DISSERTATION

THE EFFECTS OF DOCOSAHEXAENOIC ACID INTAKE DURING PREGNANCY AND LACTATION ON INFANT GROWTH AND NEUROCOGNITIVE DEVELOPMENT AND THE ASSOCIATED EFFECTS OF GENETIC VARIANTS OF THE FADS1 FADS2 GENE CLUSTER

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ABSTRACT

THE EFFECTS OF DOCOSAHEXAENOIC ACID INTAKE DURING PREGNANCY AND LACTATION ON INFANT GROWTH AND NEUROCOGNITIVE DEVELOPMENT AND THE ASSOCIATED EFFECTS OF GENETIC VARIANTS OF THE FADS1 FADS2 GENE CLUSTER

Maternal docosahexaenoic acid (DHA) intake during pregnancy and/or lactation has been positively associated with infant growth and neurocognitive development. However, randomized controlled clinical trials (RCT) report mixed results. Several RCT that failed to demonstrate an effect of DHA supplementation have found correlations between DHA status and cognitive benefits, possibly due to a failure to account for total maternal DHA intake. The majority of studies to date investigating neurocognitive development have not examined the effect of supplementing women with DHA during both pregnancy and lactation and fail to determine the effects of maternal genetic variation on infant neurocognitive development. Single nucleotide polymorphisms (SNPs) within the fatty acid desaturase (FADS)1 FADS2 gene cluster encoding for Δ5- and Δ6-desaturase enzymes were previously reported to be associated with altered omega-3 (n-3) and omega-6 (n-6) fatty acid proportions of erythrocyte, plasma phospholipids and breastmilk, possibly effecting DHA transfer to the infant.

This study was conducted to determine the relationship between DHA intake in women obtaining varying amounts of DHA daily during pregnancy and lactation and infant neurocognitive development in the first year of life and the association of maternal SNPs in the FADS1 FADS2 gene cluster. One hundred and fifteen pregnant women were randomized to

receive purified tuna oil supplement containing 300 mg of DHA and 67 mg EPA per day or an identical placebo (Sunola oil) for the last trimester of pregnancy through the first 3 months of lactation in a double-blinded placebo controlled clinical trial. Two SNPs in the FADS1 FADS2 gene cluster, rs174575 and rs174561, were genotyped from maternal DNA and erythrocyte, plasma phospholipid and breastmilk fatty acids and daily DHA intake from food frequency questionnaires (FFQ) were analyzed. Neurocognitive development of the infants was measured using the Mental Development Index (MDI) of the Bayley Scales of Infant Development III (BSID-III) at 4 and 12 months of age. Gestational length in days was calculated based upon last menstrual period and birth date. Infant birth weights and lengths were obtained from pediatric medical records at delivery and at 2 months of age.

Total daily DHA intake was estimated to range from 18 mg to 1.374 g per day calculated from all sources of DHA, including food and supplementation. Data was analyzed based on treatment group, placebo versus DHA, and by total daily DHA intake broken into three groups: low = 0-299 mg per day DHA, medium = 300-599 mg per day DHA, high = ≥600 mg per day DHA. DHA portion of 2 month breastmilk fatty acids directly correlated with daily DHA intake ($r=0.37$, $p=0.0002$). Erythrocyte and breastmilk DHA proportions significantly increased in women homozygous for the major allele (SNP rs174575, $p=0.0002$ and $p=0.030$, respectively; SNP rs174561, $p=0.003$ and $p=0.040$, respectively) in the high daily DHA intake compared to the low intake group. However, daily DHA intake had no effect significantly increasing DHA proportions in women homozygous for the minor alleles of both SNPs studied. Infants born to mothers in the high DHA intake group showed significantly higher scores on the 12 month cognitive scale of the MDI of the BSID-III ($p=0.018$), compared to the low intake group. No significant differences were seen between treatment groups or DHA intake groups on any of the

4 month infant cognitive testing. Genotype had no direct effect on BSID-III scores, however, ANCOVA for 12 month cognitive MDI subtest showed a statistically significant interaction between SNP rs174575 genotype and daily DHA intake group (p=0.023). Additionally, infants born to mothers in the DHA treatment group had an increase of 4.5 days in gestational age (p=0.048) and significantly lower incidence of preterm birth (5%; n=3) compared to infants born to mothers in the control group (18%; n=10; χ^2=4.97, p=0.026). No significant differences were seen between treatment groups or DHA intake groups in infant growth measurements at birth or at 2 months of age, although 2 month breastmilk DHA proportion of fatty acids was negatively correlated with 2 month weight (r= -0.22, p=0.048).

An intake of 600 mg of DHA per day or greater during the third trimester of gestation throughout the first three months of breastfeeding was associated with improved infant neurocognitive development. Genetic variants of the FADS1 FADS2 gene cluster influence erythrocyte and breastmilk fatty acids, and increased DHA intake does not effectively increase DHA proportions in minor allele carriers. Additionally, DHA supplementation increased gestational length and decreased preterm birth risk, however, did not affect infant birth weights. DHA supplementation during pregnancy and lactation could be beneficial for improving the neurocognitive development of infants, however, genetic variation may affect DHA transfer to the infant.

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Whatever you do, do it well.

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CHAPTER 1

INTRODUCTION

Project rationale

Docosahexaenoic acid (DHA; 22:6) is an omega-3 (n-3) long chain polyunsaturated fatty acid (LCPUFA) essential for central nervous system and retinal development that accumulates rapidly during the last trimester of gestation through the first 2 years of life.[1,2] DHA is transferred to the fetus via the placenta and after birth through breastfeeding.[3] Early neurocognitive development depends upon an adequate supply of DHA, and, thus, is influenced directly by the nutritional status of the mother during pregnancy and lactation.[4-6] Evidence suggests the conversion of DHA from n-3 precursors in humans is extremely limited, therefore, it is optimal to receive preformed DHA directly from the diet, either from fish and seafood or supplementation.[7,8]

Observational and randomized controlled trials (RCT) have reported maternal diets high in DHA during pregnancy are positively associated with improvements in health outcomes of human infants, including increased gestational age, decreased preterm birth, improved infant growth and enhanced neurocognitive development.[9-13] However, results of recent studies are mixed due to varying amounts of DHA, supplementing for different lengths of time and possible genetic variation influences on the DHA content of breastmilk.[14-19] Several RCT that failed to find a significant effect of DHA treatment often have found a significant association between DHA status and infant neurocognitive development.[20-23] Additionally, non-significant RCT rarely account for all sources of DHA in the maternal diet outside of the study supplement.

Accounting for DHA intake from food allows for a more accurate daily DHA intake to be estimated in RCT, which could dramatically affect results determining an association between DHA intake and improved infant growth and neurocognitive development.

Several recent studies have demonstrated single nucleotide polymorphisms (SNPs) in the fatty acid desaturase (FADS)1 and FADS2 gene cluster are associated with altered proportions of DHA in erythrocyte, plasma phospholipids and breastmilk. The Δ5- and Δ6-desaturase enzymes essential to endogenous DHA synthesis are encoded by fatty acid desaturase (FADS)1 and FADS2, respectively, which are arranged in a head-to-head orientation on chromosome 11q, with a third desaturase gene, FADS3, which function is currently unknown.[24] DHA proportions in erythrocyte, plasma and breastmilk fatty acids were lower in women homozygous for the minor alleles of 2 SNPs studied, FADS2 rs174575 and FADS1 rs174561, compared to women homozygous for the major allele.[18] Additionally, several recent studies have determined an association between SNPs within the FADS1 FADS2 gene cluster and child cognitive abilities, with major allele carriers showing significant advantages over minor allele carriers.[25-27]

The purpose of this study was to examine the effects of DHA intake during pregnancy and lactation on infant growth and neurocognitive development during the first year of life and the possible effects of SNPs within the FADS1 FADS2 gene cluster. The Omega Smart Baby Project was a double-blinded, randomized, placebo controlled clinical trial in which 115 pregnant women were randomized to receive 300 mg of DHA or identical placebo for the last trimester of pregnancy through the first 3 months of breastfeeding.

Hypotheses

The following hypotheses were addressed in this study:

1. Increased maternal DHA intake during pregnancy and lactation will result in higher scores on the Bayley Scales of Infant Development III (BSID-III), indicating enhanced neurocognitive development.
2. Increased maternal DHA intake during pregnancy will result in longer gestational lengths, decrease in preterm birth incidence and increased infant birth weights.
3. As maternal DHA intake increases, breastmilk DHA will also increase.
4. Minor allele carriers of SNPs within the FADS1 FADS2 gene cluster will have decreased breastmilk DHA compared to major allele carriers.
5. Major allele carriers of SNPs within the FADS1 FADS2 gene cluster will have infants with enhanced neurocognitive development compared to minor allele carriers.

CHAPTER 2

REVIEW OF LITERATURE

Docosahexaenoic acid

Long chain polyunsaturated fatty acids (LCPUFA) are essential constituents of all human cells. LCPUFA are incorporated into cell membranes, cholesterol esters, triglycerides and numerous other molecules providing both structural and functional roles throughout the body.[28] The majority of all LCPUFA in the human body are classified as either omega-3 (n-3) or omega-6 (n-6), based upon the location of the first unsaturated bond from the methyl end of the fatty acid. The 18 carbon LCPUFA of the n-6 series, linoleic acid (LA; 18:2), and the n-3 series, α-linolenic acid (ALA; 18:3), cannot be synthesized by the human body and are, therefore, essential to humans and must be obtained through diet.[28]

The n-6 fatty acid LA is found in grains and vegetable oils and is the precursor of the 20 carbon n-6 fatty acid arachidonic acid (AA; 20:4).[29] Humans synthesize AA through a process of enzymatic elongation and desaturation, as shown in **Figure 2.1**, and also can obtain it preformed in the diet through red meat and meat products.[30] The n-3 fatty acid ALA is found in plant oils and seeds and is an important precursor to LCPUFA, which include ecosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6). Metabolic conversion of ALA to DHA is shown in **Figure 2.1**. Once ingested, ALA can be further metabolized by Δ6-desaturation, subsequent elongation and Δ5-desaturation to form EPA on the endoplasmic reticulum.[28] EPA is then converted to docosapentaenoic acid (DPA; 22:5) via the addition of 2 carbons and subsequently, DPA undergoes further elongation and Δ6-desaturation. Upon transport to peroxisomes, ß-

oxidation ensues to shorten the chain by two carbons yielding the end product of DHA.[28] Furthermore, enzymes within this metabolic pathway also require iron, zinc, vitamin B6 and vitamin E, therefore, it is possible that micronutrient intake may also play a role in the formation of LCPUFA.[31]

While the synthesis of EPA and DHA from dietary ALA can occur in humans, in most individuals the conversion of DHA is estimated to be <0.1 to 1%.[8,28] However, women of child-bearing ages may convert ALA to DHA more effectively than men to compensate for the increased need of DHA during fetal development. Isotope labeled ALA was converted into DHA greater in women than in men, and DHA concentrations of subcutaneous adipose tissue were lower in men than in women.[32,33] It is proposed that estrogen may increase the activity of enzymes within the DHA synthesis pathway, consistent with findings that DHA concentration in plasma phospholipids was higher in women taking oral contraceptives containing estrogen compared to women not on contraceptives.[32,34] According to research conducted by the International Society for the Study of Fatty Acids and Lipids (ISSFAL) and European Influence of Dietary Fatty Acids on the Pathophysiology of Intrauterine Foetal Growth and Neonatal Development (PERILIP) group, ALA is not converted to DHA at levels that can be physiologically beneficial, especially during periods of increased need.[35-38]

The rate-limiting step in the conversion of ALA to DHA in humans appears to be the preliminary desaturation step of 18:3 via Δ6-desaturase, with the second Δ6-desaturase step from 24:5 fatty acid to DHA precusor 24:6 also limiting the completion of the metabolism to DHA.[24,39] Low Δ6-desaturase activity could be due to numerous common single nucleotide polymorphisms (SNPs) decreasing enzyme activity in humans, consequently preventing adequate DHA synthesis and increasing dietary need.[40] Additionally, recent research has shown that high

LCPUFA diets may in fact increase LCPUFA degradation and possibly decrease enzymatic activity of desaturases involved in EPA and DHA synthesis.[24] Increased dietary DHA intake alters gene expression within the n-6 and n-3 synthesis pathways, possibly providing feedback regulation of LCPUFA synthesis.[41] Since the synthesis pathways for both n-6 and n-3 LCPUFA compete for the same desaturase enzymes at multiple steps, it is possible that altering enzyme activity through feedback mechanisms and precursor ratios may affect downstream fatty acid proportions, thus reinforcing the need for obtaining EPA and DHA preformed directly from the diet.

Preformed DHA and EPA in the human diet are largely supplied by the consumption of fish, especially fatty fish, and seafoods.[28] While precursor n-3 fatty acid ALA is found in vegetable sources, DHA and EPA are not present in common vegetable fats and oils, including nuts, seeds and grains. Poultry, if consumed in large quantities can provide a significant source of DHA and EPA, depending upon DHA content of the chicken feed.[42] Additionally, many manufacturers are currently supplementing common functional foods with n-3 fatty acids. Dairy, poultry, eggs, cereals and even peanut butters are now commonly enriched with n-3s and DHA specifically, which can provide lower but significant dietary sources.[28] Yet, many of the current products marketed do not contain enough n-3 to provide any necessary benefit or do not properly protect the LCPUFA from oxidative damage. In the last 5-10 years, fish oil and DHA supplements have become one of the most common forms of dietary DHA sources.[28] This is especially true for pregnant and lactating women, where DHA supplements may provide a safer means for obtaining DHA compared to consuming fish and seafood possibly contaminated with mercury. In 2001, the U.S. Food and Drug Administration (FDA) released a well publicized advisory recommending pregnant women avoid consuming long-lived predatory fish that contain

the highest levels of methyl mercury, and to limit ingestion of all other fish each week, to minimize mercury exposure that could cause developmental delays in the fetus.[43] After the federal recommendations were disseminated, fish consumption by U.S. pregnant women decreased, but as awareness of possible health benefits DHA and fish oil can incur upon the developing fetus increase, obtaining DHA through purified supplements may also increase.[44]

Anthropologic data indicates the evolution of modern man coincides with an increase in marine foods high in n-3 fatty acids in the diet and the presence of DHA in neuronal tissues.[45,46] However, the current diet of mankind has shifted away from seafood and toward a diet high in saturated fats and n-6 fatty acids, mostly due to the domestication of livestock and the agricultural and industrial revolutions.[46] Numerous studies show increasing dietary ALA intakes will not increase DHA in blood lipids, indicating the diet directly induces compositional changes within cell membranes and suggests the need for obtaining preformed DHA directly from the diet.[3,28,35,37] In 2002, the Institute of Medicine (IOM) concluded that insufficient data was available to set an official recommended daily amount or adequate intake (AI) for DHA or EPA, yet, years later many of the IOMs concerns have been resolved through recent research.[47] The majority of recent dietary recommendations are for combined EPA+DHA without specific advice for each individual fatty acid, yet research clearly provides evidence that individual daily recommended intakes (DRI) are necessary for both EPA and DHA.[47,48]

Currently, North American intakes of EPA and DHA are approximately 100-150 mg per day, low according to recent research.[47] Researchers estimate the average American should consume greater than 250 mg of EPA+DHA per day upwards to 1 g per day, with increased amounts during pregnancy.[47,48] The PERILIP group recommends pregnant and lactating women receive at least 200 mg per day of preformed DHA directly from the diet, through

supplementation or consuming one to two portions of fatty fish high in n-3 LCPUFA per week.[38] The 2010 Dietary Guidelines for Americans recommend a maternal dietary intake of approximately 250 mg of DHA per day from at least two servings of seafood per week during pregnancy and lactation to see associated improvements in infant health outcomes.[49] Yet, DHA intake in the United States is estimated to be only 50 mg per day during pregnancy and lactation, lower than the 70 mg per day average of women of child-bearing age, due in part to government advisories warning about methyl mercury content of some fish.[44,50]

Mechanisms of action

DHA is abundant in the phospholipid bilayer of all cells and a major structural component of the brain, cerebral cortex, retina and skin.[51] Lipids constitute 50-60% of the dry weight of the human brain and DHA is the most abundant n-3 fatty acid, comprising 10-20% of total brain fatty acids.[52,53] The human brain is unable to synthesize DHA or EPA from precursor n-3 fatty acids, therefore, it must obtain them from elsewhere in the body.[54] To maintain LCPUFA levels, the brain depends on the transfer of LCPUFA from plasma, which is directly affected by LCPUFA from the diet, mobilization of fat stores or synthesis in the liver.[4] However, previously it was not clear how DHA could pass the blood brain barrier (BBB), since the majority of DHA in the blood is incorporated into phospholipids, triglycerides and cholesterol esters within plasma proteins, which are too large of complexes to cross the BBB.[54,55] Recently, Ouellet et al. using the *in situ* brain perfusion technique demonstrated radiolabeled DHA and EPA in free form rapidly transported through the BBB in mice, comparable to highly diffusible drugs.[56] Oulellet et al. also determined that bound to proteins, such as albumin, reduced, but did not completely prevent, the passage of DHA through the BBB and long-term high DHA dietary intake (~0.9 g per kg per day) reduced the uptake of DHA into the mouse brain.[56] Although the

mechanisms are not completely understood, once in the brain, DHA has effects on changes in membrane fluidity, including regulating the activity of membrane, neuronal development and plasticity, receptor mediated signaling, the production of anti-inflammatory lipid mediators and act as direct ligands to transcription factors of genes involved in inflammation, oxidative stress, neurogenesis, differentiation and fatty acid metabolism.[57-59]

Cellular membranes are complex in structure and LCPUFA have an important role in modulating the fluidity of the lipid bilayer. Plasma membranes contain specialized microdomains, called lipid rafts, that are comprised of tightly packed combinations of cholesterol, glycosphingolipids and glycolipids containing saturated fatty acyl residues that directly influence membrane fluidity and membrane protein trafficking.[60,61] The incorporation of DHA in the membrane bilayer creates a more fluid, permeable membrane and displaces saturated fatty acids, thus disrupting lipid raft formation and maintaining optimal neural membrane fluidity.[61] Additionally, improved fluidity of increased DHA within the membrane positively impacts the speed of signal transduction and neurotransmission, possibly leading to increased cognitive function.[62] Increased dietary intake of DHA can directly alter membrane composition and distribution, whereas increased ALA consumption alone does not alter membrane phospholipids.[63] DHA is rapidly incorporated into phospholipids, however the distribution between phospholipid classes is not equal, with preferential incorporation into phosphatidylethanolamine (PE) first, which is incorporated into white matter of the brain, neural tissue and the spinal cord, and to a lesser extent phosphatidylcholine (PC) or phosphatidylserine (PS), which is found in brain gray matter and rod photoreceptors of the retina.[60] Improving membrane fluidity throughout the brain from the presence of increased DHA could result in improved neuronal development as well as enhanced cognitive function.

LCPUFA are insoluble in the cellular environment, therefore, fatty acid binding proteins (FABP) are necessary to transport LCPUFA into the cell and to appropriate intracellular compartments for use.[64] FABP are a family of small cytosolic proteins coded by multiple genes. Multiple types of FABP have been identified, including brain FABP (B-FABP), which is present in radial glial cells and in the astrocytes, specialized glial cells that exert many essential and complex functions throughout the brain, of developing and adult brains.[65] B-FABP is believed to be important in early neurogenesis, the process in which neurons are created including the development of axons and dendrites, and neuronal migration and expression in the developing brain parallels early neuronal differentiation.[65] Research in mice indicates mutations impairing B-FABP results in a reduction of DHA with a corresponding increase in AA in astrocytes.[66] The decrease in brain DHA is directly associated with increased anxiety and fear memory, as well as spatial learning and memory impairments later in life.[66-68] Impairing DHA delivery into brain cells via B-FABP mutations suggest DHA plays an important role in early neuronal development, including influencing cognitive as well as behavioral functions of the brain. Additionally, recent research has linked polymorphisms within genes encoding for FABP with impaired activation of desaturase enzymes involved in LCPUFA synthesis, possibly further influencing the effects of LCPUFA throughout the body.[69] Fatty acid transport proteins (FATP) and placental membrane FABP (FABPpm), located exclusively in the microvillous membranes of placental syncytiotrophoblasts, are responsible for the transfer of LCPUFA and other free fatty acids from the maternal blood stream to the growing fetus.[70,71] Through the use of radiolabeled fatty acids, Campbell et al. demonstrated FABPpm preferentially binds maternal AA and DHA, compared to other fatty acids, for transport to the fetus.[72] This emphasizes the importance for the fetus to receive preformed AA and DHA from maternal supplies.

LCPUFA, especially DHA, are known to effect gene expression throughout the central nervous system, influencing neurogenesis, synaptogenesis and neurochemistry.[41,57] LCPUFA are ligands for the nuclear receptor peroxisome proliferator activated receptors (PPAR).[73,74] Although it is rarely found in the adult brain, PPARγ is expressed abundantly in the developing brain and appears to be important in regulating stem cell proliferation in early brain development.[75] An increase in DHA within the brain subsequently increases the transcription of PPARγ.[75] In addition to PPAR, DHA and AA are ligands for brain retinoid X receptor (RXR).[74] Once bound by ligand, PPAR heterodimerizes with RXR forming a complex that is then able to bind specific regions of DNA, regulating gene transcription controlling numerous cellular activities.[76] DHA directly affects the activity of RXR, which, along with retinoic acid receptor (RAR), have important roles in neurogenesis, neuronal differentiation and neurotransmission specifically within the hippocampus.[74,77] Several recent animal studies have linked DHA specifically to dopamine and serotonin production as well as to influencing their respective receptors and secondary messengers to facilitate neurotransmission.[78-80] Human infants deficient in LCPUFA have decreased dopaminergic responses compared to infants with normal LCPUFA levels.[81]

In addition to neuronal development, receptor-ligand interactions between DHA and PPAR interfere with the activation of nuclear transcription factor kappa-light-chain-enhancer of activated B cells (NFκB) proinflammatory signaling pathway, influencing the suppression of apoptotic pathways and proinflammatory cytokines, chemokines and enzymes, such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS).[57,82-85] LCPUFA, including DHA, inhibit dimerization and activation of Toll-like receptors (TLR), proteins that induce inflammatory pathways, such as NFκB, and mediate the expression of cytokines within

the developing brain.[86] Additionally, NFκB can be activated by proinflammatory cytokines such as interleukin (IL) 1 and tumor necrosis factor α (TNFα).[87] Recent research determined DHA directly inhibited TNFα mediated signaling pathways and also interacts with G-coupled protein receptor (GPR) 120, inducing anti-inflammatory pathways responsible for further inhibition of both TLR and TNFα pro-inflammatory pathways.[88,89] In addition, LCPUFA regulate the inflammatory processes of COX-2 converting AA to prostaglandins (PG) and subsequent pro-inflammatory eicosanoids.[90] Lee et al. recently reported EPA and DHA significantly reduced COX-2 expression, thus reducing PGE_2 and IL-6.[91] COX-2 expression, IL-6, TNFα and PGE_2 production have been associated with cognitive impairment and have been implicated in possibly causing preterm birth by degrading the uterine lining and stimulating uterine contractions.[92-94] The protection DHA may provide by inhibiting NFκB pro-inflammatory signaling pathway and COX-2 mediated inflammation could have vast impacts on neural development, maturation and possibly even gestational length.

While DHA is important for neurocognitive development and cognitive function, it is also important to note that it is essential the fetus receive AA for proper development. AA is n-6 fatty acid converted from LA using the same Δ6-desaturase enzyme used in the conversion of ALA to DHA, and competition exists between the n-6 and n-3 metabolic pathways.[1] AA concentrations are subject to nutritional control, and there could be a risk in reducing AA levels in the fetus from increased DHA and n-3 fatty acids intake, disrupting the proper n-6:n-3 ratio for fetal development.[95,96] Like DHA, AA is highly concentrated in the brain and retina of infants, with the majority of accumulation occurring in the last trimester through the first year of life.[1] Additionally, much like DHA, AA plays a critical role in membrane structure and fluidity, and can activate PPARγ to promote neurogenesis.[97] AA is also converted into prostaglandins,

eicosanoids and numerous signaling molecules utilized throughout the body and contribute to the development of innate immunity.[91] AA is essential for fetal growth and proper brain development and high maternal status of AA in plasma phospholipids is positively associated with fetal growth, infant birth weight and postnatal growth.[98-100] Additionally, lower levels of AA in cord blood and infant plasma phospholipids are associated with reduced performance on intelligence quotient (IQ) tests, motor development tests and behavioral measures.[101-104] These findings indicate that AA is an important fatty acid and should possibly be considered when studying fatty acids and neurocognitive development.

