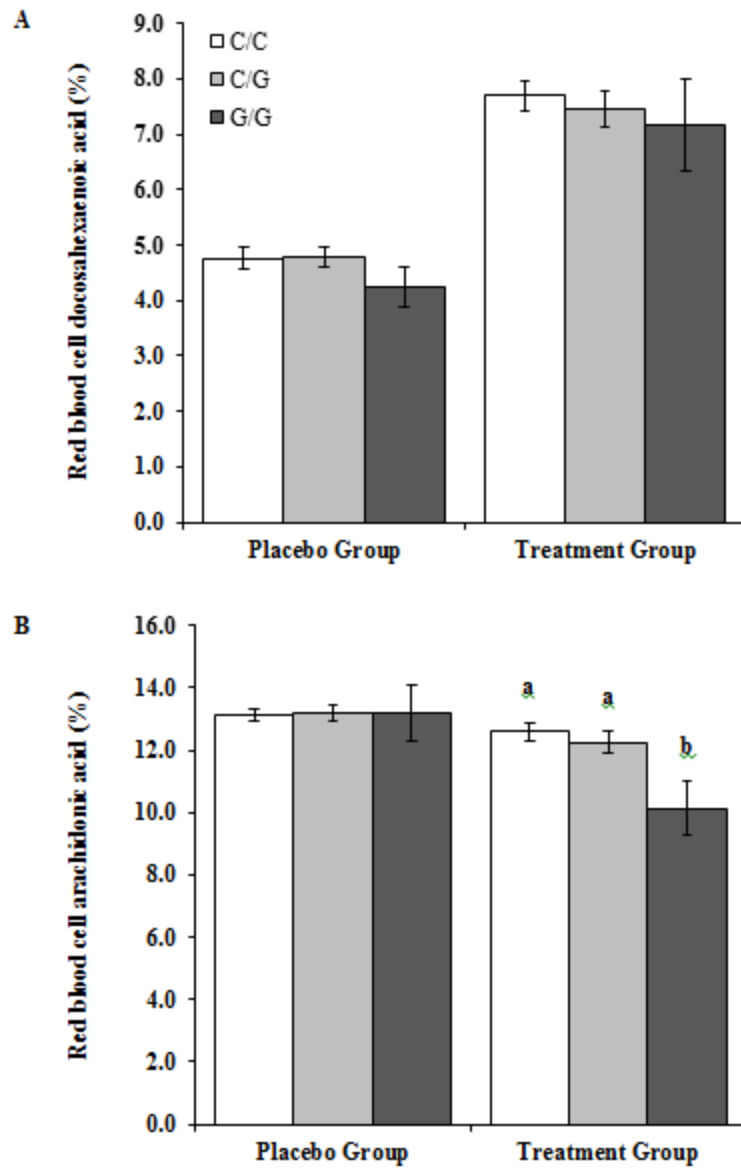


Figure 2.3. Proportion of (A) docosahexaenoic acid and (B) arachidonic acid in RBC phospholipids across FADS2 rs174575 genotypes at delivery. Columns bearing different letters are significantly different ($P < 0.05$; ANCOVA, Fisher's Least Significant Difference). Bars represent means \pm SE.

Compared to the placebo group, RBC-ARA was not reduced in major allele homozygotes assigned to the treatment group ($P = 0.899$).

At delivery, FADS2 genotype did not influence RBC-DHA in women assigned to the placebo ($P = 0.403$) or treatment ($P = 0.754$) groups (**Figure 2.3A**). Analogous to FADS1, FADS2 genotype also did not influence RBC-ARA in the placebo group ($P = 0.972$), but was related to lower ARA among women assigned to the treatment group ($P = 0.029$) (**Figure 2.3B**). Minor allele homozygotes had a lower proportion of ARA than major allele homozygotes ($P = 0.008$) and heterozygotes ($P = 0.023$). Again, RBC-ARA was not reduced in major allele homozygotes in the treatment group when compared to that in the placebo group ($P = 0.125$).

DISCUSSION

To our knowledge, this study is the first to examine the interaction between FADS genotypes and DHA supplementation in pregnant women. We show that DHA supplementation increases RBC-DHA to similar proportions, regardless of FADS1 rs174553 genotype (**Figure 3A**) and amplifies the supplementation-associated reduction in RBC-ARA among FADS minor-allele carriers (**Figures 3B and 4B**). For the first time, we show that FADS major allele homozygotes do not experience a reduction in RBC-ARA with DHA supplementation.

Similar to the findings of previous studies (84, 85), we found that the FADS1 rs174553 minor allele decreases the proportion of DHA and ARA in maternal RBC phospholipids (**Figure 3**). Moltó-Puigmartí *et al.* recently observed an association

between FADS2 rs174575 genotype and the proportion of plasma phospholipid DHA and ARA (83). Although not significant, a similar trend was observed with minor allele homozygotes having a lower proportion of RBC-DHA and ARA (**Figure 4**).

A number of studies have revealed a reduction in the concentration of plasma and erythrocyte ARA with DHA supplementation (105-108). Interestingly, we found that FADS minor alleles exaggerated the observed supplementation-associated reduction in RBC-ARA in a dose-response manner, whereas RBC-ARA was not reduced among FADS major allele homozygotes (**Figures 5B and 6B**). In light of a recent animal study designed to examine the effect of dietary α -linolenic acid on FADS expression (109), it is possible that DHA supplementation decreases the expression of the Δ -5 and Δ -6 desaturase, further reducing ARA synthesis. Specifically, the researchers found that a diet containing very low levels of PUFAs elevated the expression of FADS2 relative to that with higher PUFA diets (109). A recent study in preterm infants utilizing stable isotope technology provides supporting evidence to this hypothesis (110). Compared to infants fed a formula devoid of LC-PUFAs, those fed 0.97% and 0.64% n-6 and n-3 LCPUFAs by weight (111) showed a dramatic reduction in endogenous LC-PUFA synthesis by 7 months of age (110). Thus, it seems likely that substrate availability plays an important role in the regulation of FADS gene expression. Our findings suggest that individuals more susceptible to reduced enzymatic function (FADS minor allele carriers) are more affected by supplementation in contrast to major allele homozygotes.

A limitation of this study is that it used a sample of convenience from a trial powered to examine the influence of prenatal DHA supplementation on birth outcomes.

Few women had two copies of the minor allele for the genes examined. Although we found highly significant differences, small sample size is a potential concern..

The results of the present analysis have important implications for the design of future studies intended to assess the influence of DHA supplementation on maternal and infant outcomes. Overall, our results suggest DHA supplementation compensates for the lower proportion of RBC-DHA observed among FADS1 minor-allele homozygotes, but exaggerates the reduction in RBC-ARA among FADS1 and 2 minor-allele carriers. As the effects of reduced RBC-ARA on the growing fetus and child are not fully understood, it is possible that an optimal level of DHA supplementation exists, beyond which less advantageous outcomes are observed. ARA is considered important for fetal and infant growth and development (58, 59) and it is currently added to US infant formulas with DHA. When given in combination with DHA supplementation, there is limited evidence for a beneficial effect of ARA on brain development and function (51). However, low ARA status may be involved in the development of neuromental disorders such as schizophrenia (65). Geppert *et al.* recently investigated the effect of a fish oil/evening primrose oil blend (456 mg DHA, 72 mg eicosapentaenoic acid, and 353 mg γ -linolenic acid/day) on plasma fatty acid composition in non-pregnant women (108). The oil blend was well tolerated and increased plasma DHA without reducing the concentration of ARA in plasma phospholipids (108). If future studies demonstrate that the observed reduction in ARA status that accompanies DHA supplementation in minor-allele carriers adversely affects development, a fish oil/evening primrose oil blend may be a viable alternative. Future studies should elucidate the potential effects of reduced maternal

ARA status on pregnancy and developmental outcomes in infants. As the ideal level of intake is likely to be genotype-specific, studies should also include various FADS genotypes as covariates of projected outcomes.