DHA during pregnancy

Several studies have investigated maternal DHA levels in plasma phospholipids, total plasma lipids and erythrocytes during pregnancy.[4,105] In healthy pregnant women not taking any supplements, an initial increase in DHA occurs until the 18th week of gestation.[4] Otto et al. supplemented women with 570 mg DHA per day starting at the 16th-20th week of gestation and showed a significant increase in maternal DHA levels at weekly blood draws in both plasma and erythrocyte phospholipids after 4 weeks when compared to the control group.[6] As DHA increases in the fetus during the third trimester, maternal DHA supplies are depleted, as indicated by a decrease in the percent total fatty acids of DHA on maternal RBC.[106] Currently, during the last trimester it is estimated the fetus acquires 67-75 mg per day of DHA *in utero*, accounting for 80% of brain DHA accumulating between week 26 and 40 of gestation.[67,107,108] DHA accumulates rapidly in the infant brain between the 28th week of gestation and two years of age, during which the neurological system grows rapidly.[2]

Pregnancy is a period of increased metabolic demands and maternal nutrition plays a critical role in fetal growth and development.[109] During gestation, the fetus has limited to no metabolic capability to convert ALA to DHA, therefore the fetus is entirely dependent on maternal sources of DHA.[110] The placenta uptakes DHA circulating in the maternal bloodstream, which is influenced by maternal DHA synthesis, DHA from the diet and enhanced mobilization of DHA from maternal adipose stores.[4] Whether the placenta is involved in DHA synthesis or if the selective transport of DHA to the fetus relies completely on maternal DHA stores is currently unknown. The amount of DHA synthesized or released from adipose tissue is unclear, but a strong link between maternal circulating DHA and dietary DHA intake during pregnancy suggests that diet may be the main source of DHA for the fetus.[4,14,67,111,112]

Gestational length and preterm birth

Preterm birth, defined as a birth of less than 37 weeks gestational age, is a major public health concern and is estimated to account for 3 million infant deaths worldwide every year.[113] Children born preterm are at an increased risk for a wide range of health problems, including neurodevelopmental and attention difficulties, memory and learning problems, visual deficits and pulmonary problems. Several studies determined increased intake of seafood and DHA during pregnancy promote decreased risk for preterm birth, increased gestational length, and increased infant birth weight.[114-117]

Researchers in the 1980s first observed women living in the Faroe Islands, where high fish consumption is normal, had longer gestational lengths and delivered infants with higher birth weights (~200 g) at term when compared to infants born to women in Denmark.[118] In a prospective cohort study by Olsen et al., increased fish and seafood intake during pregnancy

reduced the incidence of preterm birth and decreased the risk of having a low birth weight baby in 8729 women surveyed. Olsen et al. found an increase of 4 days in gestational length, only a 1.9% incidence of preterm delivery (compared to 7.1% in the group never consuming fish) and a decreased risk for having a low birth weight baby [odds ratio (OR)=3.57; 95% confidence interval (CI) 1.14-11.14] in women who consumed fish during pregnancy compared to those never eating fish.[115] However, in an earlier observational study, Olsen et al. showed positive association between the levels of LCPUFA in erythrocytes and a 5.7 day increase in gestational length in pregnant Danish women, yet no association was found in Faroese women (0.7 day increase in gestational length), who have a higher daily intake of fish and seafood compared to Danish women.[119] It is possible that the effect of LCPUFA on gestational length may meet a saturation point, where no further effect of increased n-3 fatty acids can be detected.[115,120]

Numerous randomized controlled trials (RCT) have attempted to validate the effect DHA supplementation, usually in the form of fish oil or purified DHA, has on increasing gestational length. The use of DHA supplementation in RCT has grown in recent years and with supplements ranging from 100 mg to 5 g of EPA+DHA per day.[121,122] In a RCT, Olsen et al. reported a reduction in the odds of delivering earlier than 40 weeks in pregnant women supplemented with 100 mg EPA+DHA per day for an average of 20 weeks.[123] In a U.S. trial, 350 women were randomized to consume DHA enriched eggs (133 mg DHA) or normal eggs daily after the 24th week. Upon adjusting the data for confounders, Smuts et al. discovered a significant 6 day increase in gestational length in the women consuming the DHA enriched eggs compared to the normal egg group (276.5 vs 270.5 days; p=0.009).[124] In the Fish Oil Trials in Pregnancy (FOTIP) study in Europe, 232 women at high risk for preterm birth supplemented

with 900 mg DHA per day versus olive oil control starting at the 20th week of gestation saw a reduction in preterm deliveries from 33% to 21% (OR=0.54, 95% CI 0.30-0.98; p=0.05).[125]

Additionally, to determine the effect of DHA on gestational length, Olsen et al. randomized 533 women to receive fish oil, olive oil or no oil from 30 weeks gestation until delivery and found women on the fish oil supplementation had approximately 4 day increase in gestation compared to the control groups (283.3 vs 279.4 days; p<0.006).[126] An Australian study of 2399 pregnant women randomized to receive a supplement containing 800 mg DHA per day or a vegetable oil control supplement found that fewer preterm births occurred in the DHA group compared to control (1.09% vs 2.25%; p=0.03) and the DHA group also saw a reduction in the risk of having a low birth weight baby (3.41% vs 5.27% control; p=0.03).[127] Although Helland et al. did not find significant differences between supplemented and control groups in gestational length or birth weight in 341 subjects (n=148 cod liver oil 1.18g DHA, 0.8g EPA versus corn oil control n=137), when comparing the quartile with the highest concentration of DHA in umbilical plasma phospholipids with the lowest quartile, the upper quartile had a gestational length increase of 7.1 days (282.5 days vs 275.4 days).[15]

Several meta-analyses have also examined the effect of DHA supplementation during pregnancy and gestational length and infant birth weight. A positive correlation between fish intake during pregnancy and an increase in gestational length was reported in Secher's review of well-conducted observational studies, RCT, and meta-analyses.[128] In the meta-analysis of RCT, Szajewska et al. reported only a modest 1.6 day increase in gestation in women supplemented with marine oils containing DHA (1278 infants from 6 trials).[129] In their systematic review and meta-analysis, Salvig and Lamont reported results indicating a 4.5 day longer gestation and 71 g higher birth weight of infants in women who received LCPUFA (1187 women, 3 trials) as well

as a reduction of preterm births (<37 weeks), which was previously undetermined by other reviews.[130]

In Makrides' 2006 Cochrane review, which covered 6 trials involving 2783 pregnant women, an increase of 2.6 days in gestation (95% CI 1.03-4.07 days; 3 trials, 1621 women) occurred in women supplemented with marine oil compared to placebo or no treatment. [116] Additionally, marine oil supplemented mothers were 31% less likely deliver preterm (<34 weeks) and delivered slightly heavier infants (47 g difference, 95% CI 1-93 g; 3 trials, 2440 women) and the relative risk for preterm birth before 34 weeks gestation was lower (RR 0.69, 95% CI 0.49-0.99; 2 trials, 860 women).[116] Most recently, Imhoff-Kunsch et al. 2012 systematic review, which included 15 RCT examining the effect of LCPUFA in pregnancy, determined an outcome similar to Makrides' with women receiving LCPUFA having a 26% lower risk of early preterm delivery (<34 weeks) (RR=0.74; 95% CI 0.58-0.94), as well as a decreased chance of delivering a low birth weight baby (RR=0.92; 95% CI 0.83-1.02).[131]

However, several well-designed trials and observational studies have found no beneficial effects of fish oil supplementation on gestational length.[132-136] After supplementing 233 pregnant women with 1.62 g EPA and 1.08 g DHA per day, Onwude et al. determined no difference in gestational length, birth weight or hypertension/preeclampsia in an intention-to-treat analysis between placebo and treatment groups.[133] In a RCT of a subgroup of 3098 pregnant women in the Danish National Birth Cohort with low fish intake and no fish oil or DHA supplementation, Knudsen et al. reported no difference in gestational length between five treatment groups randomized to receive 0.1 g, 0.3 g, 0.7 g, 1.4 g or 2.8 g EPA+DHA per day during pregnancy and the control group.[132] An observational study of 2109 pregnant women from a U.S. birth cohort enrolled in Project Viva from 1999 to 2002 found no association between marine n-3

PUFA and seafood intake during pregnancy and gestational length or preterm birth risk.[137] Similarly, in a population based sample of 965 pregnant Danish women, no association was detected between n-3 fatty acid intake and gestational length or birth weight.[138]

Although conflicting results do exist, accumulating evidence from both RCT and meta-analyses indicate DHA positively affects gestational length and possibly subsequent infant birth weights, while reducing the risks for both preterm birth and low birth weight infants. While several studies indicated no benefit of DHA supplementation during pregnancy, it is important to note that dietary DHA was often not controlled or accounted for in participants, some sample sizes were low, and DHA concentrations were low or not analyzed by tertiles or quartiles of total daily DHA intake.

Infant neurocognitive development

It is well established that DHA is important in fetal neurological development.[58,81,139] DHA is preferentially transported to the fetus during the last trimester of gestation, which coincides with fetal retinal and brain development, specifically with a growth spurt in gray matter.[5,67,98] Neurogenesis is at its maximum during the last trimester of gestation through two years of age. Several studies have shown a variety of visual, cognitive and behavioral impairments when infants are deficient in DHA during this critical development period.[140] Recent animal studies have suggested DHA exposure *in utero* influence neural differentiation, synaptogenesis and neurotransmitter target finding, and also indicate that once the window for development is past, deficits are not always reversible later.[5,53,79]

Numerous animal studies have provided direct evidence for the importance of DHA in the development of the brain. Maternal diets deficient in n-3 fatty acids have resulted in

decreased neurogenesis and altered morphology of structures within the rat brain. Coti Bertrand et al. determined brains of embryonic pups of mothers eating a diet deficient in n-3 fatty acids had altered brain morphology and a 55-65% reduction in brain DHA content, with a compensatory increase in brain AA.[141] Cao et al. showed inhibited neurite growth and synaptogenesis in the hippocampi of embryos of female rats fed n-3 deficient diets. By contrast, brains of embryos from dams receiving adequate DHA in their diets exhibited increased neurite growth and synapsin formation, providing further evidence DHA may play a direct role in improving cognitive function within the hippocampus.[142] Studies in nonhuman primates show feeding diets with a high ratio of LA to ALA causes a significant decrease in the normally high brain DHA accumulation that occurs during development and results in less mature development of visual acuity as well as altered attention and behavior, suggesting slower brain maturation.[143-145] Levant et al. found rats fed a low ALA diet had a significant reduction of brain DHA, altered brain serotonergic system, increased hypothalamic pituitary axis response to stress, and altered dopaminergic function within the brain, indicating brain DHA depletion may be related to neurotransmitter programming and subsequent related behavioral changes that may not be reversible after the development stage is complete in early infancy.[146-148] In a recent review, Davis-Bruno et al. concluded deficiencies of DHA during the critical time period of brain growth may result in neuronal development deficits that are not corrected by later increases in dietary DHA, suggesting a crucial time frame may exist for receiving adequate DHA for optimal brain development.[149]

Observational longitudinal studies in humans support a relationship between DHA status during pregnancy and infant neurocognitive development. Cord blood DHA status was determined in Inuits from Artic Quebec, a population with typically high fatty fish intake, and

DHA levels were associated with improved cognitive, visual and motor development during the first year of life.[12] Several large birth cohorts, including the Avon Longitudinal Study of Parents and Children (ALSPAC), Project VIVA and the Danish National Birth Cohort, have further supported the importance of DHA during pregnancy, determining positive associations between maternal fish intake during pregnancy and the cognitive abilities of the children.[11,13,150] Through the ALSPAC cohort, Hibbeln et al. demonstrated women consuming greater than 340 g of fish per week had a decreased risk of their children performing poorly on standardized IQ tests when compared to mothers with low seafood intake during pregnancy.[11] Using a prospective U.S. cohort of 341 mother-child pairs, Oken et al. reported maternal intake of greater than 2 servings of fish per week during pregnancy is positively associated with improved child cognitive test performance on the Peabody Picture Vocabulary Test (PPVT) and Wide Range Assessment of Visual Motor Abilities (WRAVMA) at 3 years of age, whereas maternal fish intake ≤2 servings/week was not associated with a benefit.[13] A recent prospective longitudinal observational study of 154 Inuit children, with regularly high dietary fish intake, reported higher umbilical cord blood DHA concentrations were associated with improved performances on memory assessments at school age (mean 11.3 years).[151] While results from observational studies are important, it should be noted that such studies are limited and often cannot adjust for complex confounding variables that influence early cognitive development.

RCT are necessary to establish a relationship between maternal DHA intake during pregnancy and lactation and infant neurological development. Numerous recent RCT have associated increased maternal DHA intake during pregnancy with improvements in cognitive development of the offspring, however, many results are mixed. In a RCT, Dunstan et al. supplemented pregnant women with high doses of DHA at 2.2 g per day beginning at 20 weeks

gestation through delivery and assessed child cognitive development using several cognitive assessment tests at 2.5 years of age and determined children born to the supplemented group had significantly higher scores on hand-eye coordination subtests compared to the control group ($p=0.02$), however, no other significant differences were apparent in other tests.[9] Colombo et al. supplemented pregnant women with DHA eggs (135 mg DHA per egg) or normal eggs (35 mg DHA per egg) and assessed the effects of maternal DHA intake on look duration and attention span of 75 infants at 5 time points during the first 18 months of life.[21] Children whose mothers had the highest DHA status at delivery showed the shortest look duration at 4 and 6 months and, although this advantage was absent at 8 months, at 12-18 months infants born to mothers with the highest DHA levels demonstrated more mature attention spans and were less easily distracted when compared to infants born to mothers with low DHA status.[21]

In a double-blind, placebo-controlled RCT, Judge et al. supplemented pregnant women beginning at 24 weeks gestation through delivery with cereal bars containing 300 mg DHA consumed an average of 5 days per week or placebo bars. Judge et al. found infants born to mothers in the DHA supplemented group performed significantly better on problem-solving tasks in the Infant Planning Test at 9 months ($p=0.017$ total intention score; $p=0.011$ total intentional solutions) when compared to infants born to mothers of the placebo group, yet no differences were seen in any measure of the Fagan Test of Infant Intelligence (FTII).[16] Additionally, Judge et al. measured infant sleep/wake patterns on postnatal days 1 and 2 and, after controlling for ethnic variation, discovered significantly fewer quiet sleep (QS) arousals on day 1 ($p=0.006$) and day 2 ($p=0.011$) and less arousals in active sleep (AS) on day 1 ($p=0.012$) in the infants born to mothers in the DHA supplemented group compared to the placebo group.[152] Cheruku et al. also determined newborn sleep organization to be associated with maternal DHA

status during pregnancy, showing infants born to mothers with plasma phospholipid DHA >3.0% total fatty acids had significantly lower ratio of AS to QS and less AS compared to infants of low DHA status mothers.[153] Increased quiet sleep with less arousals in both quiet and active sleep in infants are characteristic of more mature neurological development, less negative emotionality and indicative of improved cognitive and developmental outcomes at 5 years of age.[154]

Using the BSID-II to assess the impact of DHA supplementation in pregnant women and infant neurocognitive development in a developing country, Tofail et al. randomly assigned women to receive 4 g of fish oil supplement per day (1.2 g DHA and 1.8 g EPA) or soy oil control from 25 weeks gestation until delivery. BSID-II was performed on a total of 249 infants born within the trial at 10 months of age, and no significant differences were determined between treatment groups.[155] However, it should be noted that the study took place in the developing country of Bangladesh, where 28% of mothers in the study suffered from maternal under-nutrition, were likely to have low DHA stores and possibly lack additional essential nutrients, and where the BSID has not been validated or standardized. Additionally, the investigators explained while they achieved a sample size with >80% power, the study attrition of 38% from the original randomized sample could have significantly impacted study outcomes. Subjects lost could have benefited more from the study intervention because they were at significantly greater risk for neuro-developmental problems due to increased poverty, lower birth size, and increased complications during pregnancy and at birth when compared to subjects that remained in the study.[155]

Another large RCT supplemented 315 pregnant women in Spain, Germany and Hungary with 1 sachet per day of a 15 g milk-based supplemented containing modified fish oil providing 500 mg DHA and 150 mg EPA, 400 μg per day of 5-methyltetrahydrofolate, both or placebo

from 20 weeks gestation until delivery. Neurological development was examined focusing on the detection of minor degrees of neurological function using the Hempel minor neurological dysfunction (MND) at 4 years of age on 175 children born within the study and with the Touwen MND at 5.5 years of age on 157 children, both reaching sample sizes with a power of >80%.[156] Escolano-Margarit et al. reported no significant differences between any of the treatment groups within the study, indicating DHA supplementation during pregnancy may not benefit or harm the long-term neurologic development of children.[156] However, it should be noted that higher DHA levels in fetal and maternal blood are correlated with each other and were also related to improved performance on the neurological assessments at 5.5 years of age.[156,157] In a follow-up study of 154 children from this same birth cohort, Campoy et al. observed no significant differences in the Kaufman Assessment Battery for Children (K-ABC) at 6.5 years of age between study intervention groups.[20] Yet, interestingly, children with a Mental Processing Composite (MPC) score greater than the 50th percentile were born to mothers with significantly higher percent of DHA concentrations in erythrocyte phospholipids ($p<0.001$), indicating prenatal DHA status may have subtle positive effects on the neurological development of the offspring.[20]

Similarly, van Goor et al. investigated the effects of maternal DHA supplementation during both pregnancy and lactation on infant neurological development as well as whether additional AA supplementation could further modulate the effects of DHA. Women were supplemented with 220 mg DHA (n=42), 220 mg of both DHA and AA (n=41) or control soyabean oil per day from 15.6 to 17.4 weeks gestation through 3 months postpartum and infant neurological development was tested at 2 weeks and 12 weeks after birth using a sensitive general movement quality assessment. Supplementation with DHA alone resulted in mildly

abnormal general movements in early infancy compared to the DHA+AA and control groups.[158] In a later follow-up evaluation of children born in the trial, no differences were found between any of the supplemented groups on the Hempel MND and BSID-II at 18 months of age, indicating maternal DHA or DHA+AA supplementation did not influence toddler neurocognitive development.[159] However, it is interesting to note that van Goor et al. did find umbilical blood DHA concentrations were lower in children with simple MND and umbilical blood AA concentrations were higher in children with complex MND compared to children with normal neurological condition.[159]

To assess the effects of DHA supplementation during both pregnancy and lactation, Helland et al. supplemented pregnant women in a RCT in Norway, beginning at 18 weeks gestation through 3 months postpartum with cod liver oil containing 1.18 g DHA per day or corn oil and initially found no improvements in cognitive development of children at 6 and 9 months of age when compared to control.[15] However, a follow-up study of a subset of 34 infants from the study determined that children born to mothers on the cod liver oil had higher composite score on K-ABC at 4 years of age ($p=0.049$).[10] Yet, in an additional follow up at 7 years of age, scores on the K-ABC did not differ between groups, indicating the positive effect from early DHA exposure may not remain through school age.[160] In the double blind, multicenter, RCT DOMInO study, 2320 women were randomized to receive DHA rich fish oil providing 800 mg DHA and 100 mg EPA per day or vegetable oil control supplements beginning at approximately 22 weeks gestation until delivery. Neurodevelopment was assessed on a total of 694 children born within the study using the BSID-III and Makrides et al. did not determine any significant differences in any neurocognitive development outcomes between treatment groups.[127] Yet, the researchers did note that fewer children born to mothers in the DHA supplemented group had

delayed cognitive development (BSID scores <85) compared to the control group, indicating early DHA supplementation may have an effect on preventing cognitive delays.[127]

Most of the evidence for the effect of DHA on infant cognitive development is provided by studies focusing on DHA supplementation during pregnancy, however some studies have examined possible infant benefits of DHA exposure after birth. Postnatal DHA supplementation is most often studied through the use of DHA enriched formulas or maternal DHA supplementation during lactation. Preterm infants are at the greatest risk for neurocognitive development impairments, possibly because they lack the opportunity to accumulate large amounts of DHA commonly occurring during the last trimester of gestation. Therefore, many compelling studies on the effects of DHA on infant neurocognitive development are performed in preterm infants. For instance, O'Connor et al. determined preterm infants fed formula supplemented with DHA in the form of fish oil (DHA 0.26% fatty acids of formula) had higher BSID-II scores and MacArthur Communicative Inventory scores at 12 months of age when compared to age-matched infants that were not supplemented with DHA.[161]

In the DIAMOND study infants enrolled at 1-9 days of age were provided with infant formulas containing one of four levels of supplemented DHA (0% fatty acid DHA, 0.32% DHA, 0.64% DHA or 0.96% DHA) until the infants were 12 months of age. Cognitive function of the 131 children was assessed by BSID-II at 18 months of age and Drover et al. determined significantly higher BSID Mental Development Index (MDI) scores for infants supplemented with any level of DHA (all three groups combined), compared to the no DHA formula group (104.1 vs 98,4; p=0.02).[162] In another RCT in which infants were supplemented with DHA formula (35% fatty acid DHA) or control formulas, Birch et al. demonstrated a mean increase of 7 points on the MDI of the BSID-II.[163] A recent meta-analysis of prevention trials, showed a 3.44

point advantage (95% CI 0.57-6.31) on the BSID-II of preterm infants fed LCPUFA supplemented formulas compared to infants on control formula.[17]

Unlike the controlled DHA concentrations found in infant formulas, the concentration of DHA in breastmilk varies widely worldwide and is strongly associated with maternal DHA intake.[105] Breastmilk DHA levels and infant neurocognitive development have been shown to be positively associated in numerous recent studies.[5,28,68,164] Using multiple regression analysis, Lassek and Gaulin looked at Programme for International Student Assessment (PISA) math scores in 2009 as an index of cognitive performance in children from 28 countries and country-specific breastmilk DHA levels and found DHA was significantly associated with math scores ($p=0.006$), greater than any other control variables including per capita Gross Domestic Product and per pupil education expenditures.[165]

Focusing on lactation and DHA supplementation, the DINO trial investigated the effects of postnatal DHA exposure in very preterm infants (<33 weeks gestation) and infant neurocognitive development.[23] Mothers were supplemented with 3 g DHA per day, a high amount of DHA designed to match the degree of *in utero* accumulation of DHA, 3 g of placebo soy oil, or, if formula was required, a preterm formula containing a matching DHA composition or control formula.[23] Makrides et al. determined no significant differences in infant BSID-II MDI scores at 18 months of age between treatment groups, although significant reductions were seen in the proportion of children with cognitive delays (BSID <85) in the DHA supplemented groups compared to controls.[23] To look at varying DHA supplementation during lactation, Gibson et al. assigned breastfeeding women to 200 mg, 400 mg, 900 mg or 1300 mg DHA per day or placebo for the first 12 weeks postpartum and determined infant erythrocyte DHA status at 12 months was associated with higher BSID-II MDI scores at 1 years of age, however not at 2 years of

age.[22] With continued DHA supplementation during the first 4 months of lactation, Jensen et al. reported children born to women supplemented with 200 mg per day of DHA had significant increases in the BSID-II Psychomotor Development Index (PDI) at 30 months of age compared to children of vegetable oil control supplemented mothers ($p<0.01$).[164] Yet, there were no statistically significant differences between supplementation groups on all BSID testing at 12 months or on the BSID MDI at 30 months. In a follow-up at 5 years of age, Jensen et al. found that children of DHA supplemented mothers performed better on the Sustained Attention Subscale of the Lieter International Performance Scale ($p<0.008$) than children of mothers supplemented with vegetable oil, however, no significant differences between groups were seen on other neuropsychological tests.[166]

While formal meta-analyses could not be performed in 2 recent systematic reviews due to differing infant outcome measurements, both Larque et al. and Lo et al. concluded there is currently insufficient evidence that increased DHA status results in neurocognitive improvements and most studies failed to show sustained benefits.[167,168] However, both reviews did note that children with low DHA status appeared to benefit the greatest with increased DHA during gestation and lactation and DHA did appear to show the most improvements in preventing mild to moderate cognitive delays. Many of the trials reviewed had methodologic problems, issues with study designs, cognitive tests and time frames, and a lack of consistent LCPUFA supplementation. In the 2013 systematic review and meta-analysis, Gould et al. determined similar limitations in performing meta-analysis with a lack of standard cognitive tests throughout the studies, and stated current evidence does not conclusively show a benefit of DHA on neurocognitive outcomes, but also does not disprove the possibility of a benefit.[169] Yet, Gould et al. determined evidence from 2 of the 11 RCT reviewed showed cognitive scores of children 2-5

years old were increased with LCPUFA supplementation during pregnancy or pregnancy and lactation (p=0.01), though the risk for bias is high.[169] Similarly, in reviewing evidence from recent RCT, Dziechciarz et al. and Luchtman and Song found a lack of clear and consistent benefit for n-3 fatty acid supplementation in pregnancy on infant cognitive development.[170,171] The researchers noted that while individual trials reported improvements in some cognitive tests, the improvements were not consistent across trials and did not persist over time.

Two recent Cochrane reviews focused on the benefits of LCPUFA and DHA supplemented infant formulas and cognitive development. Schulzke et al. reviewed 17 trials of LCPUFA supplemented infant formulas and did not find any benefit of supplementation on neurocognitive development in infants born preterm, however, it should be noted in most cases the studies included in the review used a mixture of n-3 and n-6 fatty acids within the formulas.[172] Simmer et al. included 15 studies using infant formulas supplemented with DHA alone or DHA plus AA and concluded that results from the most well-conducted RCT did not show beneficial effects of LCPUFA supplementation of infant formulas on neurodevelopment of the infants born at term.[173] Makrides et al. recent review determined developmental benefits of LCPUFA supplementation in infant formulas are consistently observed in preterm infants, while supplementation in term infants remains inconclusive.[17] The researchers emphasized the need for continued well-designed RCT focused on determining effective timing and dose of DHA supplementation. Similarly, a Cochrane systematic review of 6 RCT focusing on n-3 supplementation during lactation found supplementation had no significant effect on infant neurocognitive development.[174] Current evidence does not conclusively support or refute supplementation of n-3 fatty acids postnatally through infant formulas or breastmilk has a positive effect on infant neurocognitive development.