CHAPTER 3:

FADS2 GENE VARIANT INFLUENCES THE PROPORTION OF DOCOSAHEXAENOIC ACID (DHA) IN HUMAN MILK

ABSTRACT

FADS1 and FADS2 encode the rate-limiting enzymes responsible for endogenous docosahexaenoic acid (DHA) synthesis. Single nucleotide polymorphisms (SNPs) in FADS1/2 influence the proportion of blood lipid and human milk DHA, and human milk feeding confers an IQ-point advantage to children carrying the major allele for a SNP in FADS2. Previous studies have not controlled for maternal DHA status to isolate the effect of FADS SNPs on human-milk DHA. This study aimed to determine if SNPs in maternal FADS1 rs174553 and FADS2 rs174575 alleles influence the proportion of human-milk DHA in a group of supplemented women with variable status, after controlling for the proportion of DHA in maternal red blood cells (RBCs). The study population consisted of a subset of women enrolled in an NICHD-funded Phase-III clinical trial designed to determine the effects of consuming 600 mg/day DHA throughout gestation on maternal and infant/toddler outcomes. Women provided blood and milk samples the morning after and six weeks following parturition, respectively. Milk- and RBC-DHA were quantified by gas chromatography in comparison with weighed standards. Genomic DNA was extracted from buccal collection brushes, and genotyping performed with TaqMan SNP Genotyping Assays. The concentration of milk DHA was higher among women assigned to the treatment group than those assigned to the placebo ($P < 0.001$). However, when controlling for RBC-DHA to eliminate the influence of DHA supplementation and dietary intake, FADS2 minor allele homozygotes had a lower proportion of milk DHA than major-allele carriers ($P = 0.033$). These results

support the hypothesis that polymorphisms in FADS2 affect DHA in human milk and may account for the observed IQ advantage among major-allele carriers.

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n-3) is an omega-3 polyunsaturated fatty acid that accumulates rapidly in the human brain during the last intrauterine trimester and the first 2 years of life (92, 112). Throughout gestation, DHA is provided by placental transfer and accumulates in the fetal brain in a manner that is dependent on maternal status (31). After birth, human milk and supplemented formulas serve as the primary source of this important fatty acid. Several observational studies in humans have linked breastfeeding to positive developmental outcomes (88-91), and human milk-fed infants have a greater proportion erythrocyte- and cortical-DHA relative to those fed with unsupplemented formulas (92). While breastfeeding is often correlated with a more favorable socioeconomic environment, a recent randomized, controlled trial found that postnatal DHA supplementation lowers infant heart rate and increases sustained attention, independent of environmental factors (53). This suggests a significant relationship exists between infant cognition and postnatal, dietary exposure to DHA.

While DHA can be provided directly by the diet via animal fats, it is also synthesized endogenously from its essential dietary precursor, α -linolenic acid (18:3n-3). The conversion pathway consists of a succession of desaturations and elongations, and two key enzymes, Δ -5 and Δ -6 desaturase (encoded by FADS1 and FADS2, respectively) are thought to govern the rate of synthesis (**Figure 1.1**). Previous studies have demonstrated single nucleotide polymorphisms (SNPs) in FADS1 and 2 influence the proportion of blood lipid and human milk DHA (82, 83, 85) and Caspi *et al.* found that human milk feeding confers a 6.4 to 7.0-IQ-point advantage only among children

carrying the major allele for a SNP in FADS2 (80). Interestingly, one study revealed that the proportion of DHA in plasma phospholipids increases with dietary intake, irrespective of the genotype, while DHA proportions in milk increase only in FADS major-allele carriers (83). While these findings suggest that genetic variation in FADS1 and FADS2 may affect the incorporation of DHA in human milk, these investigators have not controlled for the proportion of DHA in maternal red blood cells (RBCs), a reliable indicator of DHA status. Thus, it is possible that errors in self-reported dietary intake influenced the observed gene-diet interaction.

The objective of the present study was to determine if SNPs in maternal FADS1 rs174553 and FADS2 rs174575 influence the proportion of human-milk DHA in a group of women with variable status, after controlling for the proportion of DHA in maternal RBCs. Although we will effectively eliminate the influence of group assignment in our statistical analyses, strength of utilizing the present cohort for this objective is the wide range of intake achieved by DHA supplementation and variable compliance among subjects.

SUBJECTS AND METHODS

SUBJECTS

The study population consisted of a subset of women enrolled in an NICHD-funded Phase-III clinical trial (NCT00266825), designed to determine the effects of consuming 600 mg/day of DHA throughout gestation on maternal and infant/toddler outcomes. A total of 350 women were enrolled in the trial. Those who provided both

milk at 6 weeks postpartum and a DNA sample were included in the current analysis (n=103). Women were eligible for enrollment if they were English-speaking, between 16 to 35.99 years of age, and in their 8th to 20th week of gestation. Subjects were excluded if they were expecting multiple infants or had any serious health condition likely to affect the growth and development of their fetus or the postnatal growth and development of their newborn infants. This included, but was not limited to, subjects with cancer, lupus, hepatitis, HIV/AIDS, and those with pre-pregnancy or gestational diabetes mellitus at enrollment. As morbid obesity and elevated blood pressure present a high risk for co-morbid conditions independent of and including obstetric complications, women were also excluded if they had a baseline BMI ≥ 40 or systolic blood pressure ≥ 140 mm Hg. Subject characteristics, including maternal race and education, were obtained via questionnaire at enrollment. The research protocol and informed consent forms adhered to the Declaration of Helsinki (including the October 1996 amendment) and were approved by the Institutional Review Board/ethics committee at the University of Kansas Medical Center (HSC #10186).

SUPPLEMENTATION

Women assigned to the treatment group received capsules of a marine algae oil source of DHA (DHASCO, Martek Biosciences, Columbia, MD) (200 mg DHA/capsule), while those in the control group received capsules containing half soybean and half corn oil (Martek Biosciences, Columbia, MD). All subjects were asked to consume three 200 mg capsules daily throughout gestation. A monthly supply of capsules was mailed

directly to subjects. A self-addressed, stamped envelope was also provided to return any remaining capsules from the previous month. The University of Kansas Hospital Investigational Pharmacy recorded the number of remaining capsules, and the weekly and overall capsule intake of each subject was calculated at the end of the treatment phase.

ANALYSIS OF RED BLOOD CELL AND HUMAN MILK DHA

Women provided blood and milk samples the morning after and approximately six weeks following parturition, respectively. Human milk was collected in a sterile 4-oz general purpose specimen container and stored at -80°C until analysis. Blood samples were collected by venipuncture into 2 mL K_2EDTA tubes (BD Vacutainer, Franklin Lakes, NJ). Plasma and RBCs were separated by centrifugation ($3000\times g$, 10 minutes; 4°C), frozen, and stored under nitrogen at -80°C until analysis. Lipids were isolated according to a modification of the Folch protocol (101), and RBC lipids were fractionated (102) by thin-layer chromatography. Milk total lipids and RBC phospholipids were transmethylated with boron trifluoride-methanol (103), and the resulting fatty acid methyl esters (FAME) were separated using a Varian 3900 gas chromatograph with an SP-2560 capillary column (100 m, Sigma Aldrich) and a Star 6.41 Chromatography Workstation for peak integration and analysis as previously reported (104). Injector and detector temperatures were programmed at 260°C . The temperature program for the 41-minute column run was: 140°C , 5 minutes; 4°C increase/minute to 240°C ; 240°C , 11 minutes. Individual peaks were identified and quantified by comparison with qualitative standards (PUFA No. 1 Marine Source 100 mg; PUFA No. 2 Animal Source 100 mg; Sigma

Aldrich) and a weighed standard mixture (Supelco 37 Component FAME mix, Sigma Aldrich) was employed to determine a final weight percent of total fatty acids.

GENOTYPING

FADS1 rs174553 and FADS2 rs174575 SNPs were selected among those previously studied for their relatively common minor allele frequencies (33% and 24%, respectively) and observed association with blood lipid and human milk DHA (80, 82-85). DNA was collected using buccal collection brushes during the follow-up phase of the primary trial. Genomic DNA was extracted using the Gentra Puregene Buccal Cell Kit (QIAGEN, Hilden, Germany), and genotyping was performed with made-to-order TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) using real-time polymerase chain reaction. Five-microliter total reactions were prepared according to manufacturer instructions, and individual genotypes were determined with StepOne Software (Version 2.0; Applied Biosystems).

STATISTICAL ANALYSIS

The human milk-feeding and formula-feeding cohorts enrolled in the original trial were compared using Student's *t*-test. Student's *t*-test was also used to compare the proportion of milk DHA between the treatment and placebo groups. Linear regression was used to determine the main effect of FADS minor alleles on milk DHA. Here, we controlled for the proportion of DHA in maternal RBCs to eliminate the effect of group

assignment, account for errors in reported intake, and eliminate the influence of variable compliance to the supplementation protocol.