An emerging research topic is whether boys and girls have different DHA requirements and thus would respond and benefit differently to DHA supplementation. Previous studies have documented lower neurodevelopment scores for boys compared to girls in early childhood, especially in preterm infants.[175] Using stepwise regression analysis, Lassek and Gaulin looked at 4154 American children aged 6-16 in the U.S. Third National Health and Nutrition Examination Survey (NHANES III), and after controlling for a variety of confounders, discovered a positive relationship between n-3 fatty acid intake of 1 g or greater and cognitive test scores in both males (0.19 point increase) and females (0.38 point increase). However, in female children, the positive effects were twice as strong as in males ($p=0.001$), suggesting female children may benefit more cognitively from increased n-3 intakes.[176] Utilizing the Danish National Birth Cohort, Lauritzen et al. investigated the effects of supplementing breastfeeding mothers below the median fish intake with 1.5 g of fish oil containing 900 mg DHA per day and infant cognitive development.[177] No significant differences were found in infants born in the study at 4 months, 1 year and 2 years of age between treatment groups on any cognitive development test, however, when analyzing based upon gender, a significant positive association between fish oil supplementation and problem solving on the Infant Planning Test at 9 months of age was found in girls, where no positive association was determined in boys. Additionally, it should be noted that word comprehension at one year of age was inversely associated with 4 month infant erythrocyte DHA concentration and at one year of age, passive vocabulary was lower in children born to the fish oil supplement group compared to the olive oil control ($p<0.05$), however no differences were found by 2 years of age.[177]

Supplementing early preterm infants (<33 weeks gestation) with high dose DHA (~1% total fatty acids) formula from day 2-4 until term age, at 18 months resulted in no significant

differences in overall neurocognitive development as shown on the BSID MDI. However, when separated the data by gender, the MDI scores for girls with high DHA supplementation, through DHA supplemented mothers breastmilk or DHA enriched formula, were significantly higher than girls on control formula (p=0.03; adjusted mean difference 4.7; 95% CI 0.2-9.2), with no difference discovered among the boys.[23] Additionally, higher DHA supplementation reduced the risk of mild [BSID developmental quotient (DQ) <85; p=0.01] and severe (BSID DQ<70; p=0.02) cognitive delay for girls at 18 months of age, while no effect was noted for boys.[23] A review of two large-scale intervention trials of DHA supplementation of preterm infants found significant differences between boys and girls in response to DHA supplementation and neurocognitive development.[178] These studies indicate that boys and girls respond differently to DHA supplementation and opens up a new avenue of research in the field of DHA and neurocognitive development.

Breastmilk and DHA

Important cognitive, visual, and motor development milestones are reached within the first two years of life and the lipids of breastmilk are of critical importance by providing not only a major energy source, but also LCPUFA that infants are unable to synthesize and are required for normal growth. The American Academy of Pediatrics recommends exclusively breastfeeding infants for the first 6 months, with continuation of breastfeeding for 1 year or longer.[179] Compared to infants who never breastfed, infants exclusively breastfed for 4-6 months showed significantly lower incidences of several illnesses, including respiratory tract illnesses, gastrointestinal illnesses and dermatological conditions.[180,181] Additionally, breastfed infants perform better on visual and cognitive development tests compared to formula-fed infants.[182-184] Breastfed infants were found to have improved psychomotor development at 4 months and

higher IQ throughout childhood when compared to infants fed formula lacking LCPUFA[185]. Lucas et al. concluded that children breastfed as infants had significantly higher IQ scores at 7.5 to 8 years of age than those not breastfed.[186] A recent meta-analysis of controlled clinical trials concluded that after controlling for confounding factors, IQ in breastfed infants was higher than formula fed infants beginning as early as 6 months of age.[187] This beneficial effect of breastfeeding was still present at 15 years of age, with the cognitive benefits increasing with increased breastfeeding duration.

The major components of breastmilk are proteins, carbohydrates and lipids, with lipids representing the second largest fraction at 40 g/L.[188] DHA in breastmilk ranges from 0.17% to 1.0% of total fatty acids and research indicates lactating women supplemented with DHA see increasing levels of breastmilk DHA based upon supplement concentrations.[189,190] DHA, like other fatty acids, is rapidly transferred into milk with no known selectivity between fatty acids. Breastmilk DHA can be drastically altered within 2-3 days based upon maternal intake of DHA.[191,192] Studies using stable isotope tracers have provided evidence of DHA from the maternal diet transfers directly into breastmilk.[193] Breastmilk DHA content has been shown to increase by 76% and infant plasma phospholipid DHA by 35% when the mother is exclusively breastfeeding and supplemented with 200 mg of DHA per day.[164] However, supplementing with an ALA source, such as flax seed oil, with no preformed DHA, breastmilk DHA content does not increase.[194] Infant formulas, while mimicking components of human breastmilk, often lack LCPUFA or contain fatty acid ratios not as beneficial to infant development as compared to breastmilk, and, consequently, may not be able to provide the same benefits to the infant as breastmilk.[195]

After birth, breastmilk or formula are the sole sources of n-3 and n-6 fatty acids to the infant, therefore, it is essential for development that the infant receive adequate amounts of these important LCPUFA. The concentrations of DHA in breastmilk are correlated with both visual acuity and neurocognitive development.[164,190,196] Piglets fed formula containing corn oil and no DHA showed marked deficits in DHA accretion in the brain and retina.[197] An autopsy study of human infants, Makrides et al. reported lower brain and erythrocyte DHA in infants who had been fed formula lacking DHA compared to breastfed infants ($p<0.005$). In addition, Makrides et al. also found brain cortex DHA increased in breastfed infants over time ($r^2=0.72$, $p<0.01$), indicating the duration of breastfeeding affected DHA levels of the infant ($r^2=0.62$, $p<0.01$).[198] Due to early birth and decreased DHA accretion *in utero*, preterm infants are born with lower DHA levels compared to term infants and are, therefore, at an even greater need for receiving DHA externally from breastmilk to develop at a normal, healthy pace.[199] Preterm infants not breastfed and fed infant formula lacking in LCPUFA had lower DHA status, decreased growth and showed a reduction in mental development from 9 to 12 months of age.[200]

The Danish National Birth Cohort, examined 25,446 children and found both fish intake during pregnancy and the duration of breastfeeding were independently associated with improved early child development. Children born to mothers with the greatest fish intake during pregnancy (OR=1.29; 95% CI 1.20-1.38), when compared to the lowest, and who breastfed for ≥10 months (OR=1.28; 95% CI 1.18-1.38), compared to ≤1 month, had higher child development scores at 18 months of age.[150] Unfortunately, in this study, fish intake during lactation was not assessed to determine a possible influence of increased breastmilk DHA on early child development.

Maternal genetics and breastmilk DHA

The concentrations of fatty acids, including DHA, in breastmilk are highly dependent upon the mother's diet, with breastmilk levels increasing proportional to maternal dietary increases.[190] In addition to dietary supply, DHA in breastmilk can be endogenously derived from precursor fatty acids by elongation and desaturation, of which the rate-limiting enzymes in this cascade are believed to be the Δ6-desaturase enzyme.[39] The Δ6 and Δ5-desaturase enzymes are encoded by FADS2 and FADS1 genes, respectively, which are arranged in a head-to-head orientation on chromosome 11q, with a third desaturase gene, FADS3, which function is currently unknown. In recent years, several studies have shown that SNPs in the FADS1FADS2 gene cluster are associated with altered DHA concentrations in erythrocytes, plasma and breastmilk.[18,19]

Several recent studies demonstrate SNPs within the FADS1 FADS2 gene cluster significantly influence erythrocyte and plasma LCPUFA, especially DHA, concentrations.[201-205] In a genome wide association study of plasma LCPUFA concentrations, Tanaka et al. studied 1,075 participants and found the strongest association between genetics and LCPUFA proportions of plasma fatty acids was in the FADS1 FADS2 gene. The SNP with the most significant association was rs174537, in which homozygous minor allele carriers had higher plasma concentrations of LA and ALA and lower concentrations of AA, EPA, docosapentaenoic acid (DPA) and DHA.[205] Utilizing infants participating in the KOALA and LISA birth cohorts (n=879), Rzehak et al. found minor allele carriers of all five SNPs within the FADS1 FADS2 gene cluster studied (including rs174575, rs174546, rs174556, rs174561 and rs3834458) showed higher levels of precursor fatty acids LA, dihomo-γ-linoleic acid (DGLA) and ALA with decreased levels of desaturase products, AA, EPA, DPA and DHA.[204] Confirming these findings

in pregnant women, Koletzko et al. found pregnant women participating in the large ALSPAC birth cohort (n=4457) that were minor allele carriers of 12 FADS1 FADS2 SNPs studied had increases in desaturase substrates, such as LA, DGLA and ALA and, and decreased amounts of desaturase products, including AA and DHA.[202] Studying the effects of FADS SNPs on infant DHA status, Harsløf et al. found infants homozygous for the minor allele of rs174575 and rs17448 had a decrease in erythrocyte DHA status, while minor allele carriers for rs1535 had increased DHA status, when compared to wild type carriers.[206]

Xie and Innis provided evidence that SNPs in the FADS gene cluster are associated with breastmilk DHA concentrations. The researchers recruited, assessed diets and determined the presence of 6 previously identified SNPs in the FADS1 FADS2 gene cluster in 54 pregnant women. Women homozygous for the minor allele of the rs174575 SNP in the FADS2 region had lower levels of AA ($p=0.015$), docosapentaenoic acid (22:5n-3; $p=0.003$), EPA ($p=0.01$) and DHA ($p=0.044$) in breastmilk, regardless of dietary intake, compared to women homozygous for the major allele.[19] A subsequent study by Molto-Puigmarti et al. determined plasma and breastmilk DHA concentrations based on common (≥18% frequency) FADS1 FADS2 SNPs in 309 Dutch women as part of the prospective KOALA Birth Cohort Study. DHA proportions in plasma ($p<0.01$) as well as breastmilk ($p<0.05$) were lower in women homozygous for the minor allele of all three SNPs studied, FADS1 rs174561, FADS2 rs174575 and intergenic rs3834458, than in women homozygous for the major alleles.[18] This study found similar results to the study by Xie and Innis that, while breastmilk DHA levels did increase with increased dietary intake of DHA, the women homozygous for the minor alleles could not compensate for the lower incorporation of DHA into breastmilk by increasing fish and fish oil intake.

Studies have linked maternal genetic variations in the FADS1FADS2 gene cluster with child cognitive development. Data from two population-based birth cohorts (n=740) was examined in Morales et al. 2011 study in which breastfed children of mothers with SNPs coding for lower FADS1 activity, higher FADS2 activity and higher DHA levels of breastmilk showed significant cognitive advantages at 14 months on the BSID-III.[25] Caspi et al. determined SNPs within the FADS1FADS2 gene cluster interacted with breastfeeding to provide a significant IQ advantage, specifically major allele of rs174575 children showed a 6.4 IQ advantage at 7-13 years of age over children not breastfed ($p<0.001$).[26] Additionally, the study found that children who were minor allele carriers did not have any IQ advantage to being breastfed, suggesting the SNP somehow influences the positive benefits breastfeeding has on neurocognitive development. However, in a study of nearly 6000 children, Steer et al. determined no significant differences between breastfeeding and IQ based upon genetic variations, indicating the influence of genotype on IQ remains inconclusive.[27] Genetic variations within the FADS1FADS2 gene cluster have also recently been shown to have an impact on adult cognition, cardiovascular disease, eczema and numerous other atopic diseases.[201,207]

Body composition and DHA

Currently, there is mounting evidence that increased LCPUFA, specifically DHA intake, can reduce body fat in both adults and children. Health during adulthood may have origins in both fetal and early infant life, a time in which the body may be programmed both positively and negatively.[208] The relationship between breastfeeding and childhood obesity was investigated by Arenz et al. 2004 systematic review and meta-analysis in which the researchers determined after analyzing 9 studies with over 69,000 participants that breastfeeding significantly reduced the risk of obesity during childhood (OR=0.78; 95% CI 0.71-0.85).[209]

More specifically to LCPUFA, numerous animal studies have shown diets high in LCPUFA result in lower body fat accumulation and reduces obesity.[210,211] In humans, DHA has been shown to reduce body fat by increasing oxidation and suppressing fat deposition.[212] DHA supplementation in the maternal diet may influence the growth and deposition of adipose tissue both in childhood and adulthood.[213] Donahue et al. determined higher maternal DHA+EPA intake during pregnancy and higher DHA plus EPA umbilical cord plasma phospholipids were associated with lower adiposity in children at 3 years of age, as measured by skinfold thickness [-0.31mm (95% CI -0.58,-0.04mm) for maternal diet and -0.91mm (95% CI -1.63,-0.20mm) for cord plasma] and odds of obesity (OR=0.68; 95% CI 0.50-0.92).[213]

Pedersen et al. showed a significant association between breastmilk DHA and child body mass index (BMI) from 2 to 7 years of age in 222 mother-infant pairs in the Copenhagen Prospective Study on Asthma in Childhood birth cohort.[214] In a double blind RCT in Germany, Bergmann et al. supplemented pregnant women beginning at 21 weeks gestation until the third month of lactation with a daily vitamin control or one containing 200 mg DHA and looked at the impact of DHA on postnatal growth. After adjusting for confounders, Bergmann et al. discovered the DHA group had significantly decreased BMI (p=0.037) and weight (p=0.046) of the infants, which resulted in significantly lower BMI (-0.76 kg/m^2; 95% CI -0.07, -1.46) and weight (-601g; 95% CI -171, -1030 g) of the children at 21 months of age when compared to the control group.[215] However, it is important to note that at a 6 year follow-up of these children, there was no longer any statistically significant differences between any anthropometric measures between both groups.[216] In a systematic review, Rodriguez et al. reported mixed results and concluded LCPUFA intake either maternally during pregnancy and lactation or from supplemented formulas does not adequately explain body composition differences in infants and

children.[217] While current data does not suggest or refute beneficial effects early DHA supplementation may have on body composition later in life, it is an interesting avenue for future DHA research.

Possible adverse effects

In spite of a large amount of data on the beneficial effects of n-3 fatty acids, specifically DHA, on neurocognitive development and improvements in other infant outcomes, it should be noted that supplementation of n-3 fatty acids is not totally without risk. In their review of DHA supplementation during pregnancy and lactation in animal models, Davis-Bruno et al. concluded that balancing the supplementation ratio of DHA to AA is necessary for optimal retinal and neurocognitive development, however, prolonged excessive DHA supplementation may be detrimental by increasing oxidative stress and apoptosis, causing adverse effects on brain development, growth and even survival.[149] The beneficial aspect of LCPUFA on cardiovascular disease through the antithrombotic actions in the inhibition of fatty acid oxidation and platelet aggregation could also cause adverse effects in high risk populations, such as women with complicated pregnancies or those at risk for stroke or bleeding.[218] Furthermore, the anti-inflammatory benefits of DHA could result in a potentially harmful suppression of an appropriate immune response in an immune-compromised individual.[219] Additionally, *in vitro* studies have indicated chemical oxidation products formed from LCPUFA can produce mutagenic and carcinogenic responses.[220,221] Potentially harmful oxidation products can occur within cells and also through improper storage of LCPUFA, such as exposure to UV light or high temperatures, and without naturally occurring protective antioxidants that accompany LCPUFA in foods with high endogenous LCPUFA, such as fish and seafood, LCPUFA supplementation could be a health concern.[219,222] While there are no current reports in the literature of LCPUFA

supplementation and adverse birth outcomes, it is important to consider possible harmful outcomes when supplementing high-risk populations or when supplementing high doses, over long time periods.

Discussion and conclusions

Omega-3 fatty acids are important for human life, and DHA in particular must be obtained preformed from the diet, either from seafood or fish oil supplements. During pregnancy, DHA requirements increase as the fetus grows and more DHA is transferred from maternal supplies into the fetus for neural growth and development. Increased DHA status during pregnancy is associated with increased gestational age, decrease risk for preterm birth, and increase infant birth weight, all much to the improvement of infant health. While RCT are increasingly reporting varying results on the benefits of DHA supplementation early in development, both epidemiological and interventional studies provide evidence that DHA has an important role in healthy growth and neurocognitive development. Additionally, while human milk is important for providing growth and neurocognitive benefits alone, increasing the portion of DHA in the total fatty acids of milk provides even greater benefits to the child through improved cognitive and behavioral development, and even possibly reducing BMI and the risk of obesity later in life.

While DHA has been shown to improve attention, information processing speed, and learning and memory, it is important to emphasize many studies have failed to provide conclusive results on the benefits of DHA during early development.[144,158,164,182,223-227] The majority of trials to date often have relatively small sample sizes (n<100 per group) with high attrition rates (up to >80%), often far above the follow-up rate of ≥80% that is considered the

minimum acceptable for minimizing attrition bias.[228] Studies are often powered improperly to determine large differences rather than the modest effects that would be expected in DHA intervention trials. Additionally, supplementation varies greatly between studies, in amount, length of supplementation and time frame, usually either pregnancy or lactation. It is important to note that there is currently no standard supplementation guideline for which studies should be designed, with optimal supplementation time frames and dosages currently unknown for infant development.

Contradictory findings amongst RCT could be due to several important factors that should be considered, including the source of supplemented DHA, too low of a supplemented dose to see any beneficial effect, and the DHA supplementation period was short or only during pregnancy or only during lactation. Additionally, most of the current intervention studies are conducted in populations with variable average DHA intakes from food and regularly fail to take into account dietary intakes of DHA aside from the treatment provided. Consumption of DHA directly from food sources can have a drastic effect on the overall DHA status of individuals and could possibly skew results to be non-significant, especially when comparing groups only by treatment and not DHA dosing effects.

When focusing on neurocognitive development, inconsistency in study results could be due to the lack of globalized standard tests used to measure cognitive developments, especially in infants and young children. Standardized infant development tests, such as the BSID, use a variety of tasks to assess cognitive function and may provide a reliable estimate of later cognitive ability. The BSID is widely accepted and used throughout cognitive studies, and scores have been associated with IQ scores at 4 years of age in DHA supplementation published studies.[229] Studies that assess more specific aspects of cognitive functions, such as BSID subtests, means-

end problem solving and attention tests, have reported more enhanced improvements in children supplemented with DHA.[10,16,162,229]

However, current assessments of early cognitive function, such as the BSID or FTII, may not be sensitive enough to detect the specific effects DHA supplementation may have on certain aspects of cognitive development at certain time points. For instance, both Jensen et al. and Helland et al. reported no effects of maternal DHA supplementation on infant neurocognitive scores during the first year of life, yet found significant improvements once the children reached preschool ages.[10,15,164,166] In addition to measurement means, the time at which to test children also remains highly debatable, with conflicting results at all ages, there appears to currently not be a defined proper cognitive testing age that is predictive of later IQ and long-term DHA supplementation effects. It is possible that the cognitive assessments currently used in RCT may not be ideal for the ages they are utilized or that the benefits of DHA supplementation early in development are not observable until cognitive function further develops and becomes more complex. Additionally, the use of the BSID as a reflection of infant cognitive development may no longer be appropriate. The BSID was originally designed as a measurement of infant cognitive development and to identify developmental delays, the test is not longer conclusively associated with school age IQ.[230,231] Recently, more specific tests of attention and information processing have shown to possibly be betters measures of infant cognitive function and better predictors of later childhood IQ than the BSID and such measures should be considered when developing future research studies.[232,233]

Much remains to be learned regarding the physiological effects of DHA on the developing child. It is clear by a growing body of evidence that increasing maternal DHA intake during pregnancy and lactation and prolonging breastfeeding may benefit child development.

Currently, low levels of dietary DHA consumption throughout westernized countries are of a primary concern and low DHA status in pregnant and lactating women may lead to suboptimal neurological development of the developing infant. While therapeutic recommendations exist for pregnant and lactating women based upon recent research, the recommendations are not standardized and the Institute of Medicine has currently not made any official dietary recommendations for Americans.[47,49] In addition to promoting increased DHA intake during pregnancy, recommendations should be specified to continue increased DHA consumption during lactation, and breastfeeding should be promoted and supported to optimize a variety of child health outcomes.

Future research should focus on optimizing supplementation recommendations, both dosing and duration, considering both short- and long-term effects of DHA on both preterm and term infants to improve infant development. Additionally, inter-individual differences, such as maternal and infant genetic variations in fatty acid desaturase activities and gender, and their impact on DHA developmental outcomes should be studied further. Future intervention studies should increase supplementation times to include both pregnancy and lactation, promote breastfeeding, control for dietary DHA intake in addition to supplementation, and should also be designed to examine not only visual and cognitive development, but also consider growth, body composition and possibly many other attention, behavioral and health disorders.

In conclusion, increased DHA status of pregnant and lactating women through seafood or DHA supplement intake could positively impact numerous infant outcomes, including increased gestational age, decreased preterm birth risk, improved infant growth and enhanced neurocognitive development. However, with much variation in the research, the current evidence does not conclusively support or refute that increased DHA intake during pregnancy and/or

lactation positively benefits any infant development outcome and continued research is necessary to determine if a true association exists.

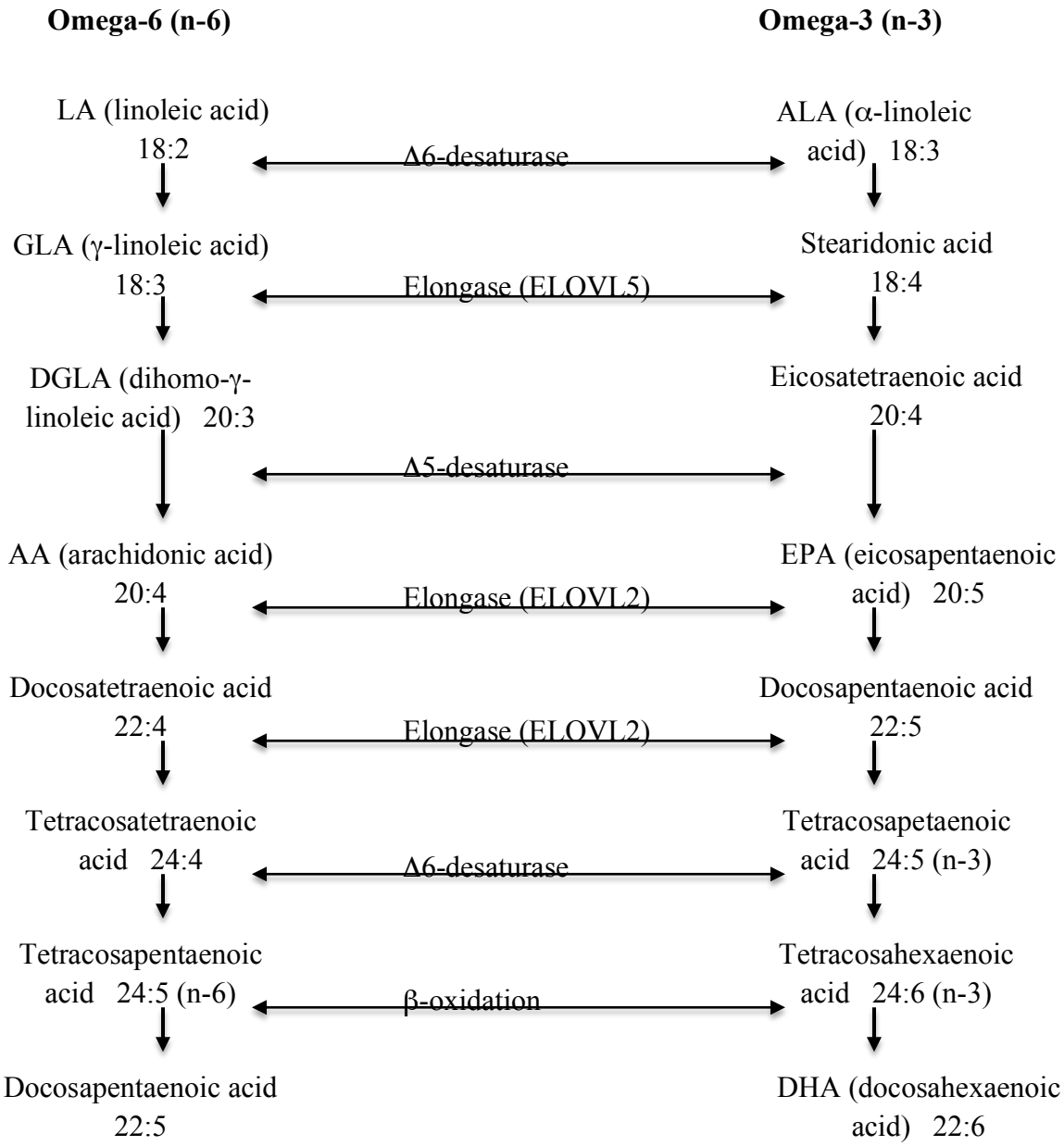

Figure 2.1. Diagram of omega-6 and omega-3 fatty acid metabolism.

CHAPTER 3

METHODS

This chapter describes the methods used in this study.

Subjects

The Omega Smart Baby Project was a double-blinded, randomized, placebo controlled clinical trial. 115 women with singleton pregnancies were randomized to receive 300 mg of purified DHA or placebo between February 2010 and October 2011. Women were recruited from Associates and Family Medicine and the Women's Clinic of Colorado in the Fort Collins, Colorado area. A trained paraprofessional breastfeeding coach recruited women on or before their oral glucose screening appointment, between 24-28 weeks of gestation. Included women were 18-42 years of age with singleton pregnancies and were willing to breastfeed exclusively for the first 3 months of life. Exclusion criteria included maternal age <18 years, multiple fetuses, diabetes, HIV positive status, chronic illnesses or other conditions that could preclude breastfeeding and any known allergies to seafood or fish oils. The Institutional Review Board at Colorado State University approved all protocols and procedures (Appendix A). All women provided written informed consent prior to participation (Appendix B).

Women were randomized to receive the study supplement of highly purified tuna fish oil containing 300 mg DHA and 67 mg EPA provided as HiDHA tuna oil (Nu-Mega Oil, Clover Corportation, Ltd; Appendix C) prepared as one hard capsule or Sunola, a high oleic acid sunflower oil placebo. The dose was selected based on previous pilot data with a power of 0.05% at the 80% confidence level. A block randomization procedure was used to assign women to

treatment. Women were provided with the study supplement at their oral glucose screen at 24-28 weeks of gestation. Women were instructed to keep supplements in the provided container in a freezer to maintain quality and begin taking one capsule per day for the remainder of their pregnancy and until 3 months of breastfeeding were complete. Study supplements were provided in two opaque bottles with the study label attached, the first given at the entrance into the study and second at the first meeting between the breastfeeding coach and mother after giving birth.