As the percentage of DHA in human milk was positively skewed, a log transformation was employed to normalize the distribution before model building. After examining all possible interactions and collapsing binary variables for FADS major alleles, a first-order model was selected with the percentage of DHA in maternal RBCs, maternal FADS1 SNP rs174553, and maternal FADS2 SNP rs174575 serving as the predictor variables. FADS SNPs were defined by the following binary variables: 1 = minor allele homozygote, 0 = major allele carrier.

Model assumptions were verified using the Kolmogorov-Smirnov, Shapiro-Wilk, and Breusch-Pagan tests. The effect of multicollinearity was examined, and the absence of outliers and influential observations was confirmed by assessing studentized deleted residuals, leverage values, Cook's distance, DFFITS, and DFBETAS. All data were analyzed with SPSS Statistics 17.0 software (SPSS, Chicago, IL), and P -values ≤ 0.05 were considered significant.

RESULTS

Compared to the formula-feeding cohort, the subset of human milk-feeding women ($n = 103$) included in the current analysis were more likely to be Caucasian ($P < 0.001$), had a higher median income by zip code ($P < 0.001$), were older ($P < 0.001$), and had a greater level of education at the time of enrollment ($P < 0.001$) (**Table 3.1**). No differences in maternal RBC-DHA were detected. Among the subset of women who fed

human milk for at least 6 weeks, the observed maternal genotypic and minor allele frequencies for each SNP are provided in **Table 3.2**.

The concentration of human-milk DHA was higher among women assigned to the treatment group than those assigned to the placebo ($n = 130$; $P < 0.001$). The mean (\pm SE) proportion of milk DHA (%) among supplemented and unsupplemented women was 0.34 (± 0.02) and 0.24 (± 0.02), respectively. However, when controlling for RBC-DHA to eliminate the influence of DHA supplementation and dietary intake, FADS2 minor allele homozygotes had a lower proportion of milk DHA than major-allele carriers ($P = 0.033$) (**Table 3.3**). The mean (\pm SE) proportion of milk DHA (%) among FADS major allele carriers and minor allele homozygotes is displayed in **Figure 3.1**.

The selected first-order regression model appeared to be appropriate and fit the data well. Stepwise selection, forward selection, and backward elimination produced the same model, each controlling for the proportion of DHA in maternal RBCs (criteria for entry: $F = 0.15$; criteria for removal: $F = 0.20$). The normality and constancy of variance assumptions were satisfied, and the multicollinearity effect was not serious. No outlying or influential observations were noted.

Table 3.1: Comparison of breast and formula-feeding subject characteristics¹

	BF (n = 103)	FF (n = 102)	P-Value
Mean Age at Enrollment (years ± SE)	27.5 ± 0.4	24.3 ± 0.3	< 0.001
Maternal Race			< 0.001
Caucasian (%)	80.2	48.7	
African American (%)	18.9	50.3	
Other (%)	0.9	1.0	
Education at Enrollment (years ± SE)	15.5 ± 0.2	12.8 ± 0.2	< 0.001
Median Income by Zip Code (\$USD ± SE)	52.6 ± 1.9 k	40.7 ± 1.2 k	< 0.001
Mean Proportion RBC-DHA (% ± SE)	6.3 ± 0.2	5.9 ± 0.2	0.125

¹Values were analyzed using Student's *t*-tests; BF, breastfeeding cohort; DHA, docosahexaenoic acid; FF, formula-feeding cohort; RBC, red blood cell

Table 3.2: Maternal FADS1 rs174553 and FADS2 rs174575 genotypic frequencies¹

Gene	SNP	M/m Alleles	Genotype (Number of Subjects)		MAF	
FADS1	rs174553	A/G	A/A (53)	A/G (41)	G/G (9)	0.29
FADS2	rs174575	C/G	C/C (58)	C/G (38)	G/G (7)	0.25

¹M/m, major/minor alleles; MAF, minor allele frequency; SNP, single nucleotide polymorphism

Table 3.3: Regression coefficients for model designed to examine the main effect of maternal FADS minor alleles on breast-milk DHA¹

Model	B	SE	t	P-Value
Constant	-0.837	0.047	-17.935	< 0.001
Maternal Red Blood Cell DHA (%)	0.033	0.007	4.719	< 0.001
Maternal FADS1 rs174553*	0.138	0.101	1.365	0.175
Maternal FADS2 rs174575*	-0.146	0.068	-2.156	0.033

¹DHA, docosahexaenoic acid

*Binary variable definition: minor allele homozygote = 1; major allele carrier = 0

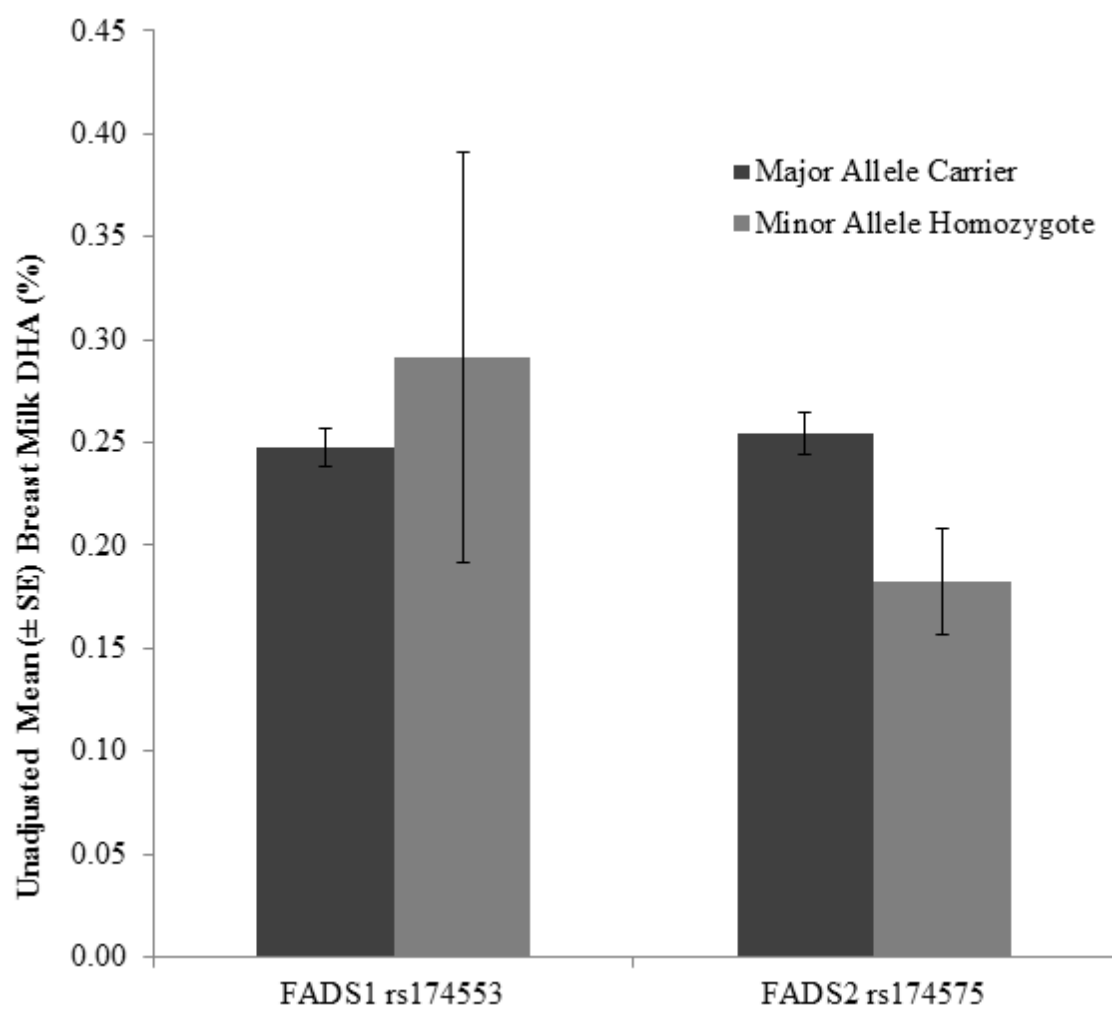


Figure 3.1. Unadjusted mean (\pm SE) proportion of breast milk DHA (%) among FADS1 rs174553 and FADS2 rs174575 major allele carriers and minor allele homozygotes.

DISCUSSION

To our knowledge, this is the first study to determine the main effect of FADS minor alleles on human-milk DHA after controlling for the proportion of DHA in maternal RBCs. The results of the present study support the hypothesis that polymorphisms in FADS2 affect DHA in human milk and, hence the transfer of that DHA to the growing infant. Moltó-Puigmartí *et al.* observed that the proportion of DHA in plasma phospholipids in pregnant women increases with dietary intake, irrespective of the FADS genotype, while DHA proportions in milk increase only in major-allele carriers (83). Here, even after controlling for the proportion of DHA in maternal RBCs (which eliminated the effect of potential errors in self-reported dietary intake), a significant diet-gene interaction remained. Regardless of DHA status, women carrying two copies of the minor allele for FADS2 SNP rs174575 had a lower proportion of milk DHA than major-allele carriers. As DHA continues to accumulate in the human brain after birth (92, 112) and postnatal supplementation has shown benefits on the Brunet-Lezine Scale (49, 50), Bayley Scales (51), and Weschler Primary Preschool Scale of Intelligence (52), this may have important implications for early cognitive development.

Caspi *et al.* previously demonstrated that the association between breastfeeding and IQ is moderated by FADS2 rs174575 (80). Specifically, the investigators found that human milk feeding confers a 6.4 to 7.0-IQ-point advantage only among children carrying the major allele (80). The current results suggest that the observed advantage among major-allele carriers is a surrogate for maternal genotype and the resulting proportion of human-milk DHA. Based on the current findings, infants nursing from

women carrying two copies of the minor allele would likely consume a lower concentration of DHA than those nursing from major-allele carriers. In light of recent studies examining the effects of postnatal DHA supplementation, these children might also be expected to perform more poorly on specific (53) and general measures (49-52) of cognitive function. If verified, genotype-specific dietary recommendations to enhance growth and cognitive development in at-risk infants may be warranted. However, in this case, at-risk infants would be identified by maternal genotype, and the child, rather than the mother, would receive DHA supplementation.

Overall, the results of the present study demonstrate that FADS2 rs174575 influences the proportion of human-milk DHA. Additional observations should be collected to validate the predictive ability of this model. Data splitting for model validation would have severely reduced the number of minor allele homozygotes for model building and, thus, was not employed. Future studies should evaluate whether maternal FADS2 rs174575 genotype influences cognitive function and development among exclusively human milk-fed infants.

CHAPTER 4:

FADS2 rs174575 GENOTYPE MODERATES THE ASSOCIATION BETWEEN INFANT FEEDING AND A MEASURE OF DEVELOPMENTAL STATUS

ABSTRACT

Human milk compared to infant formula feeding confers an IQ advantage to children carrying the major allele for a single nucleotide polymorphism (SNPs) in FADS2. A recent report found that the maternal FADS2 minor allele reduces DHA transfer in human milk, suggesting the maternal FADS2 genotype could possibly underlie the previous finding. We tested if toddler performance on the Bayley Scales of Infant Development Mental Development Index (BSID MDI) at 18 months was predicted by either maternal or child FADS1 rs174553 or FADS2 rs174575 genotype in breastfed and formula-fed infants exposed to variable amounts of DHA in utero. The study population consisted of a subset of mother/infant dyads enrolled in an NICHD-funded Phase-III RCT. Women provided blood samples the morning after parturition. RBC-DHA was quantified by gas chromatography. Genomic DNA was extracted from buccal collection brushes and genotyping performed with TaqMan SNP Genotyping Assays. MDI was assessed at 18 months of age. We found that MDI was not related to maternal FADS1 or FADS2 genotypes. Human milk-fed (but not formula-fed) infants carrying two copies of the FADS2 minor allele had a lower MDI at 18 months than major allele carriers ($P = 0.007$). The infant's FADS2 rs174575 genotype moderates the association between breastfeeding human milk feeding and an early measure of global developmental status.

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n-3) is an omega-3 polyunsaturated fatty acid that accumulates rapidly in the human brain during the last intrauterine trimester and the first 6-10 months after birth (92, 112). Prior to birth, DHA is provided by placental transfer and accumulates in the fetal brain in a manner that is dependent on maternal status (31). Several observational studies in humans have linked higher intrauterine DHA exposure to a number of positive developmental outcomes, such as improved cognitive and visual function in children (29, 30, 41), and a recent randomized, controlled trial found that postnatal DHA supplementation lowered infant heart rate and increased sustained attention at 4, 6, and 9 months of age (53).

While DHA can be provided directly by the diet via animal fats, including fish, fish oils, and specialty egg and dairy products, it is also synthesized endogenously from its essential dietary precursor, α -linolenic acid (18:3n-3). The conversion pathway consists of a succession of desaturations and elongations, and two key enzymes, Δ -5 and Δ -6 desaturase (encoded by FADS1 and FADS2, respectively) are thought to govern the rate of synthesis (**Figure 1.1**).

Caspi *et al.* recently demonstrated that the observed association between breastfeeding and IQ is moderated by FADS2 rs174575 genotype (80). Specifically, they found that adults carrying a copy of the major allele have a 6.4 to 7.0-IQ point advantage if fed human milk compared to formula in infancy (80). We and others have observed that the proportion of DHA in plasma and red blood cell (RBC) phospholipids increases with dietary intake and DHA status, irrespective of the genotype, while the DHA

proportion in milk increases only in FADS2 rs174575 major-allele carriers (83). In light of this information, and knowing Caspi *et al.* did not have data on maternal genotype (80), we hypothesized offspring genotype serves as a surrogate for maternal genotype, and the influence of FADS2 on cognition is conferred via differences in breast-milk DHA. Previous studies have not concurrently examined the influence of maternal and infant FADS genotypes on infant cognition in breastfed and formula-fed cohorts to substantiate this hypothesis. The objective of the present study was to determine if toddler performance on the Bayley Scales of Infant Development Mental Development Index (BSID MDI) at 18 months was predicted by either maternal or child FADS1 rs174553 or FADS2 rs174575 genotype in breastfed and formula-fed infants exposed to variable amounts of DHA in utero.

SUBJECTS AND METHODS

SUBJECTS

The study population consisted of a subset of mother/infant dyads enrolled in an NICHD-funded Phase-III clinical trial (NCT00266825), designed to determine the effects of consuming 600 mg/day of DHA throughout gestation on maternal and infant/toddler outcomes. A total of 350 women were enrolled in the trial. Those who provided a DNA sample and whose infants completed the 18-month BSID II MDI assessment were included in the current analysis (**Figure 2.1**). Women were eligible for enrollment if they were English-speaking, between 16 to 35.99 years of age, and in their 8th to 20th week of

gestation. Subjects were excluded if they were expecting multiple infants or had any serious health condition likely to affect the growth and development of their fetus or the postnatal growth and development of their newborn infants. Subject characteristics, including maternal race and education, were obtained via questionnaire at enrollment. The Peabody Picture Vocabulary Test (PPVT), a measure of receptive vocabulary for Standard English and a screening test of verbal ability was also administered. The research protocol and informed consent forms adhered to the Declaration of Helsinki (including the October 1996 amendment) and were approved by the Institutional Review Board/ethics committee at the University of Kansas Medical Center (HSC #10186).

SUPPLEMENTATION

Women assigned to the treatment group received capsules of a marine algae oil source of DHA (DHASCO, Martek Biosciences, Columbia, MD) (200 mg DHA/capsule), while those in the control group received capsules containing half soybean and half corn oil (Martek Biosciences, Columbia, MD). All subjects were asked to consume three 200 mg capsules daily throughout gestation. A monthly supply of capsules was mailed directly to subjects. A self-addressed, stamped envelope was also provided to return any remaining capsules from the previous month. The Investigational Pharmacy recorded the number of remaining capsules, and the weekly and overall capsule intake of each subject was calculated at the end of the treatment phase.