A food frequency questionnaire (FFQ) validated against erythrocyte DHA was used to estimate total DHA intake from food, prenatal vitamins and other sources (Appendix D). The FFQ included sources of DHA intake from fish and seafood, DHA enriched food products such as eggs or milk, and any fish oil or DHA supplements, including all prenatal vitamin information. Information was quantitated using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 22. The FFQ was validated, with estimated DHA intake per week positively correlating with the percent total DHA in erythrocyte fatty acids ($r=0.37$, $p=0.0002$). Total weekly and daily DHA intake from the FFQ was calculated and verified with a 7-day diet record data (Appendix E). The 7-day diet record information was analyzed using Nutritionist Pro (Axxya Systems, Stafford, TX) software and total daily DHA intake from food was calculated. Participants received a printed copy of their 7-day diet record analysis as a benefit to the study. Total dietary intake of the infants was gathered at 6 and 10 months of age in a 24-hour recall. Sociodemographic data, including age, race and marital status, were collected from each woman at the entrance into the study (Appendix F). Additionally, the date of the last menstrual period (LMP) and estimated due date were collected. Self-reported pre-pregnancy weight and height was used to determine maternal pre-pregnancy

body mass index (BMI). Infant birth information was gathered by the breastfeeding coaches at the first visit after delivery.

Sample collection and fatty acid analyses

A baseline blood sample was collected from women during their oral glucose screen between 24-28 weeks of gestation and stored briefly at 4^{o}C until separated. Blood samples were collected in a Vacutainer containing EDTA and separated by centrifugation within 24 hours into erythrocyte, plasma and buffy coat fractions and stored at -80^{o}C until further analysis. Breastmilk samples were obtained at 2 and 4 months postpartum and expressed using manual or electric breast pumps into sterile collection cups and transported on ice to the laboratory and immediately frozen and stored at -20^{o}C. Lipids were extracted from maternal erythrocytes, plasma, and 2 and 4 month breastmilk samples using a modified Folch extraction (chloroform:methanol 2:1 v/v). Plasma phospholipids were further separated by thin layer chromatography and all lipid extracts were resuspended in 0.5 ml chromatographic grade hexanes. Fatty acid methyl esters (FAME) were prepared on all lipid extracts by direct transmethylation using 14% boron trifluoride in methanol (Sigma Chemical Co, St. Louis, MO). FAME were analyzed via gas chromatography on an Agilent 6890 chromatograph, using a ramped temperature program and flame ionization detection and a J&W DB225 column. Individual fatty acids were identified by comparison of retention times with known FAME standards (Nuchek Prep, Elysian, MN).

Selection of SNPs and genotyping

Two SNPs in the FADS1 FADS2 gene cluster were included for SNP analysis, FADS1 rs174561 and FADS2 rs174575. The SNPs were chosen based upon previously reported

associations with differences in erythrocyte, plasma phospholipid and breastmilk DHA proportions, as well as associations with later child cognitive development. Additionally, the minor allele frequency for both SNPs was previously described as at least ≥ 18%, therefore, the expected number of subjects homozygous for the minor alleles in this study population would exceed 10.[18,19,26,27] DNA was extracted from the buffy coat (white blood cells) collected from baseline maternal blood with the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). SNPs were genotyped with TaqMan SNP Genotyping assays (Applied Biosystems, Foster City, CA) with real-time polymerase chain reaction according to the manufacturer instructions using a StepOnePlus Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

Breastfeeding

Exclusive breastfeeding was supported using a peer coaching model adapted from the in-home breastfeeding support program, developed in North Carolina and tested in a WIC population. This model was adapted to a middle class, educated population and updated to meet current breastfeeding guidelines and policies.[234,235] The program paired each pregnant woman with a well-trained paraprofessional breastfeeding coach, which provided support to the mother on an ongoing basis throughout lactation. The coach provided breastfeeding education prior to delivery, both in person and via phone or email, and began postnatal visits within days of delivery, with monthly contact throughout the study duration of 12 months. Coaches provided education, answered questions, assessed breastfeeding and provided recommendations and assistance to resolve any problems and helped the mothers reach their own personal breastfeeding goals beyond that of the study. In addition to the free breastfeeding support provided, as incentive to the study, each mother was provided with a complementary manual breast pump and had access to a medical grade electric pump if deemed necessary by the coach.

Exclusivity of breastfeeding was monitored and recorded by the coaches as reported by the mothers. Exclusive breastfeeding was classified as no food or formula other than breastmilk for the first 3 months of life. Mothers were educated and encouraged to exclusively breastfeed through 6 months of life by their coaches and educated on proper progression of introducing and increasing infant appropriate foods according to the USDA Start Healthy Feeding Guidelines.[236]

Infant cognitive testing

The BSID-III Mental Development Index (MDI) subscales were administered on infants at 4 and 12 months of age by a trained technical assistant overseen by a licensed clinical psychologist in the Psychology Department at CSU. The BSID-III was selected as a measurement of neurocognitive development due to its ease of administration and for comparison to previous studies. The BSID-III MDI cognitive, language, social-emotional and general adaptive behavior scales were completed. The MDI has a mean score of 100 with standard deviation (SD) of 15 and includes items to measure performance in areas of knowledge, memory, problem solving, sensory perception, and early language. The BSID is widely accepted and used throughout cognitive studies, the test has excellent within age test-retest reliability and scores have been associated with intelligence quotient (IQ) scores at 4 years of age in DHA supplementation published studies.[229] All BSID scores reported are composite scores and are, therefore, age corrected for each scale. To control for confounding effects of maternal IQ, mothers completed a maternal IQ test using the abbreviated Wechsler Adult Intelligence Scale (WAIS), including the block design and vocabulary subtests, which are highly correlated with full scale IQ (FSIQ; r^2=0.84).[237] To control for the confounding effect the degree of stimulation in the home environment may have on cognitive development, the mothers completed a self-administered Home Screening Questionnaire (HSQ) at 9 months of age (Appendix G).[238] Infant

characteristic questionnaire was completed by the mothers at 10 months of age to measure parental perception of infant temperament, focusing on difficult temperament (Appendix H).[239]

Gestational length and infant anthropometric measurements

Gestational length was calculated in days using the LMP method and infant date of birth. Infant anthropometric data was obtained from pediatric medical records. The mother reported infant birth weight, length and head circumference measurements to the breastfeeding coach at the first contact just after birth, and reported 2 month weights and heights after the 2 month well baby visit. Anthropometric data at birth and 2 months of age was normalized by expressing them as z scores based on term infants of the same age and sex by using parameters provided in the data files from the Centers for Disease Control and Prevention (CDC) growth charts released in 2000 (www.cdc.gov/growthcharts). Pre-pregnancy maternal BMI was calculated based on self-reported information on the study entrance form.

Data analysis

Participating women, all data collectors and investigators were blinded to supplement allocation until all study children were 12 months of age and had completed the cognitive testing. After all study data was collected, the study was unblinded only to study investigators for analysis, all other personnel collecting data will remain blinded because this cohort of children may be assessed for future development. Intent-to-treat (ITT) procedures were maintained throughout. Power was based on a previous pilot trial determining the effect DHA supplementation during pregnancy has on improved infant BSID scores at 4-6 months, 32 infants per supplementation group are necessary to reach a power of 0.05% at the 80% confidence level. Total daily DHA intake was calculated for each woman by adding estimated dietary DHA

intakes, maternal DHA supplementation (if applicable) and DHA study supplement. Data was analyzed based on treatment group, placebo versus DHA, as well as daily DHA intake groups broken into three groups: low = 0-299 mg per day DHA, medium = 300-599 mg per day DHA, high = ≥600 mg per day DHA. DHA cut-off points for the intake groups were determined on the basis of those falling below, at the median recommended intake and above the currently recommended intake.[47,48]

Statistical analysis was completed using SPSS statistical software (SPSS, Chicago, IL) and GraphPad Prism V4.0 (GraphPad Software, San Diego, CA). Results are expressed as means ± SD, unless otherwise specified. Deviations from Hardy-Weinberg proportions for the genotypes of each SNP were tested using chi-square tests.[240] Two-tailed *t* test was used to analyze results based upon treatment and one-way analysis of variance (ANOVA) was used to compare means between all daily DHA intake groups. The data for each SNP was analyzed separately and categorized based upon genotype (homozygous for the major allele, MM; heterozygous, Mm; homozygous for the minor allele, mm) and/or daily DHA intake group (low, medium or high) or treatment group (placebo or DHA). Tukey's post hoc tests were conducted on all statistically significant ANOVA results to determine if differences existed among group means. Correlations were computed using Pearson's correlation coefficient. Statistical differences in fatty acid proportions and BSID-III scores were also tested with a two-way ANOVA and ANCOVA to determine possible interactions between SNP genotype and daily DHA intake level. Data was adjusted for potential confounders, when appropriate, including, maternal age at delivery, maternal BMI, maternal IQ, breastfeeding duration, gestational age and the effects of the home environment on cognitive development via the HSQ.

CHAPTER 4

EFFECTS OF DOCOSAHEXAENOIC ACID INTAKE DURING PREGNANCY AND LACTATION ON INFANT GROWTH AND NEUROCOGNITIVE DEVELOPMENT: A RANDOMIZED CONTROLLED TRIAL[1]

*Background***:** Maternal docosahexaenoic acid (DHA) intake during pregnancy and/or lactation has been positively associated with infant growth and cognitive development. However, the majority of studies to date have not examined the effect of supplementing mothers with DHA during both pregnancy and lactation and fail to account for total maternal DHA intake.

*Aims***:** To determine the effect of increasing DHA intake during pregnancy and lactation on infant neurocognitive development in the first year of life.

*Study Design***:** A randomized, double-blinded, placebo-controlled design was used.

Subjects: 115 pregnant women were randomized to receive purified tuna oil supplement containing 300 mg of DHA and 67 mg EPA per day or an identical placebo (Sunola Oil) for the last trimester of pregnancy through the first 3 months of lactation.

Outcome Measures: Neurocognitive development was measured using the Bayley Scales of Infant Development III at 4 and 12 months of age.

Results: Infants born to mothers with >600 mg DHA/day showed significantly higher scores on the 12 month cognitive scale of the BSID-III ($p=0.018$) compared to infants born to mothers with <300 mg DHA/day. Infants born to mothers in the DHA treatment group had an increase of 4.5

[1] In preparation for *Early Human Development*

days in gestational age (p=0.048) and significantly lower incidence of preterm birth (5%; n=3) compared to infants born to mothers in the control group (18%; n=10; χ^2=4.97, p=0.026).

Conclusions: An intake of ≥600 mg DHA/day during the third trimester of gestation throughout the first three months of breastfeeding was associated with enhanced neurocognitive development of the infants.

Introduction

Docosahexaenoic acid (DHA), a 22 carbon, six double bond omega-3 long chain polyunsaturated fatty acid (LCPUFA) is present in high concentrations in brain and neural tissue. Accumulation is greatest during the third trimester of gestation through the first 2 years of life.[2] During the last trimester, it is estimated the fetus acquires 67-75 mg per day of DHA, accounting for 80% of brain DHA accumulation between week 26 and 40 of gestation.[67,107] Prior to birth, the fetus acquires DHA through placental transfer of DHA, which is influenced by DHA in the maternal diet, enhanced mobilization of DHA from maternal adipose stores, and maternal DHA synthesis.[4] Synthesis of DHA from the essential dietary precursor n-3 alpha-linolenic acid (ALA) occurs in humans, but the conversion is estimated to be only <0.1-1%.[7,8] Women of child-bearing age may convert ALA to DHA more effectively due to the proposed influence of estrogen on DHA synthesis, yet the conversion may be insufficient to meet needs during periods of rapid growth and development.[7] Low conversion rates suggest a requirement for preformed DHA obtained directly from the diet or supplements, during pregnancy and lactation. While no current daily U.S. recommended intake exists for DHA, the European Influence of Dietary Fatty Acids on the Pathophysiology of Intrauterine Foetal Growth and Neonatal Development (PERILIP) group recommends pregnant and lactating women receive at least 200 mg per day of

preformed DHA, through supplementation or consuming one to two portions of fatty fish high in n-3 LCPUFA per week.[38]

Studies have demonstrated numerous benefits of maternal fish and seafood intake and/or DHA supplementation during pregnancy, including increased gestational age, decreased preterm birth incidence, and improved infant growth and cognitive development.[9,11,13,115,118,124,137,138] The large Avon Longitudinal Study of Parents and Children demonstrated that women consuming greater than 340 g of fish per week gave birth to children with decreased risk for poor performance on age appropriate tests of cognitive development compared to mothers with low seafood intake during pregnancy.[11] Randomized controlled trials (RCT) provide some evidence linking increased DHA intake during pregnancy with enhanced neurologic development and improved cognitive measurement scores, however, such trials report varying results.[9,10,16,20,127,155,160] Furthermore, postnatal DHA supplementation studies have shown no conclusive effect of DHA intake on infant growth and neurocognitive development and, when significant, the most dramatic effects are seen in preterm infants.[22,23,161-164] Postnatally, DHA is obtained from breastmilk or enriched infant formulas, and breastmilk DHA concentration is directly influenced by maternal intake.[190,192]

Several systematic reviews and meta-analyses have reported that the benefits of DHA supplementation on neurocognitive development are currently inconclusive.[167-170] Such studies remain mixed due to the large variation in DHA supplementation length and dose as well as varying cognitive tests. Yet, many studies that report inconclusive results between DHA treatment groups often have found a significant association between DHA status and infant neurocognitive development.[20-23] Additionally, non-significant studies tend to analyze results by treatment groups and rarely account for DHA intake from food, which would allow for analysis

by daily dose of DHA that could dramatically affect results determining an association between DHA supplementation and improvements in infant growth and neurocognitive development.

The present study was designed to examine the effects of DHA intake during pregnancy and lactation on infant neurocognitive development using the BSID-III at 4 and 12 months of age. Increasing maternal intakes of DHA during the important period of brain growth in the last trimester of gestation through the first few months of lactation was hypothesized to improve scores on the BSID-III at 4 and 12 months. Additionally, increasing maternal DHA status would increase breastmilk DHA, and correlate with improvements in neurocognitive developments in infants.

Methods

Subjects

The Omega Smart Baby Project was a double-blinded, randomized, placebo controlled clinical trial. 115 women with singleton pregnancies were randomized to treatment groups between February 2010 and October 2011. A trained paraprofessional breastfeeding coach recruited women from private practice gynecology and obstetric clinics in the Fort Collins, Colorado area on or before their oral glucose screening appointment, between 24-28 weeks of gestation. Included women were 18-42 years of age with singleton pregnancies and were willing to breastfeed exclusively for the first 3 months of life. Exclusion criteria included maternal age <18 years, multiple fetuses, diabetes, HIV positive status, chronic illnesses or other conditions that could preclude breastfeeding and any known allergies to seafood or fish oils. The Institutional Review Board at Colorado State University approved all protocols and procedures. All women provided written informed consent prior to participation.

Women were randomized to receive the study supplement of highly purified tuna fish oil containing 300 mg DHA and 67 mg EPA provided as HiDHA tuna oil (Nu-Mega Oil, Clover Corportation, Ltd) prepared as one hard capsule or Sunola, a high oleic acid sunflower oil placebo. The dose was selected based on previous pilot data with a power of 0.05% at the 80% confidence level. A block randomization procedure was used to assign women to treatment. Study supplements were provided in two opaque bottles, the first given at the entrance into the study and second at the first meeting between the breastfeeding coach and mother after giving birth. Women were instructed to keep supplements in the provided container in a freezer to maintain quality and begin taking one capsule per day for the remainder of their pregnancy and until 3 months of breastfeeding were complete.

A food frequency questionnaire (FFQ) validated against erythrocyte DHA was used to estimate total DHA intake from food, prenatal vitamins and other sources. The FFQ included sources of DHA intake from fish and seafood, DHA enriched food products such as eggs or milk, and any fish oil or DHA supplements. Information was quantitated using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 22. As shown in **Figure 4.1,** the FFQ was validated, with estimated DHA intake per week positively correlating with the percent total DHA in erythrocyte fatty acids ($r=0.37$, $p=0.0002$). Total weekly and daily DHA intake from the FFQ was calculated and verified with a 7-day diet record data. The 7-day diet record information was analyzed using Nutritionist Pro (Axxya Systems, Stafford, TX) software and total daily DHA intake from food was calculated. Sociodemographic data, the date of the last menstrual period (LMP) and estimated due date were collected from each woman at the entrance into the study. Self-reported pre-pregnancy weight and height was used to determine pre-pregnancy body mass index (BMI).

Sample collection and fatty acid analyses

A baseline blood sample was collected from women during their oral glucose screen between 24-28 weeks of gestation and stored briefly at 4^{o}C until separated. Blood samples were collected in a Vacutainer containing EDTA and separated by centrifugation within 24 hours into erythrocyte, plasma and buffy coat fractions and stored at -80^{o}C until further analysis. Breastmilk samples were obtained at 2 and 4 months postpartum and expressed using manual or electric breast pumps into sterile collection cups and transported on ice to the laboratory and immediately frozen and stored at -20^{o}C. Lipids were extracted from maternal erythrocytes, plasma, and 2 and 4 month breastmilk samples using a modified Folch extraction (chloroform:methanol 2:1 v/v). Plasma phospholipids were further separated by thin layer chromatography and all lipid extracts were resuspended in 0.5 ml chromatographic grade hexanes. Fatty acid methyl esters (FAME) were prepared on all lipid extracts by direct transmethylation using 14% boron trifluoride in methanol (Sigma Chemical Co, St. Louis, MO). FAME were analyzed via gas chromatography on an Agilent 6890 chromatograph, using a ramped temperature program and flame ionization detection and a J&W DB225 column. Individual fatty acids were identified by comparison of retention times with known FAME standards (Nuchek Prep, Elysian, MN).

Breastfeeding

Exclusive breastfeeding was supported using a peer coaching model adapted from the in-home breastfeeding support program, developed in North Carolina and tested in a WIC population. This model was adapted to a middle class, educated population and updated to meet current breastfeeding guidelines and policies.[234,235] The program paired each pregnant woman with a well-trained paraprofessional breastfeeding coach, which provided support to the mother

prior to delivery and on an ongoing basis throughout the study duration of 12 months. Coaches provided education, answered questions, assessed breastfeeding and provided recommendations and assistance to resolve any problems and helped the mothers reach their own personal breastfeeding goals beyond that of the study. In addition to the free breastfeeding support provided, as incentive to the study, each mother was provided with a complementary manual breast pump and had access to a medical grade electric pump if deemed necessary by the coach. Exclusivity of breastfeeding was monitored and recorded by the coaches as reported by the mothers. Exclusive breastfeeding was classified as no food or formula other than breastmilk for the first 3 months of life. Mothers were educated and encouraged to exclusively breastfeed through 6 months of life by their coaches and educated on proper progression of introducing and increasing infant appropriate foods according to the USDA Start Healthy Feeding Guidelines.[236]

Infant cognitive testing

The BSID-III Mental Development Index (MDI) subscales were administered on infants at 4 and 12 months of age by a trained technical assistant overseen by a licensed clinical psychologist in the Psychology Department at CSU. The BSID-III was selected as a measurement of neurocognitive development due to its ease of administration and for comparison to previous studies. The BSID-III MDI cognitive, language, social-emotional and general adaptive behavior scales were completed. The MDI has a mean score of 100 with standard deviation (SD) of 15. The BSID is widely accepted and used throughout cognitive studies, the test has excellent within age test-retest reliability and scores have been associated with intelligence quotient (IQ) scores at 4 years of age in DHA supplementation published studies.[229] All BSID scores reported are composite scores and are, therefore, age corrected for each scale. To control for confounding effects of maternal IQ, mothers completed a maternal IQ

test using the abbreviated Wechsler Adult Intelligence Scale (WAIS), including the block design and vocabulary subtests, which are highly correlated with full scale IQ (FSIQ; r^2=0.84).[237] To control for the confounding effect the degree of stimulation in the home environment may have on cognitive development, the mothers completed a self-administered Home Screening Questionnaire (HSQ) at 9 months of age.[238]

Gestational length and infant anthropometric measurements

Gestational length was calculated in days using the LMP method and infant date of birth. Infant anthropometric data was obtained from pediatric medical records. The mother reported infant birth weight, length and head circumference measurements to the breastfeeding coach at the first contact just after birth, and reported 2 month weights and heights after the 2 month well baby visit. Anthropometric data at birth and 2 months of age was normalized by expressing them as z scores based on term infants of the same age and sex by using parameters provided in the data files from the Centers for Disease Control and Prevention (CDC) growth charts released in 2000 (www.cdc.gov/growthcharts).

Data analysis

Participating women, all data collectors and investigators were blinded to supplement allocation until all study children were 12 months of age and had completed the cognitive testing. After all study data was collected, the study was unblinded only to study investigators for analysis. Intent-to-treat (ITT) procedures were maintained throughout. Power was based on a previous pilot trial determining the effect DHA supplementation during pregnancy has on improved infant BSID scores at 4-6 months, 32 infants per supplementation group are necessary to reach a power of 0.05% at the 80% confidence level. Total daily DHA intake was calculated for each woman by adding estimated dietary DHA intakes, maternal DHA supplementation (if

applicable) and DHA study supplement. Data was analyzed based on treatment group, placebo versus DHA, as well as daily DHA intake groups broken into three groups: low = 0-299 mg per day DHA, medium = 300-599 mg per day DHA, high = ≥600 mg per day DHA. DHA cut-off points for the intake groups were determined on the basis of those falling below, at the median recommended intake and above the currently recommended intake.[47,48]

Statistical analysis was completed using SPSS statistical software (SPSS, Chicago, IL) and GraphPad Prism V4.0 (GraphPad Software, San Diego, CA). Results are expressed as means ± SD, unless otherwise specified. Two-tailed *t* test was used to analyze results based upon treatment and one-way ANOVA was used to compare means between all daily DHA intake groups. Tukey's post hoc tests were conducted on all statistically significant ANOVA results to determine if differences existed among group means. Correlations were computed using Pearson's correlation coefficient. Data was adjusted for potential confounders, when appropriate, including, maternal age at delivery, maternal BMI, maternal IQ, breastfeeding duration, gestational age and the effects of the home environment on cognitive development via the HSQ.

Results

Subject characteristics

A total of 115 women enrolled in the Omega Smart Baby Project **(Figure 4.2**) and were included in the analysis. Of these, 55 were randomly assigned to the placebo and 60 to the DHA supplement. Study completion rates were 80% in the DHA treatment group but only 64% in the placebo group. End of study pill counts showed high rates of adherence to the study, with >90% of the women that completed the study consuming 99% of study supplements. The reasons for discontinuation in the study between the placebo and DHA treatment groups were similar and

most often due to cessation of breastfeeding before 2 months, loss of contact, or unwillingness to continue in the study. One infant died due to conditions not related to the study. Demographic data, including age, maternal weight, maternal BMI, maternal IQ or the proportion of DHA in erythrocyte and 2 month fatty acids did not differ between women that completed the study and those that did not.

Complete demographic data for each group are provided in **Table 4.1**. There were no significant differences between the placebo and DHA group, or between any DHA intake group, in maternal age, race, marital status, pre-pregnancy maternal weight and height, and maternal IQ measurements. The mean maternal age at delivery was 31.5 ± 4.4 years of age, 93% were Caucasian and 93% were married. All enrolled pregnant women are accounted for, as shown in the consort diagram in **Figure 4.2**, with 90% completing the 4 month BSID cognitive testing, and 71% completing the 12 month cognitive testing. Additionally, 81% of enrolled women were still breastfeeding at 2 months, 78% were still breastfeeding at 4 months, and 57% had continued to breastfeed until study completion at 12 months. There was no significant difference in overall breastfeeding duration between treatment groups or DHA intake groups.

DHA intake groups and fatty acid analysis

Daily DHA intake ranged from 18 mg to 1.374 g per day of total DHA consumed, including the study DHA supplement. Mean DHA intake from food of all enrolled subjects was 101 ± 99 mg per day, from additional supplementation was 209 ± 239 mg per day, and total DHA intake of all women was 468 ± 278 mg per day. Analysis of 7-day diet records determined no significant difference in macronutrient consumption, ALA, DHA, or fish intake at 2 months among the women. Total daily DHA intake at 2 months positively correlated with daily DHA

intake at baseline ($r=0.58$, $p<0.0001$), indicating daily DHA intake of women remained similar from baseline through 2 months.

Differences in placebo versus DHA treatment groups are shown in **Table 4.1**. There was no significant difference in DHA consumption from food, but total DHA intake per day was significantly higher in the DHA group compared to the control group ($p<0.0001$), indicating the study supplement did effectively increase DHA intake in the participating women. The placebo group consumed higher daily DHA when compared to the mean of the DHA treatment group through additional supplementation ($p=0.035$). Maternal erythrocyte and plasma fatty acids percent total DHA at baseline was not significantly different between treatment groups. The mean percent total DHA in 2 month breastmilk fatty acids were significantly higher in the DHA treatment group compared to placebo control ($p=0.017$). After supplementation ended at 3 months, breastmilk percent total DHA levels remained significantly different at 4 months ($p=0.0007$).

Data were analyzed on the basis of total daily DHA intake (**Table 4.1).** Of the 115 women, 27 women (23%) consumed less than 300 mg total DHA per day and were classified in the low DHA intake group, 54 women (47%) consumed between 300 and 599 mg DHA per day and were categorized in the medium DHA intake group, and 34 women (30%) consumed 600 mg or more of DHA per day and were categorized in the high DHA intake group. DHA intake from food and voluntary supplementation was significantly different ($p<0.0001$ and $p<0.0001$, respectively) between DHA intake groups, with estimated intakes significantly higher in the high DHA intake group compared to both the low and the medium intake groups (Tukey's HSD $p<0.001$). Total daily DHA intake was significantly different based upon daily DHA intake group ($p<0.0001$). DHA intake was higher in the high DHA group compared to both the low and

medium DHA intake groups (Tukey's HSD $p<0.0001$) and the medium group was also significantly higher than the low daily DHA intake group (Tukey's HSD $p<0.001$).