ANALYSIS OF RBC AND BREAST MILK DHA

Women provided blood samples the morning after parturition. Cord blood was obtained at delivery. Blood samples were collected by venipuncture into 2 mL K₂EDTA tubes (BD Vacutainer, Franklin Lakes, NJ). Plasma and RBCs were separated by centrifugation (3000×g, 10 minutes; 4°C), frozen, and stored under nitrogen at –80°C until analysis. Lipids were isolated according to a modification of the Folch protocol (101) and fractionated (102) by thin-layer chromatography. RBC phospholipids were transmethyalted with boron trifluoride-methanol (103), and the resulting fatty acid methyl esters (FAME) were separated using a Varian 3900 gas chromatograph with an SP-2560 capillary column (100 m, Sigma Aldrich) and a Star 6.41 Chromatography Workstation for peak integration and analysis as previously reported (104). Injector and detector temperatures were programmed at 260° C. The temperature program for the 41-minute column run was: 140° C, 5 minutes; 4° C increase/minute to 240° C; 240° C, 11 minutes. Individual peaks were identified and quantified by comparison with a qualitative standard (PUFA No. 1 Marine Source 100 mg; PUFA No. 2 Animal Source 100 mg; Sigma Aldrich) and a weighed standard mixture (Supelco 37 Component FAME mix, Sigma Aldrich) was employed to determine a final weight percent of total fatty acids.

GENOTYPING

FADS1 rs174553 and FADS2 rs174575 SNPs were selected among those previously studied for their relatively common minor allele frequencies (33% and 24%,

respectively) and observed association with cognition and blood lipid and breast milk DHA (80, 82-85). Maternal and infant DNA was collected using buccal collection brushes during the follow-up phase of the primary trial. Genomic DNA was extracted using the Gentra Puregene Buccal Cell Kit (QIAGEN, Hilden, Germany), and genotyping was performed with made-to-order TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA) using real-time polymerase chain reaction. Five-microliter total reactions were prepared according to manufacturer instructions, and individual genotypes were determined with StepOne Software (Version 2.0; Applied Biosystems).

STANDARDIZED ASSESSMENT

The Bayley Scales of Infant Development (2nd Edition; BSID II) (113) was administered at 18 months of age by a psychologist trained to reliability. This is a commonly-used instrument that yields IQ-like scores and assesses a number of aspects of mental and motor development in children from birth to 42 months of age. From this assessment, both motor and mental index scores are derived. For the purpose of this study, only the Mental Development Index scores (MDI) were utilized in the analysis. MDI shows considerable continuity at 18 months to preschool IQ scores (114), and Birch *et al.* recently observed a 7-point advantage on the BSID among DHA-supplemented infants (51).

STATISTICAL ANALYSIS

Subject characteristics were compared between the breast and formula-feeding cohorts using Student's *t*-tests. Linear regression was used to determine the main effect of maternal and infant FADS minor alleles on 18-month MDI. The breastfed and formula-fed groups were examined separately. To isolate the influence of FADS genotypes on MDI and achieve the object of this study, DHA-group assignment, average weekly capsule intake, and maternal education at enrollment were included as predictor variables in the selected model. This effectively eliminated the influence of DHA supplementation and socioeconomic status on MDI. As maternal PPVT scores were not collected on all subjects and its inclusion would have resulted in some sample loss, it was not used as a measure of socioeconomic status in the present analysis. For those in the placebo group, average weekly capsule intake was assigned a value of zero.

A first-order model was selected after examining all possible interactions and collapsing binary variables for FADS major alleles: 1 = minor allele homozygote, 0 = major allele carrier. (Final predictor variables: DHA-group assignment, average weekly capsule intake, maternal education at enrollment, maternal and infant FADS1 rs174553, and maternal and infant FADS2 rs174575). Model assumptions were verified using the Kolmogorov-Smirnov, Shapiro-Wilk, and Breusch-Pagan tests. The effect of multicollinearity was examined, and the absence of outliers and influential observations was confirmed by assessing studentized deleted residuals, leverage values, Cook's distance, DFFITS, and DFBETAS. All data were analyzed with SPSS Statistics 17.0 software (SPSS, Chicago, IL), and *P*-values ≤ 0.05 were considered significant.

RESULTS

Compared to the formula-feeding cohort, the subset of breastfeeding women ($n = 103$) included in the current analysis were more likely to be Caucasian ($P < 0.001$), had a higher median income by zip code ($P < 0.001$), were older ($P < 0.001$), had a higher standardized PPVT score ($P < 0.001$), and had a greater level of education at the time of enrollment ($P < 0.001$) (**Table 4.1**). Their infants also scored higher on the BSID II MDI ($P < 0.001$). No differences in postpartum or cord blood RBC-DHA were observed. Maternal and infant genotypic and minor allele frequencies for each SNP are provided in **Table 4.2** and **Table 4.3**, respectively, for breast- and formula-feeding cohorts.

In the breastfed cohort, BSID II MDI at 18 months was not significantly related to maternal FADS1 or 2 genotypes ($P = 0.315$ and 0.436 , respectively) (**Table 4.4**), but was related to the FADS2 genotype of the infant. Specifically, FADS2 minor allele homozygotes had a significantly lower MDI at 18 months than major allele carriers ($P = 0.009$; $B = -14.6$). Interestingly, in the formula-fed cohort, MDI at 18 months was not related to maternal or infant FADS genotypes (**Table 4.5**). Mean 18-month MDI among breastfed and formula-fed major allele carriers and minor allele homozygotes is displayed in **Figure 4.1**.

The selected first-order regression model appeared to be appropriate and fit the data well. The normality and constancy of variance assumptions were satisfied, and the multicollinearity effect was not serious. No outlying or influential observations were noted.

Table 4.1: Comparison of subject characteristics¹

	BF (n = 103)	FF (n = 102)	P-Value
Maternal Characteristics			
Mean Age at Enrollment (years ± SE)	27.5 ± 0.4	24.3 ± 0.3	< 0.001
Maternal Race			< 0.001
Caucasian (%)	80.2	48.7	
African American (%)	18.9	50.3	
Other (%)	0.9	1.0	
Education at Enrollment (years ± SE)	15.5 ± 0.2	12.8 ± 0.2	< 0.001
Median Income by Zip Code (USD ± SE)	52.6 ± 1.9 K	40.7 ± 1.2 K	< 0.001
Mean Standardized PPVT Score (± SE)	105.9 ± 1.2	93.6 ± 1.6	< 0.001
Mean Postpartum RBC-PL-DHA (% ± SE)	6.3 ± 0.2	5.9 ± 0.2	0.125
Infant Characteristics			
Mean Cord Blood RBC-PL-DHA (% ± SE)	6.8 ± 0.2	6.5 ± 0.2	0.289
Mean 18-Month BSID II MDI (± SE)	100.0 ± 1.2	94.2 ± 1.0	< 0.001

¹Values were analyzed using Student's *t*-tests; BF, breastfeeding cohort; BSID II MDI, Bayley Scales of Infant Development 2nd Edition Mental Development Index; DHA, docosahexaenoic acid; FF, formula-feeding cohort; PPVT, Peabody Picture Vocabulary Test; RBC, red blood cell

Table 4.2: Maternal FADS1 rs174553 and FADS2 rs174575 genotypic frequencies¹

Gene	SNP	M/m Alleles	Genotype (Number of Subjects)	MAF
BF	FADS1 rs174553	A/G	A/A (53) A/G (41) G/G (9)	0.29
	FADS2 rs174575	C/G	C/C (58) C/G (38) G/G (7)	0.25
FF	FADS1 rs174553	A/G	A/A (56) A/G (38) G/G (8)	0.26
	FADS2 rs174575	C/G	C/C (52) C/G (41) G/G (9)	0.29

¹BF, breastfed cohort; FF, formula-fed cohort; M/m, major/minor alleles; MAF, minor allele frequency; SNP, single nucleotide polymorphism

Table 4.3: Infant FADS1 rs174553 and FADS2 rs174575 genotypic frequencies¹

	Gene	SNP	M/m Alleles	Genotype (Number of Subjects)	MAF
BF	FADS1	rs174553	A/G	A/A (58) A/G (42) G/G (3)	0.23
	FADS2	rs174575	C/G	C/C (57) C/G (40) G/G (6)	0.25
FF	FADS1	rs174553	A/G	A/A (59) A/G (35) G/G (7)	0.24
	FADS2	rs174575	C/G	C/C (55) C/G (40) G/G (5)	0.25