Analysis of maternal fatty acids by daily DHA intake group showed significant differences in the proportion of DHA in baseline erythrocyte fatty acids ($p=0.0007$), with significantly higher DHA in women within the high daily DHA intake group compared to the medium intake group (Tukey's HSD $p<0.001$). Erythrocyte fatty acid DHA positively correlated with daily DHA intake ($r=0.24$, $p=0.011$; **Figure 4.3a**), indicating that increases in daily DHA intake correlated with increases in erythrocyte DHA proportion of fatty acids. 2 month breastmilk DHA was significantly different between daily DHA intake groups ($p=0.003$), with significantly higher proportions of DHA in the high intake group compared to the low DHA intake group (Tukey's HSD $p<0.01$). Additionally, daily DHA intake positively correlated with 2 month breastmilk DHA proportion of fatty acids ($r=0.37$, $p=0.0002$; **Figure 4.3b**). Increases in daily DHA intake correlated with increases in breastmilk DHA at 2 months of breastfeeding. At 4 months, after cessation of the study supplementation, breastmilk percent total DHA levels were significantly different ($p<0.0001$) due to DHA intake from food and voluntary supplementation.

Neurocognitive development

The results of the components of the 12 month BSID-III MDI are presented both by treatment type and DHA intake group in **Table 4.2**. No statistically significant differences were found on any BSID-III scale at 4 months of age, (data not shown). There were no statistically significant differences in the MDI scores at any time point, for any scale when analyzing by treatment group alone. Significant differences appear in the cognitive development of infants based upon maternal DHA intake group ($p=0.018$) at 12 months of age. Infants born to mothers consuming greater than 600 mg of DHA daily have higher scores on the 12 month cognitive

MDI test compared to both the low intake group and the medium intake group (Tukey's HSD $p<0.05$). Furthermore, there was a positive correlation between 2 month breastmilk DHA and 12 month scores on the cognitive MDI scale ($r=0.27$, $p=0.031$), as exhibited in **Figure 4.4**. No significant differences were seen between any groups on the 12 month social and general adaptation scales. Furthermore, maternal age, IQ, breastfeeding duration or HSQ did not correlate with any of the BSID-III scores. Results were not significant for any outcome analyzed based upon 4 month breastmilk samples, most likely since the sample was collected outside of study supplementation period.

Neurocognitive results were also analyzed based upon gender. There were no statistically significant differences comparing male and female infants for any of the 4 or 12 month BSID-III tests. However, significant differences in the 12 month MDI cognitive score of the BSID-III emerged in female infants based upon maternal daily DHA intake ($p=0.010$). As shown in **Figure 4.5a**, female infants born to mothers in the high DHA intake group scored 12.3 points higher on the 12 month cognitive MDI scale (118.9 ± 10.4) compared to the female infants born in the low intake group (106.6 ± 12.9; Tukey's HSD $p<0.05$) and scored 13 points higher compared to infants born in the medium daily DHA intake group (105.9 ± 12.7; Tukey's HSD $p<0.05$). In contrast, no statistically significant differences were seen in male infants within DHA intake groups ($p=0.529$; **Figure 4.5b**). There were no statistically significant differences between daily DHA intake groups for either gender on the social or general adaptation testing at 12 months, as well as on all 4 month BSID-III testing or when classifying by treatment group.

Gestational length and premature birth

Gestational length in the DHA treatment group was 4.5 days longer than the placebo group ($p=0.048$). No statistically significant differences in gestational length were seen among

daily DHA intake groups, although there was a trend of 6.1 day longer gestational length in the high daily DHA intake group compared to the low intake group (p=0.053). Total daily DHA intake positively correlated with gestational length (r=0.20, p=0.031), **Figure 4.6**. The incidence of preterm births in the study was 11%, with a significantly greater incidence in the placebo group with 18% (n=10) compared to 5% (n=3; p=0.021) in the DHA group (χ^2=4.97, p=0.026). DHA intake during pregnancy was associated with a reduction in preterm births (OR= 4.2, 95% CI 1.10-16.26; RR=3.6, 95% CI 1.06-12.54). No statistically significant differences in the incidence of preterm birth were found between daily DHA intake groups. However, when combining the medium and high daily DHA intake groups compared to the low daily DHA intake group, the incidence of preterm births significantly decreased from 22% (n=6) in the low daily DHA group to 8% (n=7) in women consuming greater than 300 mg DHA per day (χ^2=4.87, p=0.027). Women consuming 300 mg of DHA per day or greater had an associated reduction in the risk of preterm births compared to women less than 300 mg DHA per day (OR= 0.3, 95% CI 0.09-0.92; RR=0.8, 95% CI 0.68-1.04).

Infant anthropometric measurements

Infant anthropometric data are shown in **Table 4.3**. No statistically significant differences were seen in any anthropometric measurements at birth or at 2 months of age when based upon treatment group or daily DHA intake group. 2 month weight was negatively correlated with 2 month breastmilk DHA proportion of fatty acids (r= -0.22, p=0.048; **Figure 4.7**). Increases in 2 month breastmilk DHA correlated with decreases in infant 2 month weight, indicating a possible relationship between infant DHA intake and growth in early infancy. No statistically significant differences were seen between treatment group or daily DHA intake group in any of the calculated z-scores.

Discussion

The Omega Smart Baby Project significantly increased daily maternal DHA intake to effectively assess the relationship between maternal DHA intake during pregnancy and lactation and infant neurocognitive development. Pregnant women participating in the study had low baseline intakes of DHA, consuming roughly 90-100 mg of DHA per day during pregnancy and lactation from food sources. These intakes agree with findings from previous studies investigating DHA intake in pregnant women.[16,152,241] DHA from food provided 22% of the total daily DHA intake, indicating food sources of DHA impact total daily DHA and should be accounted for in RCT. While 67% of women consumed voluntary DHA supplements daily, the addition of the study supplement significantly increased total daily DHA intake by 58% in the DHA treatment group compared to the placebo group, allowing a significant treatment affect to be detected. As demonstrated in this study, it is important for researchers to quantify daily DHA intake from food as well as from self-supplementation in order to properly determine comprehensive and accurate daily DHA intake levels.

It is well established that DHA is important in fetal neurological development.[58,81,139] DHA is preferentially transported to the fetus during the last trimester of gestation, coinciding with fetal retinal and brain development, specifically with the growth spurt in gray matter and neurogenesis, which is maximal during the last trimester of gestation through two years of age.[5,67,98] Results of numerous RCT showed no effects of DHA treatment during pregnancy, however, several RCT did show a significant correlation between maternal DHA status and infant neurocognitive development.[9,10,16] The absence of consistent correlations between DHA treatment and infant neurocognitive development in RCT could be due to the lack of accurately accounting for maternal DHA intake from all sources. The current study estimated total daily

DHA intake from food and supplements throughout pregnancy and the first 3 months of lactation, which allowed for the successful detection of a dosing effect of DHA intake on infant neurocognitive development. The current study showed an association between maternal daily DHA intake of 600 mg or greater and significantly improved scores on the cognitive MDI scale of the BSID-III at 12 months of age. Similarly, a recent RCT that also accounted for daily DHA intake found infants born to women supplemented with a functional food containing 300 mg DHA per day from the 24th week of gestation through delivery performed significantly better on problem-solving tasks at 9 months of age compared to infants born to mothers of the placebo group.[16]

The timing and length of DHA supplementation are important factors in determining the effect DHA has on infant neurocognitive development. Many previous RCT stopped DHA supplementation after birth, a time of extensive brain growth for the infant. Numerous studies have shown breastfeeding improves cognitive development of infants, and increased DHA content of breastmilk is believed to be one of the major factors.[164,182,183,187,242] DHA is rapidly transferred into breastmilk with no known selectivity and is altered within 2-3 days based upon maternal intake of DHA.[190-192] This study showed DHA supplementation successfully increased breastmilk DHA and 2 month breastmilk DHA positively correlated with infant scores on the cognitive scale of the BSID-III at 12 months. To date, only 2 recent RCT supplemented DHA prenatally and maintained allocation to treatment postnatally.[15,20] The current study successfully supplemented women with DHA for over 7 months, during the last trimester of pregnancy through the first 3 months of breastfeeding. It is possible that the positive association between maternal DHA intake and infant cognitive development at 12 months of age observed in this study could be due to the prolonged DHA supplementation period during both pregnancy and lactation.

A topic of emerging interest is gender differences of DHA requirements, with boys and girls responding and benefiting differently to DHA supplementation. Through a stepwise regression analysis, the positive relationship between omega-3 fatty acid intake of 1 g or greater and cognitive test scores were found to be twice as strong in female American children in the Third National Health and Nutrition Examination Survey (NHANES III) compared to male children, suggesting female children may benefit more cognitively from increased omega-3 intakes.[176] In the current study, female infants born to mothers in the high daily DHA intake group scored significantly higher on the 12 month cognitive scale of the BSID-III MDI compared to female infants born to women in the low intake group. However, daily DHA intake did not significantly affect male cognitive test scores. Similarly, the DINO trial of early preterm infants did not find a significant effect of DHA treatment on infant neurocognitive development, yet, when focusing on gender differences, the study showed girls with higher DHA supplementation had significantly improved BSID MDI scores and a reduction in the risk of mild [developmental quotient (DQ) <85] and severe (DQ<70) cognitive delays at 18 months of age, with no observed affect in boys.[23] The reasons for these gender differences are currently unknown and research is very limited, yet, this data could open up a new avenue of research in the field of DHA and neurocognitive development.

Numerous RCT have attempted to validate the effect of DHA supplementation on increasing gestational length, while subsequently reducing the risk of preterm birth and the incidence of low birth weight infants.[15,124-127]Several systematic reviews and meta-analyses have determined a 1.6 to a 4.5 day increase in gestational length in women supplemented with DHA.[116,130,131] Additionally, many of the same reviews also determined increased DHA intake during pregnancy was associated with a reduction in preterm birth incidence up to 31% and increased infant birth weights as much as 71 g.[116,130,131] The current study showed that

supplementing with 300 mg of DHA per day during pregnancy significantly increased gestational length and decreased the incidence of preterm birth, but did not have any affect on infant birth weight. The increase in gestational length shown in this study does not explain the improvements in cognitive test scores found in infants born to high daily DHA intake women because gestational age was controlled for in the analyses and each scale of the BSID is age corrected.

The mixed results of RCT investigating effect of DHA supplementation and neurocognitive development are possibly due to several inconsistencies, including differing supplementation time periods, length of supplementation, varying amounts of DHA supplemented doses, and/or a failure to maintain assignment to treatment group postnatally. In recent RCT, the majority of DHA supplementation occurs in either pregnancy or lactation, but often not during both periods of extensive brain development and increased DHA need. Experimental groups were supplemented with a wide range of DHA, from as little as 200 mg up to 2.2 g of DHA per day, and the length of supplementation ranged from as little as 10 weeks to over 6 months. The current study chose to supplement women during the last trimester of gestation through the first 3 months of breastfeeding with a 300 mg daily dose of DHA based upon previous successful RCT and supplementation recommendations.[38,243] Many RCT did not also account for total DHA intake, such as DHA intake from voluntary supplementation or food, assuming baseline DHA intake would be low. Failing to account for DHA sources outside of the study supplement may explain the lack of treatment effects demonstrated in recent studies. Many studies without a significant treatment effect have shown a significant association between DHA status and infant development.[20-23,156,157] While no treatment effects were determined, by accounting for DHA intake from all sources in the maternal diet, the current study showed high DHA intake was associated with improved neurocognitive development.

Inconclusive RCT and meta-analyses could also be due to the large variation in the type of cognitive tests used and the age at which the tests are administered. The current study used the BSID because the test is widely accepted and commonly used throughout cognitive studies, and when administered in late infancy, has been shown to correlate with IQ scores at 4 years of age.[229] The age when cognitive testing is performed may play a large role in successfully determining a positive association between DHA supplementation and infant neurocognitive development. The current study did not find any significant associations between maternal DHA intake and infant cognitive test scores at 4 months of age, but did see positive associations at 12 months of age on the BSID-III. Similarly, several recent studies have failed to find an effect of DHA supplementation on infant cognitive development when the tests were administered <12 months of age.[21] Furthermore, it is possible that current cognitive assessments used early in infancy may not be sensitive enough to detect the specific effects DHA supplementation may have on certain aspects of cognitive development. A previous RCT showed 4 month BSID scores did not significantly relate to later cognitive performance, but the 12 month scores related significantly to preschool verbal and performance IQs as well as gross motor scores.[244]

A possible limitation to this study was a completion rate of 80% in the DHA treatment group compared to only a 64% completion rate in the placebo control group. Reasons for the differences between treatment group study completion rates are unknown. However, study numbers at completion for each group were greater than the 32 infants determined necessary to detect a significant difference between groups. Additionally, due to the frequent contact between women and their breastfeeding coaches, study adherence of women that completed the study were excellent with over 90% of women taking all of the supplied study supplements. While compliance in this study was measured by end of study pill counts and not by a physiologic measurement, baseline maternal DHA in erythrocyte fatty acids did positively correlate with

daily DHA intake reported on the baseline FFQ (r=0.24, p=0.011) and DHA content of 2 month breastmilk samples also positively correlated with estimated DHA intake on the 2 month FFQ (r=0.21, p=0.045).

Many prenatal supplements with DHA were recently introduced to the market within the last 5-10 years and women choosing to take a DHA supplement were not excluded from the study. Awareness of possible benefits DHA and fish oil can incur upon the developing fetus has increased over the last decade as more research studies provide positive associations and more obstetricians and dietitians promote DHA supplementation during pregnancy and lactation. In the current study, a large number of women were prescribed prenatal vitamins with DHA or were voluntarily supplementing with DHA or fish oil in addition to the study supplement during the study period. By accounting for this additional supplementation, the present study was able to more accurately estimate total daily maternal DHA intake and determine significant positive associations between maternal DHA intake and infant neurocognitive development beyond that of study treatment groups.

In conclusion, supplementing pregnant and lactating women with DHA could safely enhance the neurocognitive development of the infants. The current study provides information on optimal DHA intake and timing of supplementation, and may also help to impact future national standards and recommendations for DHA supplementation during pregnancy and lactation. While numerous studies remain inconclusive, more research is necessary to determine whether DHA supplementation during pregnancy, lactation or continued intake through both time periods of critical brain growth more effectively enhances infant neurocognitive development.

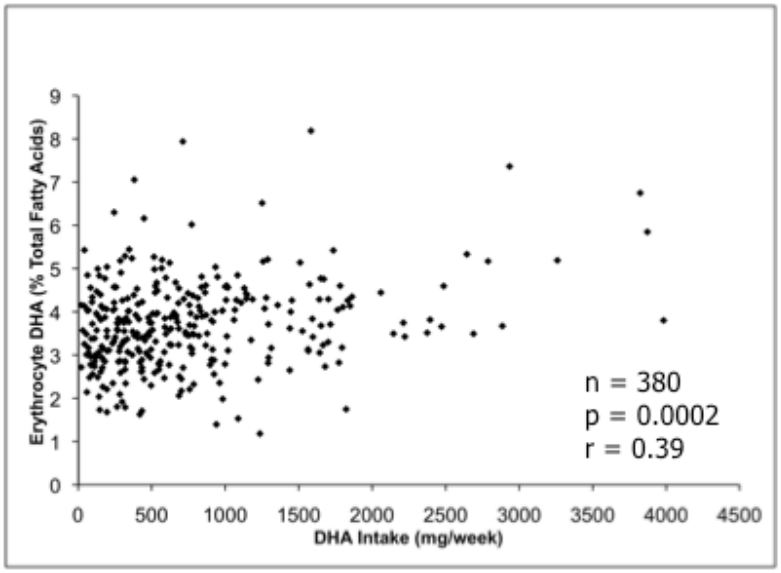

Figure 4.1. Food frequency questionnaire (FFQ) validation. Weekly estimated maternal docosahexaenoic acid (DHA) intake is positively correlated with the proportion of DHA in maternal erythrocyte fatty acids.

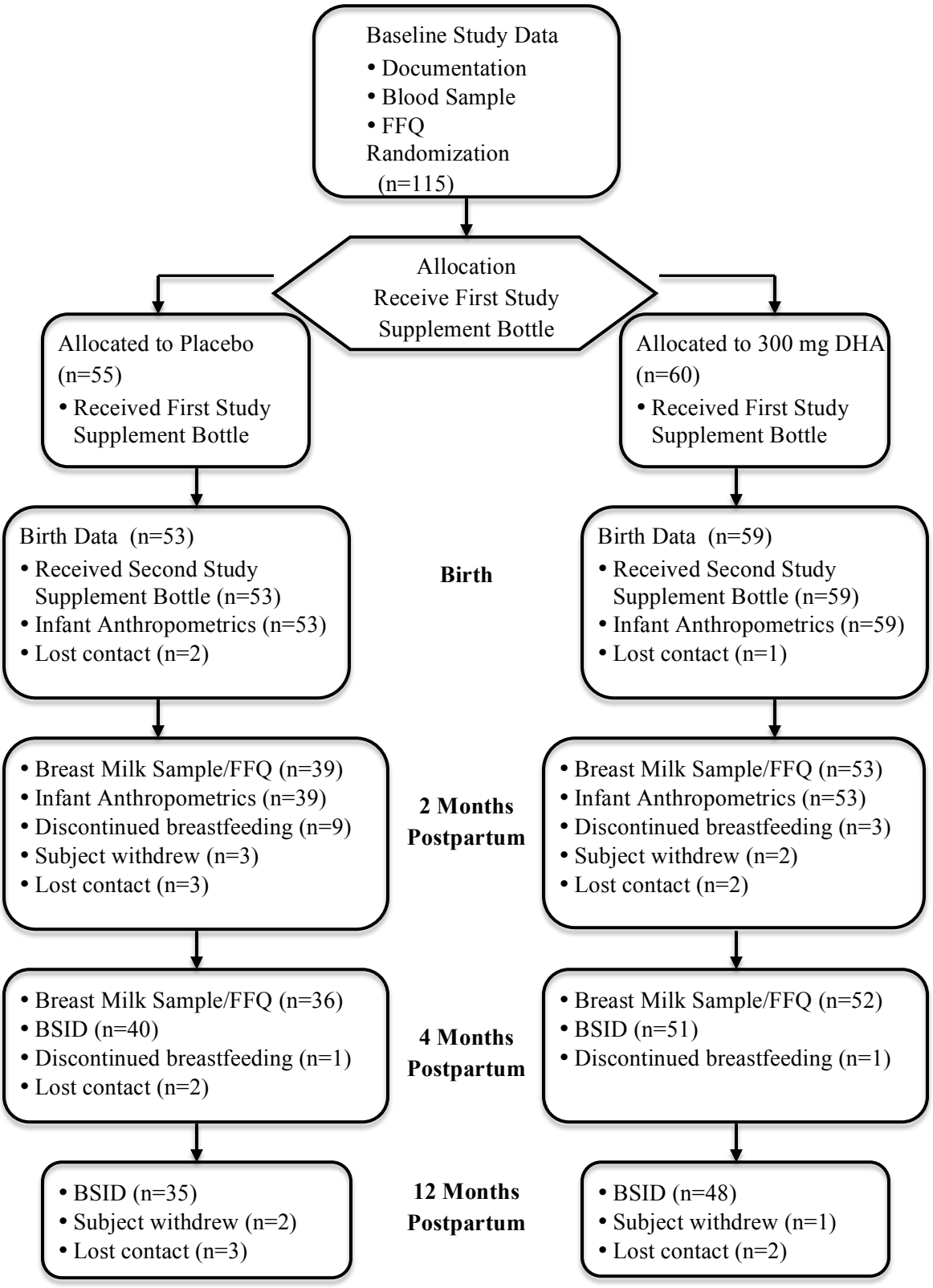

Figure 4.2. Consolidated flow diagram of study data and sample collection. DHA, docosahexaenoic acid; FFQ, food frequency questionnaire; BSID, Bayley Scales of Infant Development III.

Table 4.1

Participants characteristics.[a]

	Treatment			Daily DHA Intake			
	Placebo	DHA	p[c]	Low DHA (0-299 mg DHA/d)	Medium DHA (300-599 mg DHA/d)	High DHA (>600 mg DHA/d)	p[d]
No of participants	55	60		27	54	34	
Maternal age (y)	31.2 ± 4.4[b]	31.7 ± 4.4	0.513	30.2 ± 4.9	31.8 ± 4.4	32.0 ± 3.7	0.203
Maternal Race [n (%)]							
African American	0 (0)	1 (1.67)		0 (0)	1 (1.9)	0 (0)	
Caucasian	52 (95)	55 (92)		26 (96)	47 (87)	34 (100)	
Hispanic	2 (3)	2 (3)		1 (4)	3 (5.5)	0 (0)	
Asian	1 (2)	1 (1.67)		0 (0)	2 (3.7)	0 (0)	
Other	0 (0)	1 (1.67)		0 (0)	1 (1.9)	0 (0)	
Maternal weight (kg)	66.0 ± 10.9	67.7 ± 15.8	0.495	65.9 ± 10.4	69.1 ±16.5	64.2 ± 10.5	0.236
Maternal height (cm)	166.4 ± 5.5	165.2 ± 67	0.309	167.0 ± 4.9	164.5 ± 6.2	166.9 ± 6.8	0.105
Maternal BMI (kg/m^2)	23.8 ± 3.8	24.9 ± 6.1	0.270	23.6 ± 4.0	25.7 ± 6.2	23.0 ± 3.4	0.040[e]
Marital Status [n (%)]							
Married	51 (93)	56 (93)		25 (93)	48 (89)	33 (97)	
Committed relationship	4 (7)	1 (2)		2 (7)	4 (7.4)	0 (0)	
Single	0 (0)	3 (5)		0 (0)	2 (3.7)	1 (3)	
Maternal IQ - WAIS							
Vocabulary	42.8 ± 7.2	43.7 ± 6.7	0.579	40.4 ± 8.7	44.1 ± 6.3	44.1 ± 6.1	0.188
Block Design	52.2 ± 9.1	49.0 ± 10.6	0.164	53.7 ± 6.7	48.5 ± 10.6	51.0 ± 10.7	0.199
Maternal RBC DHA (% total fatty acids)	6.17 ± 1.47	5.75 ± 1.55	0.137	5.91 ± 1.48	5.48 ± 1.21	6.72 ± 1.70	0.0007[c]
Maternal plasma DHA (% total fatty acids)	5.17 ± 1.36	4.97 ± 1.53	0.455	4.99 ± 1.30	4.83 ± 1.43	5.47 ± 1.52	0.125
DHA study supplement [n (%)]	0 (0)	60 (100)		0 (0)	38 (70)	23 (68)	
Voluntary DHA/Fish oil supplement [n (%)]	39 (71)	38 (63)		12 (44)	32 (59)	34 (100)	
DHA intake from food (mg/d)	101 ± 95	101 ± 103	0.999	68 ± 49	78 ± 67	165 ± 137	<0.0001[e]
DHA intake from voluntary additional supplements (mg/d)	258 ± 262	165 ± 208	0.035[e]	74 ± 94	129 ± 157	447 ± 260	<0.0001[e]
Total daily DHA intake (mg/d)	359 ± 280	566 ± 238	<0.0001[e]	142 ± 83	418 ± 82	815 ± 186	<0.0001[e]
Preterm birth - <37 wks [n (%)]	10 (18)	3 (5)		6 (22)	4 (7)	3 (9)	
Late term birth - >40 wks [n (%)]	15 (27)	16 (27)		6 (22)	16 (30)	9 (27)	
Cesarean Delivery [n (%)]	12 (22)	20 (33)		5 (19)	17 (31)	10 (29)	
Gestational length (d)	274.4 ± 14.8	278.9 ± 7.8	0.048[e]	272.8 ± 16.4	277.4 ± 11.6	278.9 ± 7.8	0.135
Breastfeeding duration (weeks)	39.3 ± 18.9	41.6 ± 15.7	0.496	36.7 ± 19.8	40.6 ± 17.0	46.8 ± 12.5	0.063
2 month breast milk DHA (% fatty acid)	0.40 ± 0.45	0.62 ± 0.43	0.017[e]	0.26±0.42	0.51 ± 0.42	0.71 ± 0.44	0.003[e]

[a] WAIS, Wechsler Adult Intelligence Scale; DHA, docosahexaenoic acid.

[b] Mean ± SD (all such values).

[c] Mean differences between placebo and DHA supplemented groups were tested with two-tailed *t* test.

[d] Mean differences between daily DHA intake groups were assessed by ANOVA.

[e] Significant ($p<0.05$).

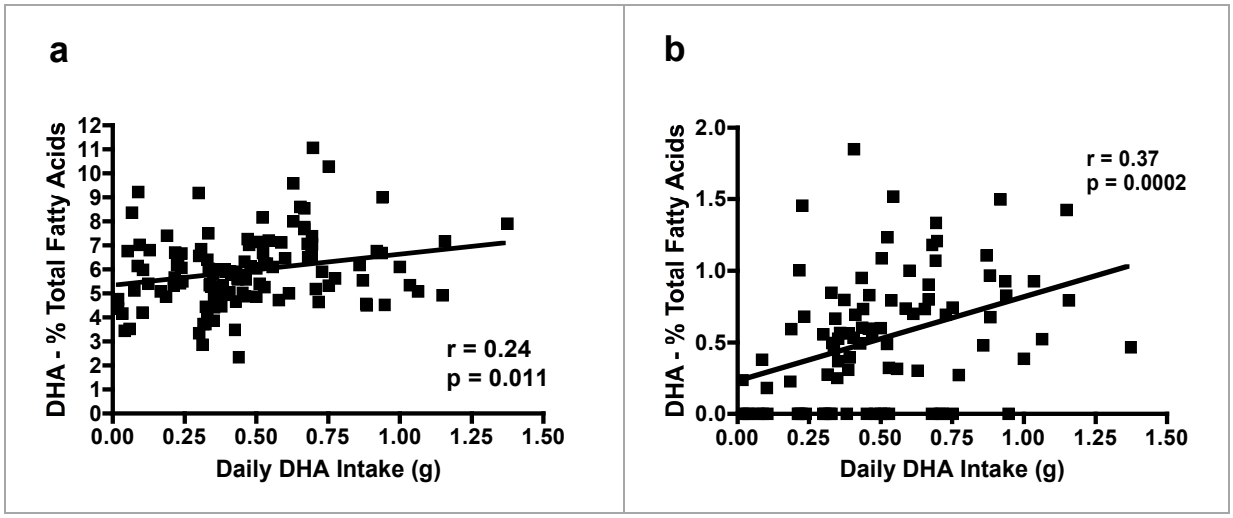

Figure 4.3. Daily maternal docosahexaenoic acid (DHA) intake. Graph **a** shows the correlation between daily DHA intake and the proportion of DHA in maternal erythrocyte fatty acids. Graph **b** shows the correlation between daily DHA intake and the proportion of DHA in 2 month breastmilk fatty acids.