¹BF, breastfeeding cohort; FF, formula-feeding cohort; M/m, major/minor alleles; MAF, minor allele frequency; SNP, single nucleotide polymorphism

Table 4.4: Regression coefficients for model designed to examine the main effect of maternal and infant FADS minor alleles on 18-month MDI in the breastfeeding cohort¹

Model	B	SE	<i>t</i>	<i>P</i>-Value
Constant	98.4	8.1	12.2	< 0.001
DHA Group Assignment[‡]	-14.1	10.2	-1.4	0.170
Average Weekly Capsule Intake	0.7	0.5	1.2	0.237
Maternal Education at Enrollment	0.2	0.5	0.5	0.654
Maternal FADS1 rs174553*	8.4	8.3	1.0	0.315
Infant FADS1 rs174553*	9.1	7.8	1.2	0.248
Maternal FADS2 rs174575*	-4.3	5.5	-0.8	0.436
Infant FADS2 rs174575*	-14.6	5.5	-2.7	0.009

¹MDI, Bayley Scales of Infant Development 2nd Edition Mental Development Index; DHA, docosahexaenoic acid

[‡]Binary variable definition: treatment group = 1; control group = 0

*Binary variable definition: minor allele homozygote = 1; major allele carrier = 0

Table 4.5: Regression coefficients for model designed to examine the main effect of maternal and infant FADS minor alleles on 18-month MDI in the formula-feeding cohort¹

Model	B	SE	<i>t</i>	<i>P</i>-Value
Constant	85.6	5.6	15.3	< 0.001
DHA Group Assignment[‡]	-4.3	5.9	-0.7	0.462
Average Weekly Capsule Intake	0.4	0.3	1.1	0.271
Maternal Education at Enrollment	0.6	0.4	1.3	0.185
Maternal FADS1 rs174553*	1.4	6.8	0.2	0.841
Infant FADS1 rs174553*	-1.3	4.5	-0.3	0.773
Maternal FADS2 rs174575*	-5.1	4.8	-1.1	0.290
Infant FADS2 rs174575*	8.7	5.5	1.6	0.117

¹MDI, Bayley Scales of Infant Development 2nd Edition Mental Development Index; DHA, docosahexaenoic acid

[‡]Binary variable definition: treatment group = 1; control group = 0

*Binary variable definition: minor allele homozygote = 1; major allele carrier = 0

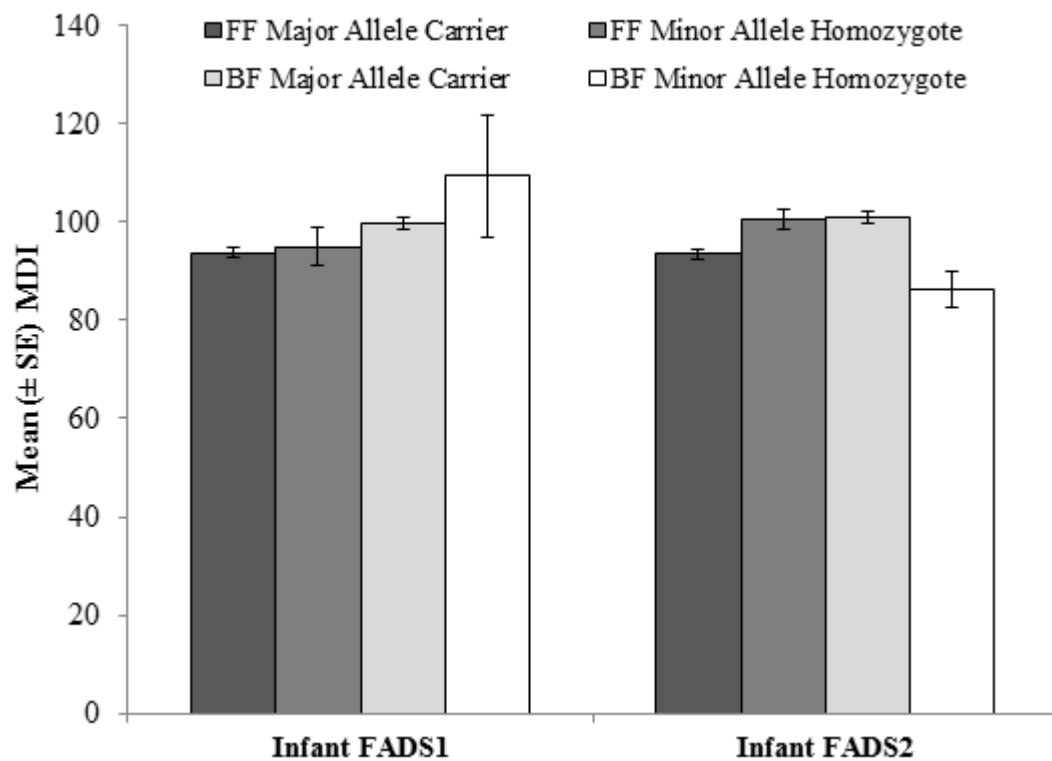


Figure 4.1. Mean 18-month MDI among breastfed and formula-fed FADS1 rs174553 and FADS2 rs174575 major allele carriers and minor allele homozygotes. BF, breastfed cohort; FF, formula-fed cohort

DISCUSSION

To our knowledge, this is the first study to concurrently examine the influence of maternal and infant FADS genotypes on a measure of infant developmental status. Caspi *et al.* previously found that adults carrying a copy of the major allele have a 6.4 to 7.0-IQ point advantage if breastfed in infancy (80), but did not have information on maternal genotype. Based on recent reports indicating that FADS2 minor allele homozygotes have a lower proportion of breast-milk DHA (83, 85) regardless of dietary intake or status (80), we hypothesized that offspring genotype was serving as a surrogate for maternal genotype, and the influence of FADS2 on cognition was conferred via differences in breast-milk DHA. The results of the present study do not support this hypothesis, but reinforce the findings of Caspi *et al.* that the FADS2 genotype of the infant moderates the association between breastfeeding and IQ. In the present study, maternal FADS genotype did not influence the 18-month MDI of human-milk fed infants when infant genotype was included as a covariate in the model. Rather, the FADS2 genotype of the infant was significantly related to MDI in the breastfeeding cohort, with minor allele homozygotes displaying a 14.6-point deficit at 18 months. Also similar to Caspi *et al.*, who showed adults' IQ was unrelated to genotype in formula-fed infants, we find offspring genotype is not related to MDI, an early test of toddler global mental and motor development, in the formula-fed cohort.

A recent report by Sauerwald *et al.* may shed light on these seemingly ambiguous findings (115). The investigators examined the effects of various levels of DHA intake on plasma and erythrocyte fatty acids and endogenous LC-PUFA synthesis in preterm

infants using stable isotope technology. Preterm infants were randomized to preterm formulas with gamma-linolenic acid (0.4%) and arachidonic acid (AA, 0.1%) but different DHA contents (0.04%, 0.33%, or 0.52%); 24 received human milk (0.51% AA and 0.38% DHA, nonrandomized) (115). They found that LC-PUFA synthesis was lower in infants fed human milk than in those fed formulas (115). This may explain why FADS2 only appears to influence BSID II MDI in breastfed infants, and the genotype of the infant has a greater influence than maternal genotype on this measure of cognitive function.

Although maternal genotype was not related to 18-month MDI in the present study, future studies examining the influence of reduced breast-milk DHA among FADS minor allele homozygotes are warranted. DHA continues to accumulate in the human brain after birth (92, 112), and postnatal supplementation has shown benefits on several measures of intelligence (49-53). Our previous findings regarding the influence of maternal FADS2 on breast-milk DHA suggest that infants nursing from women carrying two copies of the minor allele consume a lower concentration of DHA than those nursing from major-allele carriers. While maternal FADS were not related to 18-month MDI in the present study, it is possible that this reduction in breast-milk DHA may influence other measures of cognitive development. Recent studies indicate the BSID II MDI is not particularly responsive to LC-PUFA supplementation/status (116-118), and a more comprehensive and sensitive approach, in which specific measures of cognitive function are assessed, may be more appropriate.

A limitation of this study is that it used a sample of convenience from a trial powered to examine the influence of prenatal DHA supplementation on birth outcomes. In addition, the breast and formula-fed cohorts were defined according to the provision of a milk sample at 6 weeks postpartum; we did not account for overall length of breastfeeding. However, we still observed significant differences between the two cohorts that are consistent with previous research. Finally, additional observations should be collected to validate the predictive ability of the model used in this study. Data splitting for model validation would have severely reduced the number of minor allele homozygotes for model building and, thus, was not employed.

Overall, the results of the present study demonstrate that the FADS2 rs174575 genotype of the infant moderates the association between breastfeeding and MDI. Future studies should evaluate whether maternal genotype influences other, more specific measures of early cognitive development.

CHAPTER 5:

DISCUSSION AND CONCLUSION

SUMMARY OF FINDINGS

This series of studies aimed to (1) elucidate the effect of DHA supplementation and FADS1 rs174553 and FADS2 rs174575 genotypes on red blood cell (RBC) ARA and DHA in a cohort of pregnant women, (2) determine if SNPs in maternal FADS1 and FADS2 influence the proportion of breast-milk DHA after controlling for the proportion of DHA in maternal RBCs, and (3) determine if toddler performance on the Bayley Scales of Infant Development Mental Development Index (BSID MDI) at 18 months is predicted by either maternal or child genotype in breastfed and formula-fed infants.

Overall, our results suggest DHA supplementation compensates for the lower proportion of RBC-DHA observed among FADS1 minor-allele homozygotes, but exaggerates the reduction in RBC-ARA among FADS1 and FADS2 minor-allele carriers. They also demonstrate that FADS2 influences the proportion of breast-milk DHA, but suggest the FADS2 genotype of the infant is more predictive than maternal genotype of cognitive outcomes. Finally, our results support the hypothesis that the FADS2 genotype of the infant moderates the association between breastfeeding and MDI.

DOCOSAHEXAENOIC ACID (DHA) SUPPLEMENTATION DIFFERENTIALLY MODULATES ARACHIDONIC ACID AND DHA STATUS ACROSS FADS GENOTYPES IN PREGNANCY

To our knowledge, this study in pregnant women was the first to examine the interaction between FADS genotypes and DHA supplementation. We found that DHA supplementation increases RBC-DHA to similar proportions, regardless of FADS1

rs174553 genotype and amplifies the supplementation-associated reduction in RBC-ARA among FADS minor-allele carriers. We also demonstrated that FADS major allele homozygotes do not experience a reduction in RBC-ARA with DHA supplementation.

FADS2 GENE VARIANT INFLUENCES THE PROPORTION OF DOCOSAHEXAENOIC ACID (DHA) IN HUMAN MILK

This study determined the main effect of FADS minor alleles on human-milk DHA after controlling for the proportion of DHA in maternal RBCs. This eliminated the effect of errors in self-reported dietary intake and variable adherence to the supplementation protocol. Regardless of DHA status, women carrying two copies of the minor allele for FADS2 had a lower proportion of milk DHA than major-allele carriers. These results support the hypothesis that polymorphisms in FADS2 limit DHA in human milk and, hence the transfer of that DHA to the growing infant.

FADS2 rs174575 GENOTYPE MODERATES THE ASSOCIATION BETWEEN INFANT FEEDING AND A MEASURE OF DEVELOPMENTAL STATUS

To our knowledge, this is the first study to concurrently examine the influence of maternal and infant FADS genotypes on a measure of infant developmental status. The results of this study reinforce the findings of Caspi *et al.* that the FADS2 genotype of the infant moderates the association between breastfeeding and IQ. Maternal FADS genotype did not influence the 18-month MDI of human-milk fed infants when infant

genotype was included as a covariate in the model. Rather, the FADS2 genotype of the infant was significantly related to MDI in the breastfeeding cohort, with minor allele homozygotes displaying a 14.6-point deficit at 18 months. Also similar to Caspi *et al.*, who showed adults' IQ was unrelated to genotype in formula-fed infants, we found that offspring genotype is not related to MDI, an early test of toddler global mental and motor development, in the formula-fed cohort.

CLINICAL IMPLICATIONS

The results of the present analyses have important implications for the design of future studies intended to assess the influence of DHA supplementation on maternal and infant outcomes. A number of studies have observed a reduction in the concentration of plasma and erythrocyte ARA with DHA supplementation (105-108). Interestingly, we found that FADS minor alleles exaggerated the observed supplementation-associated reduction in RBC-ARA in a dose-response manner, whereas RBC-ARA was not reduced among FADS major allele homozygotes.

It is possible that DHA supplementation decreases the expression of the Δ -5 and Δ -6 desaturase, further reducing ARA synthesis in minor allele carriers. This hypothesis is supported by a recent animal study designed to examine the effect of dietary α -linolenic acid on FADS expression (109), and another in preterm infants utilizing stable isotope technology (110). In the latter, infants fed 0.97 and 0.64% n-6 and n-3 LC-PUFAs by weight (111) showed a dramatic reduction in endogenous LC-PUFA synthesis

by 7 months of age compared to infants fed a formula devoid of long-chain polyunsaturated fatty acids (110). It seems likely that substrate availability plays an important role in the regulation of FADS gene expression. Our findings suggest that those more susceptible to reduced enzymatic function (FADS minor allele carriers) are more affected by supplementation than major allele homozygotes.

As the effects of reduced RBC-ARA on the growing fetus and child are not fully understood, it is possible that an optimal level of DHA supplementation exists, beyond which less advantageous outcomes are observed. ARA is considered important for fetal and infant growth and development (58, 59), and it is currently added to US infant formulas with DHA. When given in combination with DHA supplementation, there is limited evidence for a beneficial effect of ARA on brain development and function (51). However, low ARA status may be involved in the development of neuromental disorders such as schizophrenia (65).

Moltó-Puigmartí *et al.* observed that the proportion of DHA in plasma phospholipids in pregnant women increases with dietary intake, irrespective of the FADS genotype, while DHA proportions in milk increase only in major-allele carriers (83). Here, even after controlling for the proportion of DHA in maternal red blood cells, a significant diet-gene interaction remained. Regardless of DHA status, women carrying two copies of the minor allele for FADS2 SNP rs174575 had a lower proportion of milk DHA than major-allele carriers. Thus, infants nursing from women carrying two copies of the minor allele consume a lower concentration of DHA than those nursing from major-allele carriers. As DHA continues to accumulate in the human brain after birth

(92, 112) and postnatal supplementation has shown benefits on the Brunet-Lezine Scale (49, 50), Bayley Scales (51), and Weschler Primary Preschool Scale of Intelligence (52), this may have important implications for early cognitive development.

Caspi *et al.* previously found that adults carrying a copy of the major allele have a 6.4 to 7.0-IQ point advantage if breastfed in infancy (80), but did not have information on maternal genotype. Based on our previous findings and recent reports indicating that FADS2 minor allele homozygotes have a lower proportion of breast-milk DHA (83, 85) regardless of dietary intake or status (80), we hypothesized that offspring genotype was serving as a surrogate for maternal genotype, and the influence of FADS2 on cognition was conferred via differences in breast-milk DHA. Although the results of our final analysis do not support this hypothesis, but reinforce the findings of Caspi *et al.*, it is possible that the observed reduction in breast-milk DHA may influence other, more specific measures of cognitive development. If verified, genotype-specific dietary recommendations to enhance growth and cognitive development in at-risk infants may be warranted. However, in this case, at-risk infants would be identified by maternal genotype, and the child, rather than the mother, would receive DHA supplementation.

LIMITATIONS

The present studies used a sample of convenience from a trial powered to examine the influence of prenatal DHA supplementation on birth outcomes. Few women

had two copies of the minor allele for the genes examined. Although we found highly significant differences, small sample sizes may be misleading.

In addition, the breast and formula-fed cohorts were defined according to the provision of a milk sample at 6 weeks postpartum; we did not account for overall length of breastfeeding. However, we still observed significant differences between the two cohorts that are consistent with previous research.

Finally, data splitting for model validation would have severely reduced the number of minor allele homozygotes for model building and, thus, was not employed. Additional observations should be collected to validate the predictive ability of the models used in this study.

FUTURE DIRECTIONS

Although maternal genotype was not related to 18-month MDI in the present analyses, future studies examining the influence of reduced breast-milk DHA among FADS minor allele homozygotes are warranted. DHA continues to accumulate in the human brain after birth (92, 112), and postnatal supplementation has shown benefits on several measures of intelligence (49-53). Recent studies indicate the BSID II MDI is not particularly responsive to long-chain polyunsaturated fatty acid (LC-PUFA) supplementation/status (116-118), and a more comprehensive and sensitive approach, in which specific measures of cognitive function are assessed, may be more appropriate.