Table 4.2

Neurocognitive development measured by the Mental Development Index (MDI) of the Bayley Scale of Infant Development (BSID) III of infants born to mothers classified based upon study treatment or daily DHA intake at 12 months of age.[a]

	Treatment			Daily DHA Intake Group			
	Placebo n=35	DHA n=48	p[c]	Low DHA (0-299 mg DHA/d) n=17	Medium DHA (300-599 mg DHA/d) n=40	High DHA (>600 mg DHA/d) n=26	p[d]
Cognitive	109.8 ± 12.6[b]	109.7 ± 11.3	0.959	106.1 ± 11.5	107.4 ± 10.5	115.6 ± 12.0	0.018[e]
Language	96.1 ± 9.5	98.5 ± 12.7	0.357	92.3 ± 4.6	97.5 ± 10.5	100.8 ± 14.5	0.076
Social	107.6 ± 13.1	108.4 ± 15.8	0.807	106.3 ± 15.9	108.6 ± 14.0	108.3 ± 15.5	0.089
General Adaptation	107.3 ± 10.9	108.5 ± 13.3	0.696	105.5 ± 11.4	105.7 ± 11.8	113.1 ± 12.5	0.500

[a] DHA, docosahexaenoic acid.

[b] Mean ± SD (all such values).

[c] Mean differences between placebo and DHA supplemented groups were tested with two-tailed *t* test.

[d] Mean differences between daily DHA intake groups were assessed by ANOVA.

[e] Significant ($p<0.05$).

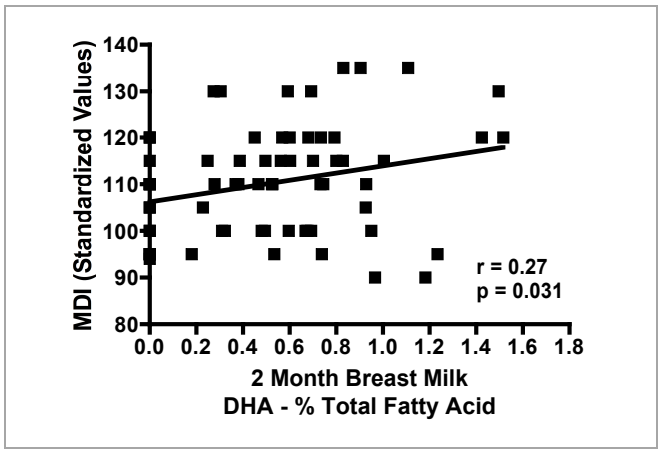

Figure 4.4. Correlation between the proportion of docosahexaenoic acid (DHA) in 2 month breast milk fatty acids and infant motor development index (MDI) scores on the cognitive scale of the Bayley Scales of Infant Development III (BSID-III) at 12 months of age.

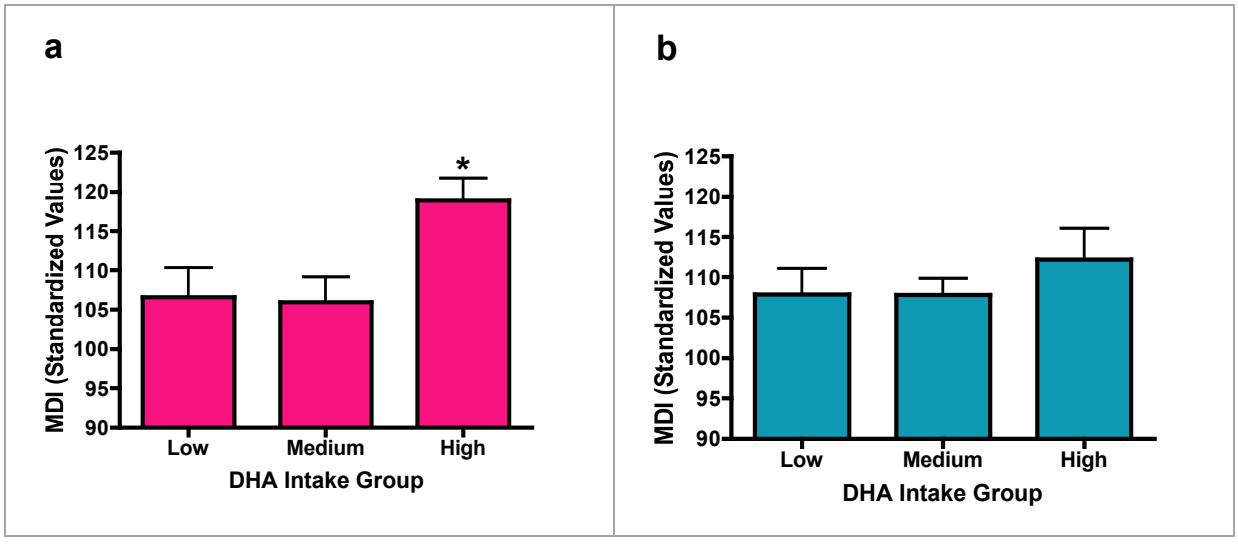

Figure 4.5. Infant motor development index (MDI) scores on the cognitive scale of the Bayley Scales of Infant Development III (BSID-III) at 12 months of age classified by gender and maternal daily docosahexaenoic acid (DHA) intake group. Graph **a** shows only female infant scores on the cognitive scale by daily DHA intake group and graph **b** shows only male infants scores. * Significantly different from low daily DHA intake group (graph **a**), $p<0.05$ ANOVA, Tukey's post hoc test.

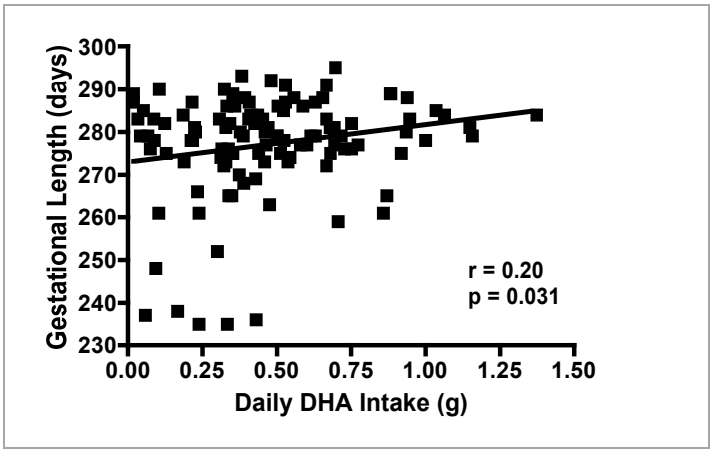

Figure 4.6. Correlation between maternal daily docosahexaenoic acid (DHA) intake and gestational length in days.

Table 4.3

Infant characteristics and anthropometric measurements.[a]

	Treatment				Daily DHA Intake			
	Placebo n=55	DHA n=60	p[c]		Low DHA (0-299 mg DHA/d) n=27	Medium DHA (300-599 mg DHA/d) n=54	High DHA (>600 mg DHA/d) n=34	p[d]
Infant males [n (% male)]	29 (53)	27 (45)			11 (41)	30 (56)	15 (44)	
Birth weight (kg)	3.3 ± 0.7[b]	3.3 ± 0.5	0.853		3.2 ± 0.8	3.4 ± 0.5	3.3 ± 0.5	0.688
Birth length (cm)	51.0 ± 3.1	50.8 ± 2.2	0.768		50.3 ± 2.9	51.2 ± 2.7	50.8 ± 2.3	0.367
Birth head circumference (cm)	34.4 ± 2.0	34.8 ± 1.3	0.144		34.2 ± 2.2	34.6 ± 1.6	34.9 ± 1.2	0.345
Birth weight-age z-score	-0.19 ± 1.02	-0.27 ± 1.10	0.699		-0.17 ± 1.03	-0.20 ± 1.11	-0.33 ± 1.02	0.821
Birth height-age z-score	0.48 ± 0.97	0.41 ± 1.06	0.732		0.40 ± 0.96	0.51 ± 1.10	0.39 ± 0.93	0.846
Birth weight-height z-score	-0.79 ± 1.36	-0.85 ± 1.14	0.807		-0.61 ± 1.26	-0.88 ± 1.14	-0.90 ± 1.42	0.606
2 month weight (kg)	5.37 ± 0.70	5.18 ± 0.49	0.153		5.34 ± 0.73	5.27 ± 0.53	5.19 ± 0.59	0.709
2 month height (cm)	59.35 ± 2.32	58.33 ± 2.72	0.071		58.92 ± 2.05	58.92 ± 2.78	58.42 ± 2.66	0.707
2 month weight-age z-score	0.34 ± 0.97	0.18 ± 0.73	0.380		0.38 ± 1.01	0.23 ± 0.75	0.20 ± 0.85	0.769
2 month height-age z-score	0.69 ± 0.85	0.38 ± 1.12	0.162		0.61 ± 0.67	0.53 ± 1.10	0.42 ± 1.11	0.822
2 month weight-height z-score	-0.76 ± 1.00	-0.58 ± 1.23	0.487		-0.53 ± 1.00	-0.74 ± 1.20	-0.61 ± 1.16	0.787

[a] DHA, docosahexaenoic acid.

[b] Mean ± SD (all such values).

[c] Mean differences between placebo and DHA supplemented groups were tested with two-tailed *t* test.

[d] Mean differences between daily DHA intake groups were assessed by ANOVA.

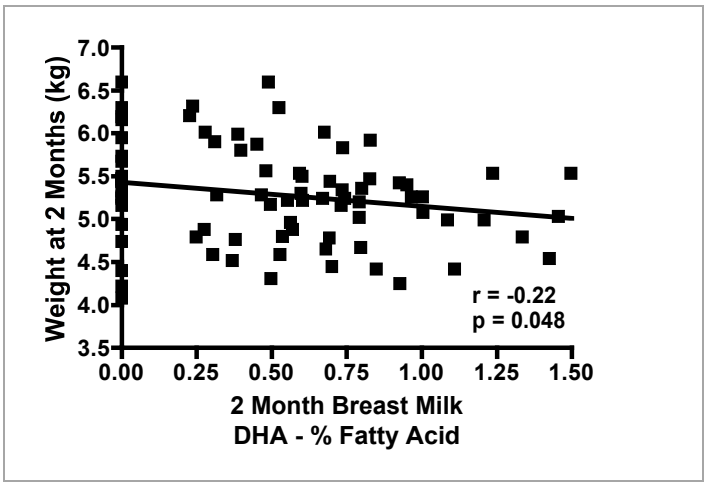

Figure 4.7. Correlation between 2 month infant weight and the proportion of docosahexaenoic acid (DHA) in 2 month breast milk fatty acids.

CHAPTER 5

MATERNAL DOCOSAHEXAENOIC ACID INTAKE DURING PREGNANCY AND LACTATION AND GENETIC VARIANTS OF THE FADS1 FADS2 GENE CLUSTER ARE ASSOCIATED WITH ALTERED FATTY ACIDS IN ERYTHROCYTES AND BREASTMILK AND INTERACT WITH INFANT NEUROCOGNITIVE DEVELOPMENT[2]

Effects of single nucleotide polymorphisms (SNPs) within the fatty acid desaturase (FADS)1 FADS2 gene cluster were previously associated with altered omega-3 (n-3) fatty acid composition, particularly docosahexaenoic acid (DHA), of erythrocyte, plasma phospholipids and breastmilk. The aim of this study was to determine if FADS SNPs influence fatty acid composition in mothers obtaining varying amounts of DHA daily and the subsequent affects on infant neurocognitive development. Women were randomly assigned to receive 300 mg DHA per day or Sunola capsules the 24-28th week of gestation through three months of lactation in a double-blinded placebo controlled clinical trial. Total daily DHA intake was estimated from all dietary sources. Fatty acids in erythrocytes and plasma phospholipids were determined in maternal baseline blood and in breastmilk from 2 month samples. Two SNP in the FADS1 FADS2 gene cluster, rs174575 and rs174561, were analyzed from maternal DNA. Infant neurocognitive development was assessed with the Bayley Scales of Infant Development-III (BSID-III) at 4 and 12 months of age. High DHA intake ≥600 mg per day increased erythrocytes and breastmilk DHA in women homozygous for the major alleles of both SNPs, but did not

[2] In Preparation to *The British Journal of Nutrition*

significantly increase DHA in women heterozygous or homozygous for the minor allele for SNP rs174575. Genotype had no direct effect on BSID-III scores, however, ANCOVA for 12 month cognitive MDI scale showed a statistically significant interaction between SNP rs174575 genotype and daily DHA intake group. Genetic variants influence erythrocyte and breastmilk fatty acids, possibly affecting DHA transfer and infant neurocognitive development.

Introduction

Docosahexaenoic acid [DHA; 22:6(n-3)] and arachidonic acid [AA; 20:4(n-6)] are the most abundant long chain polyunsaturated fatty acids (LCPUFA) in the brain.[2,3,98] DHA is important in fetal neurological development and accumulates within the brain during the third trimester of gestation through the first 2 years of life, coinciding with a growth spurt in gray matter and neurogenesis.[2,5,67,98,107] Randomized controlled trials (RCT) provide some evidence linking increased DHA intake during pregnancy and lactation with enhanced neurocognitive development, however, such trials increasingly report varying and inconclusive results.[9,10,16,21,127,155,156,160,162,164,166,168-170]

Prenatally, the fetus acquires DHA through placental transfer and, after birth, the infant receives DHA from breastmilk or enriched infant formulas.[4,196] DHA is transferred to the fetus or breastmilk from the maternal bloodstream and is influenced by DHA in the maternal diet, enhanced mobilization of DHA from maternal adipose stores, and maternal DHA synthesis.[4] Synthesis of DHA from essential dietary precursor alpha-linolenic acid (ALA) occurs in humans, yet, the conversion is estimated at only <0.1-1%.[7,8] Low rates of endogenous DHA synthesis suggest the need to obtain DHA preformed directly from the diet or supplements, especially during times of increased need, such as pregnancy and lactation. The European Influence of

Dietary Fatty Acids on the Pathophysiology of Intrauterine Foetal Growth and Neonatal Development (PERILIP) group recommends pregnant and lactating women receive at least 200 mg per day of preformed DHA, through supplementation or consuming one to two portions of fatty fish high in n-3 LCPUFA per week, for healthy child development.[38]

DHA, like other fatty acids, is rapidly transferred into breastmilk with no known selectivity between fatty acids, ranges from 0.17-1% total fatty acids and can be drastically altered within 2-3 days based upon maternal intake of DHA.[190-192] In addition to dietary supply, DHA in breastmilk can be endogenously derived from precursor fatty acids by elongation and desaturation, in which the rate-limiting step in humans appears to be the Δ6-desaturase enzyme.[39] Decreased enzyme activity could be due to numerous common single nucleotide polymorphisms (SNPs).[40] The Δ5- and Δ6-desaturase enzymes are encoded by fatty acid desaturase (FADS)1 and FADS2, respectively, are arranged in a head-to-head orientation on chromosome 11q.[24] Several studies have demonstrated women homozygous for the minor alleles of SNPs in the FADS1 FADS2 gene cluster, specifically FADS2 rs174575 and FADS1 rs174561, have lower erythrocyte, plasma and breastmilk DHA compared to women homozygous for the major allele, but results are mixed.[18,19,202,206,245] Additionally, data from population-based birth cohorts found breastfed children of mothers with SNPs coding for lower FADS1 activity, higher FADS2 activity and higher DHA breastmilk levels, as well as breast-fed children with the major allele of rs174575, showed significant cognitive advantages over minor allele carriers.[25-27]

The present study was designed to examine the effects of SNPs within the FADS1 FADS2 gene cluster in mothers supplemented and consuming varying amounts of DHA during pregnancy and lactation on fatty acid composition, particularly DHA, in erythrocyte, plasma phospholipids and breastmilk. Erythrocyte, plasma phospholipids and breastmilk DHA was

hypothesized to decrease in women homozygous for the minor alleles of SNPs in the FADS1 FADS2 gene and could not be increased by increasing daily DHA intake. The final aim was to investigate a suspected correlation between maternal genotype and daily DHA intake interaction and the neurocognitive development in infants exclusively breastfed for the first 3 months of life.

Methods

The Omega Smart Baby Project was a double-blinded, randomized, placebo controlled clinical trial in which 115 women with singleton pregnancies were randomized to receive 300 mg purified DHA or identical placebo between February of 2010 and October of 2011. Subjects were recruited on or before their oral glucose screening appointment, between 24-28 weeks of gestation, at private obstetric and gynecology and family practice clinics in Fort Collins, Colorado. Included subjects were 18-42 years of age with singleton pregnancies and were willing to breastfeed exclusively for the first three months of life. Study exclusion criteria included maternal age less than 18 years, multiple fetuses, diabetes, HIV positive status, chronic illnesses or other conditions that could preclude breastfeeding and any known allergies to fish, seafood or fish oils. The Institutional Review Board at Colorado State University (CSU) approved all protocols and procedures. All subjects provided written informed consent prior to participation in the study.

Women were randomized to receive the study supplement of highly purified tuna fish oil containing 300 mg DHA and 67 mg eicosapentaenoic acid (EPA) (HiDHA Nu-Mega Oil, Clover Corportation, Ltd) prepared as one hard capsule or Sunola, a high oleic acid sunflower oil placebo. The dose was selected based on a previous pilot data with a power of 0.05% at the 80% confidence level. A block randomization procedure was used to assign subjects to treatment.

Women were provided with the study supplement at their oral glucose screen at 24-28 weeks of gestation. Women were instructed to keep supplements in the provided container in a freezer to maintain quality and begin taking one capsule per day for the remainder of their pregnancy and until 3 months of breastfeeding were complete. Study supplements were provided in two opaque bottles, the first given at the entrance into the study and second at the first meeting between the breastfeeding coach and mother after giving birth.

A food frequency questionnaire (FFQ) validated against erythrocyte was used to estimate total DHA intake from food, prenatal vitamins and other sources. The FFQ was validated prior to study initiation and estimated DHA intake per week positively correlated with the proportion of DHA in erythrocyte fatty acids ($r=0.37$, $p=0.0002$). The FFQ included sources of DHA intake from fish and seafood, DHA enriched food products such as eggs or milk, and any fish oil or DHA supplements, including all prenatal vitamin information. Information was quantitated using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 22. Total weekly and daily DHA intake from the FFQ was calculated and verified with a 7-day diet record data. The 7-day diet record information was analyzed using Nutritionist Pro (Axxya Systems, Stafford, TX) software and total daily DHA intake from food was calculated. Sociodemographic data, including age, race and marital status, were collected from each participant at the entrance into the study.

Sample collection and fatty acid analyses

A baseline blood sample was collected from women in a Vacutainer containing EDTA during their oral glucose screen between 24-28 weeks of gestation and stored at 4^{o}C until separated. Whole blood samples were separated by centrifugation within 24 hours into

erythrocyte, plasma and buffy coat fractions and stored at -80^{o}C until further analysis. Breastmilk samples were obtained from each participant at 2 and 4 months postpartum and expressed using manual or electric breast pumps into sterile collection cups and transported on ice to the laboratory and immediately frozen and stored at -20^{o}C. Lipids were extracted from maternal erythrocytes, plasma, and 2 and 4 month breastmilk samples using a modified Folch extraction (chloroform:methanol 2:1 v/v). Plasma phospholipids were further separated by thin layer chromatography and all lipid extracts were resuspended in 0.5 ml chromatographic grade hexanes. Fatty acid methyl esters (FAME) were prepared on all lipid extracts by direct transmethylation using 14% boron trifluoride in methanol (Sigma Chemical Co, St. Louis, MO). FAME were analyzed via gas chromatography on an Agilent 6890 chromatograph, using a ramped temperature program and flame ionization detection and a J&W DB225 column. Individual fatty acids were identified by comparison of retention times with known FAME standards (Nuchek Prep, Elysian, MN).

Selection of SNPs and genotyping

Two SNPs in the FADS1 FADS2 gene cluster were included for SNP analysis, FADS1 rs174561 and FADS2 rs174575. The SNPs were chosen based upon previously reported associations with differences in erythrocyte, plasma phospholipid and breastmilk DHA proportions, as well as associations with later child neurocognitive development. Additionally, the minor allele frequency for both SNPs was previously described as at least ≥ 18%, therefore, the expected number of subjects homozygous for the minor alleles in this study population would exceed 10.[18,19,26,27] DNA was extracted from the buffy coat collected from baseline maternal blood with the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). SNPs were genotyped with TaqMan SNP Genotyping assays (Applied Biosystems, Foster City, CA) with

real-time polymerase chain reaction (PCR) according to the manufacturer instructions using a StepOnePlus Real-Time PCR instrument (Applied Biosystems, Foster City, CA).

Breastfeeding

Exclusive breastfeeding was supported using a peer coaching model adapted from the in-home breastfeeding support program, developed in North Carolina and tested in a WIC population. This model was adapted to a middle class, educated population and updated to meet current breastfeeding guidelines and policies.[234,235] In addition to the free breastfeeding support provided, as incentive to the study, each participant was provided with a complementary manual breast pump and had access to a medical grade electric pump if deemed necessary by the coach. Exclusivity of breastfeeding was monitored and recorded by the coaches as reported by participants. Exclusive breastfeeding was classified as no food or formula other than breastmilk for the first 3 months of life. Participants were educated and encouraged to exclusively breastfeed through 6 months of life by their coaches and educated on proper progression of introducing and increasing infant appropriate foods according to the USDA Start Healthy Feeding Guidelines.[236]

Infant cognitive testing

The Bayley Scales of Infant Development III (BSID_III) Mental Development Index (MDI) subscales were administered on study participant infants at 4 and 12 months of age by a trained technical assistant overseen by a licensed clinical psychologist in the Psychology Department at CSU. The BSID-III was selected as a measurement of neurocognitive development due to its ease of administration and for comparison to previous studies. For the purpose of this study, only the BSID-III MDI cognitive, language, social-emotional and general

adaptive behavior subtests were completed. The BSID is widely accepted and used throughout cognitive studies, the test has excellent within age test-retest reliability and scores have been associated with intelligence quotient (IQ) scores at 4 years of age in DHA supplementation published studies.[229] All BSID scores reported are composite scores and are, therefore, age corrected for each subtest. To control for confounding effects of maternal IQ, mothers completed a maternal IQ test using the abbreviated Wechsler Adult Intelligence Scale (WAIS), including the block design and vocabulary subtests, which are highly correlated with full scale IQ (FSIQ; r^2=0.84).[237] To control for the confounding effect the degree of stimulation in the home environment may have on cognitive development, the mothers completed a self-administered Home Screening Questionnaire (HSQ) at 9 months of age.[238]

Data analysis

Participants, all data collectors and investigators were blinded to supplement allocation until all study children were 12 months of age and had completed the cognitive testing. Intent-to-treat (ITT) procedures were maintained throughout. Power was based on a previous pilot trial determining the effect DHA supplementation during pregnancy has on improved infant BSID scores at 4-6 months, 32 infants per supplementation group are necessary to reach a power of 0.05% at the 80% confidence level. Total daily DHA intake was calculated for each woman by adding estimated dietary DHA intakes, maternal DHA supplementation (if applicable) and DHA study supplement. Data was analyzed based on treatment group, placebo versus DHA, as well as daily DHA intake groups broken into three groups: low = 0-299 mg per day DHA, medium = 300-599 mg per day DHA, high = ≥600 mg per day DHA. DHA cut-off points for the intake groups were determined on the basis of those falling below, at the median recommended intake and above the currently recommended intake.[47,48]

Statistical analysis was completed using SPSS statistical software (SPSS, Chicago, IL) and GraphPad Prism V4.0 (GraphPad Software, San Diego, CA). Deviations from Hardy-Weinberg proportions for the genotypes of each SNP were tested using chi-square tests.[240] Results are expressed as means ± standard deviation (SD), unless otherwise specified. The data for each SNP was analyzed separately and categorized based upon genotype (homozygous for the major allele, MM; heterozygous, Mm; homozygous for the minor allele, mm) and/or daily DHA intake group (low, medium or high) or treatment group (placebo or DHA). One-way analysis of variance (ANOVA) was used to compare mean fatty acid proportions between all categorized data, in which an additive model was assumed so that an increase or decrease in fatty acid proportions was tested for each increment of one minor allele (ie, homozygous for the major allele: coded 1; heterozygous: coded 2; homozygous for the minor allele: coded 3). Tukey's post hoc tests were conducted on all statistically significant ANOVA results to determine if differences existed among group means. Statistical differences in fatty acid proportions and BSID-III scores were also tested with a two-way ANOVA and ANCOVA to determine possible interactions between SNP genotype and daily DHA intake level. Correlations between the proportions of a fatty acid in erythrocyte as well as plasma phospholipids and breastmilk samples were computed using Pearson's correlation coefficient. Data was adjusted for potential confounders, when appropriate, including, maternal age at delivery, maternal IQ, breastfeeding duration, gestational age and the effects of the home environment on cognitive development via the HSQ.