Future studies should also determine whether the observed reduction in ARA status that accompanies DHA supplementation in minor-allele carriers adversely affects developmental outcomes in infants. As the ideal level of DHA intake is likely to be genotype-specific, studies should also include various FADS genotypes as covariates of projected outcomes.

CONCLUSIONS

Together, the results of the present analyses suggest that DHA supplementation compensates for the lower proportion RBC-DHA observed among FADS1 minor-allele homozygotes, but exaggerates the supplementation-associated reduction in RBC-ARA among FADS minor-allele carriers. They support the hypothesis that polymorphisms in FADS2 limit DHA in breast milk and confirm the previous observation that the FADS2 rs174575 genotype of the infant moderates the association between breastfeeding and an early measure of infant developmental status.

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APPENDIX A:

PROCEDURE FOR THE COLLECTION OF BUCCAL CELLS

1. To collect cells, place the buccal collection brush into the child's mouth and scrape the cheek pouch (the space between the gums and inner cheek) or underneath the tongue 10 times.

Important Notes:

To avoid issues of contamination, wait at least 1 hour after the child last consumed milk or food to collect the buccal cells, and allow the child to drink water before collection.

Avoid rubbing the collection brush directly on the child's teeth to minimize the amount of bacteria transferred.

Never allow the collection brush to contact any other surface, including gloved hands.

2. Once collected, cut the end of the brush into an autoclaved 1.5 mL microcentrifuge tube.

Important Notes:

To avoid contamination, always use a new pair of autoclaved scissors for each subject. Do not remove the scissors from the jar until the sample has been collected. Do not allow the blade or the tip of the scissors to touch any surface other than the collection brush.

Do not place hands inside jar containing autoclaved microcentrifuge tubes. Rather, remove the foil covering the jar and shake a tube onto the inside surface of the foil. (Avoid touching the foil to prevent contamination.) Then drop the tube onto a clean surface. Do not place any unused tubes back in the jar. Close the lid of the microcentrifuge tube immediately and do not open until sample has been collected. (DNA present in the air can easily contaminate samples.) Never touch the inside of the microcentrifuge tube, including the inside surface of the lid.

3. Label the outside of the tube with a permanent marker (include the date of collection) and store at room temperature (15 – 25°C).

APPENDIX B:

DNA PURIFICATION FROM A BUCCAL BRUSH

(ADAPTED FROM THE GENTRA PUREGENE HANDBOOK)

1. To collect buccal cells, scrape the inside of the mouth 10 times with a Buccal Collection Brush.

For best results, wait at least 1 hour after eating or drinking to collect buccal cells.

2. Remove the collection brush from its handle using sterile scissors, and place the detached head into a sterile 1.5 mL microcentrifuge tube.

DNA may be purified immediately or samples may be stored on the collection brush for up to 1 month at room temperature (15 – 25°C).

3. Dispense 300 µL Cell Lysis Solution into the microcentrifuge tube.

Samples are stable in Cell Lysis Solution for at least 2 years at room temperature.

If 300 µL of Cell Lysis Solution is not sufficient to cover the head, the protocol must be scaled up to use a larger volume. Contact QIAGEN Technical Services for more information.

4. Add 1.5 µL Puregene Proteinase K (cat. no. 158918), mix by inverting 25 times, and incubate at 55°C for at least 1 hour (up to overnight for maximum yield) to complete cell lysis.
5. Remove the collection brush head from the Cell Lysis Solution with sterile forceps, scraping it on the sides of the tube to recover as much liquid as possible.

6. If RNA-free DNA is required, add 1.5 μ L RNase A Solution and mix by inverting 25 times. Incubate for 15 minutes at 37°C. Incubate for 1 minute on ice to quickly cool the sample.

Samples can be incubated at 37°C for up to 1 hour.

7. Add 100 μ L Protein Precipitation Solution and vortex vigorously for 20 seconds at high speed.
8. Incubate for 5 minutes on ice.
9. Centrifuge for 3 minutes at 13,000 – 16,000 x g.

The precipitated proteins should form a tight pellet.

If the protein pellet is not tight, incubate on ice for 5 minutes and repeat centrifugation.

10. Pipet 300 μ L isopropanol and 0.5 μ L Glycogen Solution (cat. no. 158930) into a clean 1.5 mL microcentrifuge tube, and add the supernatant from the previous step by pipetting carefully.

Be sure the protein pellet is not dislodged while pipetting.

11. Mix by inverting gently 50 times.
12. Centrifuge for 5 minutes at 13,000 – 16,000 x g.
13. Carefully discard the supernatant.

14. Remove any remaining solution with a pipette, taking care that the pellet remains in the tube.
15. Add 300 μ L of 70% ethanol and invert several times to wash the DNA pellet.
16. Centrifuge for 1 minute at 13,000 – 16,000 x g.
17. Carefully discard the supernatant. Remove any remaining solution with a pipette, taking care that the pellet remains in the tube. Allow to air dry for up to 15 minutes.

The pellet might be loose and easily dislodged.
18. Add 25 μ L DNA Hydration Solution and vortex for 5 seconds at medium speed to mix.
19. Incubate at 65°C for 1 hour to dissolve DNA.
20. Incubate at room temperature overnight with gentle shaking. Ensure tube cap is tightly closed to avoid leakage. Samples can then be centrifuged briefly and transferred to a storage tube.

APPENDIX C:

PREPARATION OF REACTION MIX AND PLATE USING WET DNA DELIVERY METHOD

ADAPTED FROM THE TAQMAN® GENOTYPING MASTER MIX PROTOCOL

1. Calculate the number of reactions to be performed for each assay, including extra reactions (approximately one extra reaction for every 10 required reactions) to provide excess volume for the loss that occurs during reagent transfers. Include at least two no template controls (NTCs) and (if needed) at least one genomic DNA control of known genotype on each plate to ensure accurate genotype calling.
2. Calculate the volume of each reaction mix component needed for each assay by multiplying the appropriate volume from the table below by the number of reactions determined in step 1.

Reaction Components	Volume/Well (5 µL volume reaction)
TaqMan [®] SNP Genotyping Assay Mix (20X)	0.25 µL
TaqMan [®] Genotyping Master Mix (2X)	2.50 µL

3. Gently swirl the bottle of 2X TaqMan[®] Genotyping Master Mix to mix.
4. Vortex and centrifuge the 20X genotyping assay mix briefly.
5. Pipette the required volumes of 2X TaqMan[®] Genotyping Master Mix and 20X genotyping assay mix into a sterile 1.5 mL microcentrifuge tube. (Perform steps 5, 8, 9, 10, and 11 in a dead-air hood. Pipette all solutions with low-retention, aerosol-resistant pipette tips, and wipe down all pipettes with RNase Away[®] before use.)
6. Cap the tube and vortex briefly to mix the solutions.

7. Briefly centrifuge the tube to spin down the contents and eliminate air bubbles from the solution.
8. Pipette 2.75 μL of the reaction mix into each well of the reaction plate.
9. Inspect each well for volume uniformity, noting which wells do not contain the proper volume.
10. Pipette 2.25 μL of one control or diluted DNA sample into each well of the plate.
11. Cover the plate with MicroAmp® Optical Adhesive Film, and smooth firmly with a MicroAmp® Adhesive Film Applicator to prevent evaporation of the plate contents.
12. Briefly centrifuge the plate to spin down the contents and eliminate air bubbles from the solutions.

APPENDIX D:

PCR PROTOCOL FOR FADS SNP RS174575 AND RS174553 GENOTYPING

Allelic Discrimination PCR Reaction

Reaction Components	Volume/Well (5 μ L volume reaction)
Genomic DNA diluted in DNA Hydration Solution	2.25 μ L
TaqMan [®] SNP Genotyping Assay Mix (20X)	0.25 μ L
TaqMan [®] Genotyping Master Mix (2X)	2.50 μ L
Total	5.00 μ L

Thermal Cycler Conditions

Number of Cycles	Temperature	Time
1	60°C	30 seconds
1	95°C	10 minutes
55	92°C	15 seconds
	60°C	90 seconds
1	60°C	30 seconds