Results

Subject characteristics

55 women were randomly assigned to the placebo and 60 to the DHA supplement. All enrolled women are accounted for, with 71% completing the 12 month cognitive testing. The study had completion rates of 64% in the placebo group and 80% in the DHA group. End of study pill counts showed high rates adherence to the study, with >90% of the women that completed the study consuming all study supplements. The reasons for discontinuation in the study between the placebo and DHA treatment groups were similar and most often due to cessation of breastfeeding before 2 months, loss of contact, or unwillingness to continue in the study. One infant died due to conditions not related to the study. Demographic data, including age, maternal weight, maternal BMI, maternal IQ or the proportion of DHA in erythrocyte, plasma phospholipids and 2 month fatty acids did not differ between participants that completed the study and those that did not.

Complete demographic data for each group are provided in **Table 5.1**. There were no significant differences between the placebo and DHA group, or between any DHA intake group, in maternal age, ethnicity, marital status, pre-pregnancy maternal weight and height, and maternal IQ. The mean maternal age at delivery was 31.5 ± 4.4 years of age, 93% were Caucasian and 93% were married. There was no significant difference in overall breastfeeding duration between treatment groups or DHA intake groups.

Daily DHA intake groups

Daily DHA intake ranged from 18 mg to 1.374 g per day of total DHA consumed, including the study DHA supplement. Mean DHA intake from food of all enrolled subjects was

101 ± 99 mg per day, from additional supplementation was 209 ± 239 mg per day, and total DHA intake of all women was 468 ± 278 mg per day. Analysis of 7-day diet records determined no significant difference between subject macronutrient consumption, ALA, DHA, or fish intake at 2 months and 4 months. Daily total DHA intake at 2 months positively correlated with daily DHA intake at baseline ($r=0.58$, $p<0.0001$), indicating that daily DHA intake of subjects remained similar from baseline through 2 months.

Differences in placebo versus DHA treatment groups and total daily DHA intake groups are shown in **Table 5.1**. Total daily DHA intake per day was significantly higher in the DHA treatment group compared to the control group, and in the high DHA intake group compared to the low intake group (Tukey's HSD $p<0.0001$). Estimated daily DHA intake from food was not significantly different between treatment groups, but was significantly higher in the high DHA group compared to the low and medium intake groups (Tukey's HSD $p<0.01$). Additional DHA supplementation was significantly different between treatment groups and daily DHA intake groups.

DHA intake groups and fatty acid analysis

The means and standard deviations for the fatty acid composition of erythrocyte, plasma phospholipids and 2 month breastmilk samples are shown in **Table 5.2**, classified by daily DHA intake group. Results analyzed by treatment groups were not statistically significant for erythrocyte and plasma phospholipids and are not shown, (see Appendix I). Additionally, 4 month breastmilk samples were not analyzed by daily DHA intake groups since these samples were collected outside of the study supplementation period, therefore, results are not shown. One-way ANOVA revealed no statistically significant differences of any maternal plasma

phospholipid fatty acids analyzed by DHA intake group. In contrast, the analysis of maternal erythrocyte fatty acids between daily DHA intake groups showed significant differences in the percent fatty acids of DHA, with significantly higher DHA in subjects within the high daily DHA intake group compared to the medium intake group (Tukey's HSD $p<0.001$). Additionally, differences were seen between daily DHA intake groups in erythrocyte palmitic acid (16:0), oleic acid (18:1n-9) and dihomo-γ-linoleic acid (DGLA; 20:3n-6), with decreased palmitic acid and DGLA and increased oleic acid in the high daily DHA intake group compared to the low intake group (Tukey's HSD $p<0.05$). Erythrocyte DHA positively correlated with daily DHA intake ($r=0.24$, $p=0.011$), indicating that increases in daily DHA intake correlated with increases in erythrocyte DHA.

Daily DHA intake significantly influenced 2 month breastmilk fatty acid composition, as shown in **Table 5.2**. High daily DHA intake resulted in significantly increased palmitic acid and stearic acid (18:0) and lower n-6 fatty acids linoleic acid (LA, 18:2) and AA in 2 month breastmilk samples compared to the low intake group (Tukey's HSD $p<0.05$). For the n-3 family of fatty acids in 2 month breastmilk samples, subjects in the high daily DHA intake group had significantly lower ALA and significantly higher DHA compared to the low intake group (Tukey's HSD $p<0.05$). Additionally, daily DHA intake positively correlated with 2 month breastmilk DHA proportion of fatty acids ($r=0.37$, $p=0.0002$). Increases in daily DHA intake correlated with increases in the proportion of DHA in 2 month breastmilk fatty acids. Additionally, when analyzing by treatment group, the DHA group had significantly higher DHA [$t(90)=2.44$, $p=0.017$] and lower oleic acid [$t(90)=3.16$, $p=0.002$] compared to the placebo control group in 2 month breastmilk. All other fatty acids were not statistically different between treatment groups and results are not shown (see Appendix J).

Several positive correlations of 2 month breastmilk fatty acids and erythrocyte and plasma fatty acids were observed. As shown in **Table 5.2**, maternal baseline erythrocyte oleic acid, EPA and DHA positively correlated with 2 month breastmilk samples (2-tailed, $p<0.05$). Increases in erythrocyte oleic acid, EPA and DHA (**Figure 5.1**) correlated with increases in these fatty acids in 2 month breastmilk. Additionally, plasma phospholipid palmitic acid, oleic acid, LA, ALA and AA positively correlated with 2 month breastmilk samples (2-tailed, $p<0.05$). Increases in plasma palmitic acid, oleic acid, LA, ALA, and AA correlate with increases of these fatty acids in 2 month breastmilk.

SNP associations with fatty acids

SNPs rs174575 and rs174561 in the FADS1 FADS2 gene cluster were genotyped with a success rate of 100% from all 115 mothers within the study. The consort diagram in **Figure 5.2** shows the breakdown of subjects at baseline and 2 months postpartum by genotype. The genotypic distribution for both SNP did not deviate from Hardy-Weinberg equilibrium ($p>0.05$; **Table 5.3**). Minor allele frequencies analyzed for SNP rs174575 was 23% and SNP rs174561 was 24% of the study population. Of the 7 women homozygous for the minor allele of SNP rs174575, 5 were also homozygous for the minor allele of SNP rs174561 as well. Of the remaining 2 women homozygous for the minor allele of SNP rs174575, both were heterozygous for the SNP rs174561. 8 women were homozygous for the minor allele of SNP rs174561, 5 of which were homozygous for the minor allele, 1 was heterozygous and 2 were homozygous for the major allele of SNP rs174575. Out of the 7 women homozygous for the minor allele of SNP rs174575 and out of the 8 women homozygous for the minor allele of SNP rs174561, only 5 from each genotype completed the study.

Results based upon genotype alone appear to affect fatty acid composition. **Table 5.4** and **Table 5.5** show analysis of the association of the 2 SNPs, rs174575 and rs174561, with the fatty acid composition of maternal erythrocytes and plasma phospholipids, respectively, based upon genotype only. Erythrocytes and plasma phospholipids were similar and showed statistically significant differences between genotype in AA percent total of fatty acids for both SNP rs174575 and rs174561, with lower AA present in subjects homozygous for the minor alleles when compared to the major alleles (Tukey's HSD, $p<0.05$). Additionally, erythrocytes had a statistically significant difference in the n-6 fatty acid DGLA for both SNP rs174575 and rs174561, with higher DGLA in women heterozygous compared to women homozygous for the major allele (Tukey's HSD, $p<0.01$ for both SNPs).

When comparing the fatty acid composition of 2 month breastmilk samples based on genotype, as shown in **Table 5.6**, AA percent total of fatty acids did not vary based upon genotype as erythrocyte and plasma AA did, however, DHA differed among women classified by the rs174575 allele, with significantly lower DHA in heterozygous subjects (Tukey's HSD, $p<0.01$) compared to subjects homozygous for the major allele. Conversely, there was no significant difference seen in 2 month breastmilk DHA in relation to SNP rs175461 genotype. In 4 month breastmilk samples, shown in **Table 5.7**, there were no significant differences in the DHA percent total of fatty acids for either SNP, but it must be noted that this sample set was outside of the study supplementation period. It is a possibility that without the study supplement, the greater variability between subjects may have affected the possibility of achieving a statistically significant outcome. For SNP rs174561, 4 month breastmilk AA was significantly different between genotypes, with lower proportions in heterozygous and minor allele carrier subjects compared to subjects homozygous for the major allele (Tukey's HSD, $p<0.05$).

Genotype and daily DHA intake

Fatty acid composition was also analyzed by genotype based upon treatment and daily DHA intake groups. The analysis of erythrocyte, plasma and 2 month breastmilk samples, as shown in **Table 5.8**, **Table 5.9**, and **Table 5.10**, respectively, provides evidence of the effect SNP may play in regards to total daily DHA intake. It should be noted that the results for subjects homozygous for the minor allele (mm) for all sample types could not be analyzed via ANOVA due to an n=1 in one of the groups, therefore, only mean ± SD are presented for mm subjects in **Table 5.8, Table 5.9** and **Table 5.10**. Results analyzed based upon treatment, placebo versus DHA, were generally not statistically significant (unless mentioned) and therefore, data is not shown (see Appendix J). Genotypic analysis of 4 month samples based upon DHA intake group were not appropriate to be performed, since the 4 month sample was outside of the DHA study supplementation period, therefore, the same daily DHA intake classification would not apply.

Statistically significant differences were seen in the percent total of fatty acids of erythrocytes based upon daily DHA intake for homozygous major allele carriers for both SNP rs174575 and rs174561, shown in **Table 5.8**. Women homozygous for the major allele of SNP rs174575 had decreased erythrocyte palmitic acid in the high daily DHA intake group compared to the medium intake group (Tukey's HSD, $p<0.05$) and increased oleic acid in the high daily DHA intake group compared to the low intake group (Tukey's HSD, $p<0.01$). Additionally, erythrocyte DHA significantly increased in the high daily DHA intake group compared to the low intake group in women homozygous for the major allele of rs174575 (Tukey's HSD, $p<0.05$). However, no significant differences in erythrocyte DHA were noticed between DHA intake groups in women heterozygous or minor allele carriers [$t(4)=0.63$, $p=0.561$] of SNP

rs174575, (when a *t* test was performed between the medium and high daily DHA intake groups since an ANOVA could not be performed due to an n=1 in the low intake group).

For SNP rs174561, erythrocyte oleic acid and DHA significantly increased in the high DHA intake group compared to the medium intake group in women homozygous for the minor allele (Tukey's HSD, $p<0.05$). In SNP rs174561 heterozygotes, only oleic acid was significantly different for SNP rs174561, with increased erythrocyte oleic acid in the high DHA intake group compared to the medium intake group (Tukey's HSD, $p<0.05$). Analysis based on treatment group showed significant differences only in erythrocyte fatty acids in women homozygous for the minor allele, with increased stearic acid for SNP rs174575 [$t(5)=3.53$, $p=0.017$] and DGLA for SNP rs174561 [$t(6)=2.87$, $p=0.028$] in the DHA treatment group compared to the placebo control, and no significant differences between DHA proportions were seen (see Appendix J).

Plasma phospholipid fatty acid composition showed statistically significant differences with both SNPs studied based upon daily DHA intake, (**Table 5.9)**. Women homozygous for the major allele for SNP rs174575 had increased plasma oleic acid and decreased plasma DGLA in the high daily DHA intake group compared to the low intake group (Tukey's HSD, $p<0.05$). Additionally, subjects heterozygous for SNP rs174575 had significantly increased plasma EPA in the high DHA intake group compared to the low (Tukey's HSD, $p<0.05$). For SNP rs174561, only DGLA was significantly different in plasma fatty acids of homozygous major allele carriers, with decreased DGLA in the high daily DHA intake group compared to the low intake group (Tukey's HSD, $p<0.05$). There were no statistically significant differences in the proportion of DHA in plasma fatty acids based on daily DHA intake group for any genotype, although this result would be expected based upon non-genotype classified results shown in **Table 5.2**. Additionally, when analyzing based on treatment group, only women heterozygous for both SNP

rs174575 and rs174561 in the DHA treatment group had significantly lower plasma DGLA [$t(34)=2.19$, $p=0.036$; $t(39)=2.14$, $p=0.039$; respectively], with no significant differences in plasma DHA proportions (see Appendix I).

Analysis of 2 month breastmilk samples demonstrated that genetic variation within the FADS1 FADS2 gene cluster affects the fatty acid composition of breastmilk, and subsequently, influences fatty acids the infant receives. Similarly to the results classified by daily DHA intake alone shown in **Table 5.2**, as daily DHA intake increases, the breastmilk DHA also increases in subjects homozygous for the major alleles. As shown in **Figure 5.3a**, the percent total of fatty acids of DHA in 2 month breastmilk in women homozygous for the major allele of SNP rs174575 significantly increased in the high daily DHA intake group compared to the low intake group (Tukey's HSD $p<0.05$). However, the differences in 2 month breastmilk DHA in heterzygotes for SNP rs174575 were only trending between daily DHA intake group (**Figure 5.3b**) and, since an ANOVA could not be performed due to an n=1 in the low intake group, a *t* test between the medium and high daily DHA intake groups showed no significant differences between groups in women homozygous for the minor allele [$t(2)=0.70$, $p=0.554$; **Figure 5.3c**]. Additionally, women homozygous for the major allele of SNP rs174575 had significantly decreasred breastmilk palmitoleic acid and significantly increased stearic acid in the high daily DHA intake group compared to the medium intake group (Tukey's HSD $p<0.05$).

Analysis of 2 month breastmilk samples for SNP rs174561 showed the percent total of fatty acids of DHA was significantly increased in subjects homozygous for the major allele and heterozygous in the high daily DHA intake group compared to the low intake group (Tukey's HSD $p<0.05$), as shown in **Figure 5.3d** and **5.3e**, respectively. While an ANOVA could not be performed due to low incidence of SNP rs174575 in the high intake group, a *t* test between the

low and medium daily DHA intake groups showed no statistically significant difference in 2 month breastmilk DHA in homozygous minor allele carriers [$t(2)=0.16$, $p=0.891$; **Figure 5.3f**]. No other fatty acids had any significant differences based upon daily DHA intake group for both SNP rs174575 and rs174561. When analyzing by genotype and treatment group, 2 month breastmilk DHA was only significantly higher in the DHA treatment group for women homozygous for the major allele of SNP rs174575 compared to the placebo control group [$t(53)=2.60$, $p=0.012$]. The DHA treatment group also had significantly decreased 2 month breastmilk oleic acid in women heterozygous for SNP rs174575 [$t(30)=2.28$, $p=0.030$] and in women homozygous for the major allele of SNP rs174561 [$t(53)=2.28$, $p=0.027$] as well as decreased AA in women heterozygous for SNP rs174575 [$t(30)=2.57$, $p=0.015$] compared to the placebo control group (see Appendix J).

Infant neurocognitive development

Significant differences in the cognitive scale of the BSID-III MDI at 12 month were seen based upon maternal DHA intake, with infants born to mothers in the high DHA intake group scoring significantly higher than the low intake group ($p=0.018$; Tukey's HSD $p<0.05$). No significant responses were seen between any group on the 12 month language, social and general adaptation scales as well as all of the 4 month BSID-III testing. Furthermore, maternal age, IQ, breastfeeding duration or HSQ did not correlate with any of the BSID-III scores. BSID-III scores analyzed based upon SNP genotype showed no statistically significant differences for both SNP rs174575 and rs174561, as shown in **Table 5.11**. An ANCOVA for BSID-III MDI scores and daily DHA intake group was performed controlling for genotype of both SNP studied to determine if any interaction existed between SNP and daily DHA intake group on infant neurocognitive development. The ANCOVA for 12 month cognitive MDI test showed a

statistically significant interaction between daily DHA intake group and SNP rs174575 genotype, F=3.40, p=0.023. There was not a significant interaction between daily DHA intake group and SNP rs174561 genotype for the 12 month cognitive MDI test, F=0.42, p=0.793. ANCOVA for all other BSID-III MDI scales did not show a statistically significant interaction between daily DHA intake group and SNP genotype for both SNP studied.

Discussion

The effect of SNPs within the FADS1 FADS2 gene cluster on fatty acid composition of erythrocytes and plasma phospholipids seen in this study agree with several previous studies.[18,19,40,203,245] A study of European adults reported higher proportions of serum LA and ALA and lower proportions of AA and EPA in minor allele carriers of several SNPs within the FADS1 FADS2 gene cluster.[245] In the current study, decreased erythrocyte and plasma phospholipid AA in women carriers of the minor allele for both SNPs studied was identified. Additionally, increased daily DHA intake increased erythrocyte DHA in women homozygous for the major allele for SNP rs174575 and rs174561, but increasing DHA intake did not effectively increase erythrocyte DHA in women heterozygous or homozygous for the minor allele. Unlike several prior studies, FADS SNP or daily DHA intake did not significantly affect plasma phospholipids fatty acid composition in this study for reasons unknown. SNPs within the FADS1 FADS2 gene cluster may result in decreased transcription or activity of the Δ5- and Δ6-desaturases, which could explain observed decreases in desaturase products, such as AA and DHA.

Postnatally, the infant receives fatty acids essential for growth and development, including DHA, from breastmilk or enriched infant formulas. Previous studies using stable

isotope tracers have shown DHA from the maternal diet transfers directly into breastmilk and several studies have indicated FADS1 FADS2 genotype affects the proportion of DHA in breastmilk.[18,19,193] Women homozygous for the minor allele of SNP rs174575 were found in recent studies to have significantly decreased breastmilk AA, EPA and DHA compared to women homozygous for the major allele.[18,19] Although both Δ5- and Δ6-desaturases are present in the lactating mammary gland, decreased desaturase expression and/or activity due to SNPs within the FADS1 FADS2 gene cluster could explain observed decreases in desaturase products.[246] While no changes in breastmilk AA or EPA were observed based on FADS genotype in this study, a significant reduction in breastmilk DHA was observed in women heterozygous for SNP rs174575 compared to women homozygous for the major allele. However, no significant difference in 2 month breastmilk DHA was seen in women homozygous for the minor allele of SNP rs174575 or based on SNP rs174561 genotype. It is possible that significant results in carriers of homozygous minor alleles were not seen due to the low number of women in this group and the high variability of DHA proportion of breastmilk fatty acids, as demonstrated in **Figure 5.3c**.

Based the results of previous studies, it would be expected that increasing the intake of preformed DHA would help women homozygous for the minor alleles of SNPs within the FADS1 FADS2 gene cluster to compensate for lower desaturase expression and/or activity. While increased daily DHA intake resulted in significant increases in 2 month breastmilk DHA in this study (**Table 5.2**), women homozygous for the minor allele of SNP rs174575 and rs174561 did not significantly increase breastmilk DHA with increased daily DHA intake. In contrast, major allele carriers of both SNPs had a significant increase in breastmilk DHA in response to a high daily DHA intake ≥600 mg compared to low daily intake <300 mg. Similarly,

Moltó-Puigmartí et al. found proportions of DHA in breastmilk only increased with increased fish and fish-oil intake in the major allele carriers for SNP rs174575 and rs174561 and not in the minor allele carriers.[18] If incorporation of preformed DHA into breastmilk is limited based upon maternal genetic variation within the FADS1 FADS2 gene cluster, it may not be useful for mothers that are minor allele carriers of SNP rs174575 or rs174561 to increase their DHA intake for the purposes of raising breastmilk DHA. Such results may also indicate children born to mothers who are minor allele carriers of FADS1 FADS2 SNPs and have low DHA intake may be at risk for insufficient DHA supply below amounts recommended for brain and neurocognitive development.

It is possible that SNPs within the FADS1 FADS2 gene cluster may result in decreased endogenous fatty acid synthesis due to possible decreased Δ5- and Δ6-desaturase activity. The genomic structure surrounding SNP rs174575 was determined to contain binding sites for sterol regulatory element transcription factors (SREBPs) in which LCPUFA are able to modify gene expression of FADS2, thus affecting subsequent Δ6-desaturase activity, which is believed to be the rate limiting step on the metabolic pathway to producing DHA endogenously.[26,247] However, DHA synthesis does not entirely account for breastmilk DHA, as indicated by our finding and others that increasing daily DHA intake results in an increase in the proportion of DHA in breastmilk.[18,164,193] It is speculated that since the current study did not see an increase in proportions of DHA in breastmilk for women heterozygous or homozygous for the minor alleles of SNP rs174575 and rs174561 in response to increased daily DHA intake, there may be a modification in the incorporation of DHA into breastmilk as a result of genetic variation. SNP within the FADS gene cluster could be associated with fatty acid transport proteins (FATP), affecting transport of DHA from maternal supplies or dietary intake into breastmilk.[248]

Additionally, FADS1 FADS2 gene cluster markers could be linked to fatty acid binding proteins (FABP), a family of carrier proteins involved in the transfer of fatty acids between membranes. A recent study determined a polymorphism within the FABP2 gene was associated with impaired activation of Δ6-desaturase and subsequently resulted in decreased plasma AA proportions of plasma fatty acids.[69]

Higher plasma AA has been positively associated in previous studies with fetal growth and development, whereas low fetal levels have been associated with reduced performance IQ.[103,104,192,249] AA serves as a precursor for eicosanoids, signaling molecules that are important in many aspects of growth and development, including immune and inflammatory responses, in addition to digestive and neurological development.[28,250,251] Confirming prior studies in adults, several recent studies determined pregnant women that were minor allele carriers of common SNPs in the FADS1 FADS2 gene cluster had lower plasma phospholipids and breastmilk AA.[18,19] This study showed a significant decrease in erythrocyte and plasma phospholipid AA in women that were minor allele carriers of both SNP rs174575 and rs174561. However, a significant reduction in breastmilk AA was not seen in relation to SNP in this study. The decrease in AA proportions could be due to SNP affects on decreased Δ6-desaturase transcription or activation. It is currently unknown whether decreases in maternal AA proportions of fatty acids are disadvantageous to the fetus and child due to subsequent reduced eicosanoid production leading to diminished neurocognitive development.

It is well established that DHA is important in fetal neurological development.[58,81,139] DHA is preferentially transported to the fetus during the last trimester of gestation, which coincides with fetal retinal and brain development, specifically with a growth spurt in gray matter and neurogenesis.[5,67,98] While results remain mixed, previous research has shown

increased DHA intake during pregnancy and lactation and higher DHA status results in improved neurocognitive development in young children and enhanced IQ in later childhood.[10,16,17,20,22,127,155,160-165,168,169] Similarly, the current study showed an association between maternal daily DHA intake of ≥600 mg per day during pregnancy and lactation and significantly improved BSID-III MDI cognitive scale scores at 12 months of age. Additionally, this study found a significant interaction between FADS2 SNP rs174575 genotype and daily DHA intake on infant cognitive scale scores at 12 months of age. Several recent studies have discovered a possible association between FADS SNP and child IQ.[25,245,252] A recent study of large birth cohorts showed children breastfed that were major allele carriers for SNP rs174575 had a significant increase in IQ compared to carriers of the same allele that were not breastfed.[26] Additionally, the study found children who were minor allele carriers did not have any IQ advantage to being breastfed, suggesting the SNP somehow influences the positive benefits breastfeeding has on neurocognitive development. It is unlikely that the breastfeeding effects on cognitive tests and IQ are influenced directly by genetic variation, but rather the SNP may influence the availability of DHA to the child through breastmilk, subsequently affecting child cognitive development.

A potential limitation to this study was the limited number of minor allele carriers for each of the SNP investigated and a study population that was relatively homogenous in relative genetic background (94% Caucasian). Unfortunately, this study had less than the anticipated 10 homozygous minor allele carriers based upon previously described minor allele frequency of at least ≥18%.[18] Furthermore, the study was limited by only studying two SNP that were chosen based upon the effects on breastmilk fatty acid composition. Additional SNP may yield more significant results that could help to provide a clearer picture to the effect genetic variation

within the FADS1 FADS2 gene cluster has on DHA and other fatty acid proportions. Future supplementation studies could increase SNP studied as well as increase study subjects through a more diverse population to determine if the effects of our current study apply. In addition to increased subjects, additional cognitive testing of the children at a later age would be beneficial to determine if maternal genotype, AA and DHA proportions of breastmilk have an impact on child IQ and academic performance.

Numerous prenatal supplements with DHA were recently introduced to the market within the last 5-10 years and women choosing to take a DHA supplement were not excluded from the study. The increased awareness of possible benefits DHA and fish oil can incur upon the developing fetus has increased over the last decade as more research studies provide positive associations and more health care providers promote DHA supplementation during pregnancy and lactation. In the current study, all sources of DHA in the maternal diet were accounted for, allowing for a more accurate total daily DHA intake to be estimated. Therefore, positive associations between total DHA intake and maternal fatty acid composition were determined beyond that of study treatment groups or genotype alone.

In conclusion, this study confirmed previously reported associations between maternal SNPs within the FADS1 FADS2 gene cluster and fatty acid composition of erythrocytes and breastmilk, while also estimating total daily DHA intake from all sources during pregnancy and lactation. These results suggest that genetic variation may influence maternal-to-infant transfer of DHA during lactation, and possibly effect infant neurocognitive development.

Table 5.1

Participant characteristics.[a]

	Treatment			Daily DHA Intake			
	Placebo	DHA	p[c]	Low DHA (0-299 mg DHA/d)	Medium DHA (300-599 mg DHA/d)	High DHA (>600 mg DHA/d)	p[d]
No of participants	55	60		27	54	34	
Maternal age (y)	31.2 ± 4.4[b]	31.7 ± 4.4	0.513	30.2 ± 4.9	31.8 ± 4.4	32.0 ± 3.7	0.203
Maternal Race [n (%)]							
African American	0 (0)	1 (1.67)		0 (0)	1 (1.9)	0 (0)	
Caucasian	52 (95)	55 (92)		26 (96)	47 (87)	34 (100)	
Hispanic	2 (3)	2 (3)		1 (4)	3 (5.5)	0 (0)	
Asian	1 (2)	1 (1.67)		0 (0)	2 (3.7)	0 (0)	
Other	0 (0)	1 (1.67)		0 (0)	1 (1.9)	0 (0)	
Maternal weight (kg)	66.0 ± 10.9	67.7 ± 15.8	0.495	65.9 ± 10.4	69.1 ±16.5	64.2 ± 10.5	0.236
Maternal height (cm)	166.4 ± 5.5	165.2 ± 67	0.309	167.0 ± 4.9	164.5 ± 6.2	166.9 ± 6.8	0.105
Maternal BMI (kg/m^2)	23.8 ± 3.8	24.9 ± 6.1	0.270	23.6 ± 4.0	25.7 ± 6.2	23.0 ± 3.4	0.040[e]
Marital Status [n (%)]							
Married	51 (93)	56 (93)		25 (93)	48 (89)	33 (97)	
Committed relationship	4 (7)	1 (2)		2 (7)	4 (7.4)	0 (0)	
Single	0 (0)	3 (5)		0 (0)	2 (3.7)	1 (3)	
Maternal IQ - WAIS							
Vocabulary	42.8 ± 7.2	43.7 ± 6.7	0.579	40.4 ± 8.7	44.1 ± 6.3	44.1 ± 6.1	0.188
Block Design	52.2 ± 9.1	49.0 ± 10.6	0.164	53.7 ± 6.7	48.5 ± 10.6	51.0 ± 10.7	0.199
DHA study supplement [n (%)]	0 (0)	60 (100)		0 (0)	38 (70)	23 (68)	
Voluntary DHA/Fish oil supplement [n (%)]	39 (71)	38 (63)		12 (44)	32 (59)	34 (100)	
DHA intake from food (mg/d)	101 ± 95	101 ± 103	0.999	68 ± 49	78 ± 67	165 ± 137	<0.0001[e]
DHA intake from voluntary additional supplements (mg/d)	258 ± 262	165 ± 208	0.035[e]	74 ± 94	129 ± 157	447 ± 260	<0.0001[e]
Total daily DHA intake (mg/d)	359 ± 280	566 ± 238	<0.0001[e]	142 ± 83	418 ± 82	815 ± 186	<0.0001[e]
Gestational length (d)	274.4 ± 14.8	278.9 ± 7.8	0.048[e]	272.8 ± 16.4	277.4 ± 11.6	278.9 ± 7.8	0.135
Breastfeeding duration (weeks)	39.3 ± 18.9	41.6 ± 15.7	0.496	36.7 ± 19.8	40.6 ± 17.0	46.8 ± 12.5	0.063

[a] WAIS, Wechsler Adult Intelligence Scale; DHA, docosahexaenoic acid.

[b] Mean ± SD (all such values).

[c] Mean differences between placebo and DHA supplemented groups were tested with two-tailed *t* test.

[d] Mean differences between daily DHA intake groups were assessed by ANOVA.

[e] Significant ($p<0.05$).

Table 5.2

Fatty acid composition (percentage of total fatty acids identified) of maternal baseline erythrocyte, baseline plasma phospholipids and 2 month breastmilk samples classified according to daily DHA intake group (low=0-299mg, medium=300-599mg, high=>600mg per day) and Pearson's correlation coefficients between erythrocyte and breastmilk fatty acids as well as plasma phospholipids and breastmilk fatty acids.[a]

	Erythrocyte				Plasma				2 mo BM				2 mo BM Correlated	2 mo BM Correlated
Fatty Acid	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**	**p**	**with Erythrocyte - r**	**with Plasma - r**
Myristic 14:0	0.59±0.17[b]	0.65±0.33	0.55±0.20	0.172	0.07±0.15[b]	0.09±0.19	0.06±0.14	0.769	4.46±1.08	4.86±2.19	5.24±1.9	0.319	-0.09529	0.061
Palmitic 16:0	24.88±2.07	25.06±2.09	23.98±1.74	0.044[c]	31.46±1.56	32.56±6.22	32.30±4.94	0.654	18.23±4.49	15.81±4.26	20.31±4.83	0.0004[c]	-0.1686	0.249[d] (p=0.017)
Palmitoleic 16:1	0±0	0±0	0±0		0.10±0.21	0.12±0.27	0.08±0.18	0.680	2.59±0.61	3.04±0.86	2.66±1.00	0.138		0.125
Stearic 18:0	14.66±1.45	14.62±1.73	14.49±1.46	0.905	12.67±1.11	12.63±2.35	13.05±2.70	0.682	6.41±2.57	5.20±1.93	6.91±2.52	0.011[c]	-0.02949	0.138
Oleic 18:1n9	15.18±1.71	15.65±1.92	16.40±1.78	0.031[c]	9.82±0.90	9.56±1.77	10.09±1.30	0.266	39.47±3.63	39.24±3.19	38.24±4.21	0.384	0.2176[d] (p=0.037)	0.315[d] (p=0.002)
Vaccenic 18:1n7	1.53±0.35	1.65±0.60	1.64±0.56	0.602	1.52±0.20	1.55±0.34	1.54±0.33	0.943	1.69±0.49	1.82±0.25	1.83±0.27	0.289	0.1073	0.0129
Linoleic 18:2	16.85±2.78	16.95±3.03	16.82±2.55	0.976	23.22±1.96	22.86±4.14	22.31±3.15	0.579	23.09±5.14	25.44±5.86	20.86±5.80	0.004[c]	-0.1496	0.225[d] (p=0.032)
ALA 18:3	0.32±0.19	0.38±0.23	0.36±0.23	0.552	0.22±0.17	0.25±0.19	0.19±0.21	0.314	2.16±0.94	2.41±0.76	1.91±0.76	0.037[c]	0.1545	0.401[d] (p<0.0001)
DGLA 20:3	2.41±0.61	2.55±0.59	2.19±0.50	0.017[c]	3.96±0.80	3.81±0.89	3.42±1.01	0.051	0.54±0.58	0.44±0.30	0.41±0.19	0.374	0.07469	0.042
AA 20:4	14.68±1.44	14.26±2.09	14.01±1.71	0.362	10.94±1.52	10.67±2.59	10.35±2.66	0.640	0.92±0.40	1.08±0.53	0.73±0.36	0.004[c]	0.05864	0.251[d] (p=0.017)
EPA 20:5	0.55±0.36	0.62±0.60	0.54±0.38	0.713	0.48±0.40	0.47±0.41	0.70±0.74	0.125	0.08±0.14	0.08±0.22	0.12±0.15	0.516	0.2236[d] (p=0.032)	0.137
DPA 22:5n3	2.32±0.76	2.13±0.48	2.27±0.79	0.422	0.54±0.39	0.60±0.36	0.49±0.44	0.484	0.10±0.18	0.04±0.09	0.11±0.17	0.175	0.124	-0.037
DHA 22:6	5.91 ± 1.48	5.48 ± 1.21	6.72 ± 1.70	0.0007[c]	4.99±1.30	4.83±1.43	5.47±1.53	0.125	0.26±0.42	0.51±0.42	0.71±0.44	0.003[c]	0.2791[d] (p=0.007)	0.204

[a] The number of women with erythrocyte and plasma phospholipid data in the low DHA intake group, medium DHA intake group, and high DHA intake group were as follows: 27, 55, and 33, respectively. The number of women with 2 month breastmilk samples in the low, medium and high DHA intake group are as follows: 18, 30 and 44, respectively. ALA, α-linoleic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid, 2 mo BM, 2 month breastmilk samples. Differences of fatty acid proportions between intake groups were assessed using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

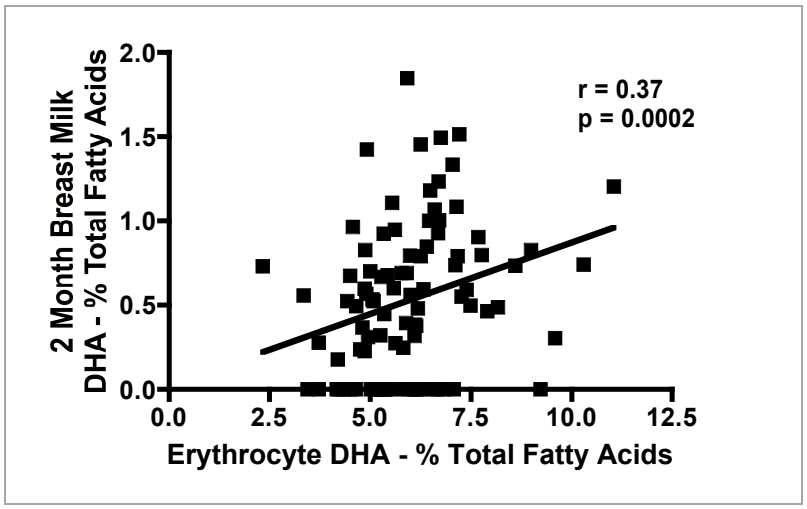

Figure 5.1. Correlation between maternal erythrocyte docosahexaenoic acid (DHA) correlation and 2 month breastmilk DHA.

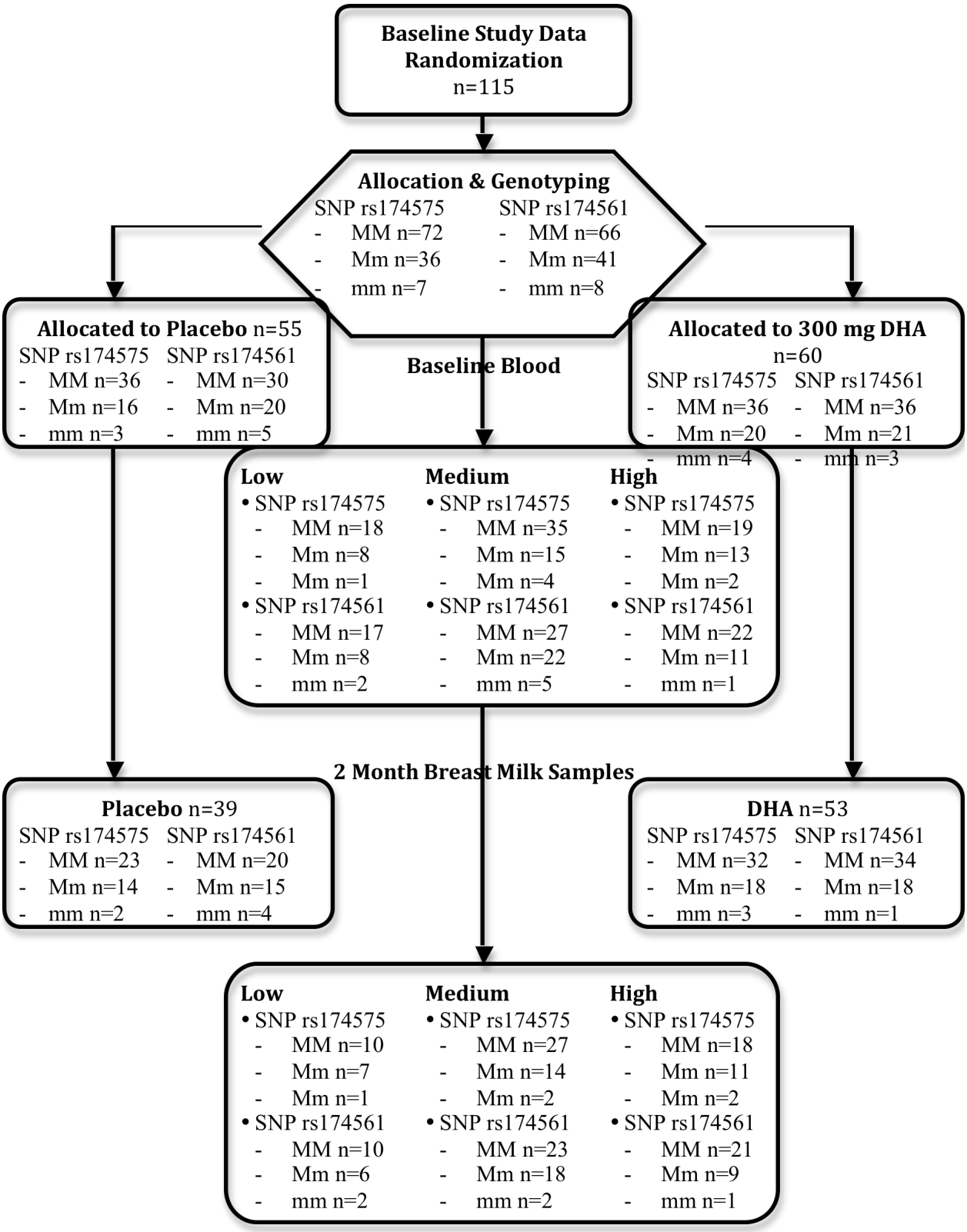

Figure 5.2. Consolidated flow diagram of study subjects by SNP genotype. DHA, docosahexaenoic acid; Daily DHA intake groups: Low=0-299 mg DHA per day, Medium = 300-599 mg DHA per day, High = >600 mg DHA per day.

Table 5.3

Characteristics of the two studied single nucleotide polymorphisms (SNPs) in the FADS1 FADS2 gene cluster.[a]

			Genotype[b]			Allele[b]			
SNP	Gene	M/m alleles	M/M	M/m	m/m	M	m	MAF	HWE[c]
rs174561	FADS1	T/C	66	41	8	107	49	25%	0.375
rs174575	FADS2	C/G	72	36	7	108	43	22%	0.343

[a] M/m, major and minor alleles; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

[b] Number of women carrying M or m, the major and minor alleles, and carrying M/M, M/m or m/m genotypes, respectively.

[c] *P* values of deviation from HWE among all subjects were tested by chi-square tests.

Table 5.4

Fatty acid (percentage of total fatty acids identified) of maternal baseline erythrocyte samples classified according to genotype for SNP rs175475 and rs174561.[a]

Fatty Acid	SNP	MM	Mm	mm	p
Myristic 14:0	rs174575	0.61±0.30[b]	0.59±0.20	0.71±0.27	0.556
	rs174561	0.62±0.29	0.58±0.22	0.67±0.27	0.574
Palmitic 16:0	rs174575	24.67±2.22	24.76±1.64	24.73±2.10	0.973
	rs174561	24.88±2.23	24.37±1.71	24.97±1.87	0.421
Stearic 18:0	rs174575	14.60±1.64	14.74±1.47	13.59±1.38	0.210
	rs174561	14.70±1.65	14.52±1.48	13.86±1.45	0.349
Oleic 18:1n9	rs174575	15.81±2.04	15.52±1.54	16.57±1.69	0.388
	rs174561	15.85±2.05	15.54±1.58	16.25±1.85	0.538
Vaccenic 18:1n7	rs174575	1.63±0.64	1.62±0.29	1.56±0.14	0.947
	rs174561	1.63±0.53	1.63±0.59	1.54±0.15	0.904
Linoleic 18:2	rs174575	16.87±3.12	16.58±2.25	18.43±1.86	0.286
	rs174561	16.56±3.12	17.06±2.09	18.55±2.05	0.148
ALA 18:3	rs174575	0.35±0.23	0.37±0.20	0.40±0.21	0.821
	rs174561	0.33±0.22	0.40±0.21	0.44±0.21	0.177
DGLA 20:3	rs174575	2.30±0.55	2.63±0.62	2.54±0.39	0.016[c]
	rs174561	2.21±0.52	2.68±0.57	2.73±0.49	<0.0001[c]
AA 20:4	rs174575	14.15±1.94	14.82±1.42	12.72±1.91	0.014[c]
	rs174561	14.43±1.89	14.36±1.63	12.55±1.82	0.022[c]
EPA 20:5	rs174575	0.64±0.54	0.46±0.41	0.63±0.26	0.204
	rs174561	0.58±0.51	0.56±0.48	0.69±0.45	0.802
DPA 22:5n3	rs174575	2.26±0.71	2.16±0.58	2.13±0.45	0.695
	rs174561	2.26±0.72	2.17±0.60	2.16±0.42	0.779
DHA 22:6	rs174575	6.10±1.57	5.68±1.45	5.85±1.43	0.407
	rs174561	5.94±1.40	6.11±1.77	5.25±0.93	0.346

[a] The numbers of women with erythrocyte data with MM, Mm and mm genotype for SNP rs174575 were as follows: 72, 36, and 7, respectively. The numbers of women with erythrocyte data with MM, Mm and mm genotype for SNP rs174561 were as follows: 66, 41, and 8, respectively. SNP, single nucleotide polymorphism; MM, homozygous for the major allele; Mm, heterozygous; mm, homozygous for the minor allele; ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table 5.5

Fatty acid (percentage of total fatty acids identified) of maternal baseline plasma phospholipid samples classified according to genotype for SNP rs175475 and rs174561.[a]

Fatty Acid	SNP	MM	Mm	mm	p
Myristic 14:0	rs174575	0.07±0.16[b]	0.09±0.19	0±0	0.411
	rs174561	0.09±0.18	0.06±0.13	0.05±0.15	0.587
Palmitic 16:0	rs174575	32.07±3.79	32.28±7.25	33.33±2.77	0.819
	rs174561	31.87±3.48	32.58±6.86	33.18±5.75	0.667
Palmitoleic 16:1	rs174575	0.10±0.23	0.11±0.24	0.09±0.24	0.977
	rs174561	0.11±0.25	0.08±0.19	0.13±0.25	0.723
Stearic 18:0	rs174575	12.71±2.02	12.80±2.57	12.88±2.67	0.968
	rs174561	12.61±1.87	12.92±2.64	12.98±2.81	0.751
Oleic 18:1n9	rs174575	9.89±1.13	9.37±1.99	10.58±1.19	0.074
	rs174561	9.91±1.14	9.46±1.89	10.20±1.38	0.213
Vaccenic 18:1n7	rs174575	1.54±0.32	1.54±0.30	1.56±0.18	0.978
	rs174561	1.53±0.33	1.54±0.30	1.60±0.11	0.819
Linoleic 18:2	rs174575	22.94±3.05	22.53±4.28	22.92±2.64	0.839
	rs174561	22.65±2.69	22.94±4.37	23.47±3.93	0.783
ALA 18:3	rs174575	0.23±0.20	0.20±0.15	0.30±0.21	0.420
	rs174561	0.21±0.20	0.22±0.17	0.30±0.20	0.455
DGLA 20:3	rs174575	3.61±0.87	3.91±1.01	4.00±0.87	0.194
	rs174561	3.63±0.86	3.83±1.03	3.95±0.87	0.435
AA 20:4	rs174575	10.71±2.35	10.91±2.41	8.37±1.54	0.031[c]
	rs174561	11.27±2.27	10.02±2.32	8.50±1.78	0.0007[c]
EPA 20:5	rs174575	0.52±0.44	0.60±0.69	0.49±0.48	0.748
	rs174561	0.54±0.50	0.54±0.58	0.59±0.52	0.966
DPA 22:5n3	rs174575	0.55±0.39	0.56±0.37	0.53±0.50	0.979
	rs174561	0.55±0.39	0.54±0.38	0.69±0.44	0.585
DHA 22:6	rs174575	5.05±1.45	5.09±1.60	4.96±0.69	0.974
	rs174561	5.02±1.28	5.26±1.59	4.35±1.89	0.250

[a] The numbers of women with plasma data with MM, Mm and mm genotype for SNP rs174575 were as follows: 72, 36, and 7, respectively. The numbers of women with plasma data with MM, Mm and mm genotype for SNP rs174561 were as follows: 66, 41, and 8, respectively. SNP, single nucleotide polymorphism; MM, homozygous for the major allele; Mm, heterozygous; mm, homozygous for the minor allele; ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table 5.6

Fatty acid (percentage of total fatty acids identified) of maternal 2 month breastmilk samples classified according to genotype for SNP rs175475 and rs174561.[a]

Fatty Acid	SNP	MM	Mm	mm	p
Myristic 14:0	rs174575	4.96±1.88[b]	4.81±1.91	5.99±2.13	0.435
	rs174561	4.89±1.79	5.08±2.16	5.00±1.40	0.903
Palmitic 16:0	rs174575	18.07±4.54	18.75±5.69	20.48±4.55	0.529
	rs174561	18.20±4.98	18.58±5.02	20.00±4.91	0.728
Palmitoleic 16:1	rs174575	2.73±0.85	2.88±1.01	2.40±0.66	0.495
	rs174561	2.76±0.97	2.79±0.82	2.77±0.80	0.991
Stearic 18:0	rs174575	6.14±2.18	6.39±2.97	6.63±1.75	0.848
	rs174561	6.15±2.50	6.39±2.50	6.54±1.76	0.874
Oleic 18:1n9	rs174575	38.63±3.55	39.01±4.26	39.42±3.95	0.850
	rs174561	38.43±3.90	39.19±3.56	40.28±4.34	0.448
Vaccenic 18:1n7	rs174575	1.79±0.25	1.83±0.44	1.71±0.12	0.723
	rs174561	1.82±0.29	1.76±0.38	1.84±0.24	0.689
Linoleic 18:2	rs174575	23.19±6.01	22.53±6.32	20.02±2.80	0.507
	rs174561	23.38±6.16	22.21±6.05	20.26±2.56	0.430
ALA 18:3	rs174575	2.24±0.85	1.95±0.79	1.91±0.40	0.246
	rs174561	2.22±0.81	2.01±0.88	1.89±0.29	0.417
DGLA 20:3	rs174575	0.46±0.37	0.45±0.26	0.27±0.25	0.456
	rs174561	0.47±0.38	0.43±0.24	0.22±0.30	0.254
AA 20:4	rs174575	0.89±0.49	0.89±0.41	0.67±0.15	0.578
	rs174561	0.89±0.49	0.88±0.43	0.76±0.21	0.824
EPA 20:5	rs174575	0.11±0.19	0.08±0.15	0.06±0.13	0.605
	rs174561	0.09±0.13	0.11±0.23	0.15±0.20	0.703
DPA 22:5n3	rs174575	0.10±0.17	0.07±0.14	0±0	0.264
	rs174561	0.11±0.17	0.07±0.13	0±0	0.219
DHA 22:6	rs174575	0.65±0.46	0.34±0.38	0.44±0.42	0.007[c]
	rs174561	0.57±0.47	0.50±0.42	0.31±0.42	0.388

[a] The numbers of women with 2 month breastmilk data with MM, Mm and mm genotype for SNP rs174575 were as follows: 55, 32, and 5, respectively. The numbers of women with 2 month breastmilk data with MM, Mm and mm genotype for SNP rs174561 were as follows: 54, 33, and 5, respectively. SNP, single nucleotide polymorphism; MM, homozygous for the major allele; Mm, heterozygous; mm, homozygous for the minor allele; ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table 5.7

Fatty acid (percentage of total fatty acids identified) of maternal 4 month breastmilk samples classified according to genotype for SNP rs175475 and rs174561.[a]

Fatty Acid	SNP	MM	Mm	mm	p
Myristic 14:0	rs174575	6.24±2.10[b]	5.73±1.61	6.12±2.67	0.539
	rs174561	6.16±2.18	6.01±1.60	5.31±1.86	0.648
Palmitic 16:0	rs174575	21.41±4.03	21.51±2.28	21.13±3.15	0.974
	rs174561	21.48±3.91	21.26±2.70	21.86±3.14	0.926
Palmitoleic 16:1	rs174575	2.38±0.71	2.72±0.76	2.31±0.42	0.102
	rs174561	2.37±0.71	2.71±0.75	2.39±0.54	0.119
Stearic 18:0	rs174575	7.15±1.69	6.88±1.57	7.73±1.12	0.515
	rs174561	7.03±1.68	7.13±1.55	7.58±1.52	0.757
Oleic 18:1n9	rs174575	37.80±4.74	37.28±3.28	38.97±4.78	0.695
	rs174561	37.24±4.92	38.29±2.53	39.14±5.40	0.417
Vaccenic 18:1n7	rs174575	1.81±0.24	1.91±0.28	2.00±0.35	0.128
	rs174561	1.80±0.25	1.94±0.28	1.89±0.10	0.055
Linoleic 18:2	rs174575	19.87±4.10	20.79±4.58	18.51±1.93	0.433
	rs174561	20.56±4.49	19.49±3.80	18.55±1.69	0.377
ALA 18:3	rs174575	1.86±0.73	1.74±0.73	1.75±0.61	0.751
	rs174561	1.84±0.65	1.80±0.87	1.69±0.38	0.887
DGLA 20:3	rs174575	0.40±0.25	0.43±0.11	0.32±0.18	0.524
	rs174561	0.44±0.22	0.37±0.20	0.35±0.09	0.287
AA 20:4	rs174575	0.59±0.22	0.60±0.17	0.43±0.25	0.254
	rs174561	0.63±0.16	0.51±0.27	0.52±0.10	0.043[c]
EPA 20:5	rs174575	0.05±0.10	0.08±0.17	0.09±0.12	0.657
	rs174561	0.06±0.13	0.04±0.13	0.14±0.13	0.323
DPA 22:5n3	rs174575	0.06±0.11	0.07±0.12	0.06±0.12	0.966
	rs174561	0.06±0.12	0.05±0.11	0.11±0.15	0.594
DHA 22:6	rs174575	0.38±0.39	0.27±0.33	0.52±0.36	0.253
	rs174561	0.34±0.35	0.35±0.43	0.48±0.17	0.721

[a] The numbers of women with 4 month breastmilk data with MM, Mm and mm genotype for SNP rs174575 were as follows: 55, 30, and 5, respectively. The numbers of women with 4 month breastmilk data with MM, Mm and mm genotype for SNP rs174561 were as follows: 54, 29, and 5, respectively. SNP, single nucleotide polymorphism; MM, homozygous for the major allele; Mm, heterozygous; mm, homozygous for the minor allele; ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table 5.8

Fatty acid composition (percentage of total fatty acids identified) of maternal baseline erythrocyte samples classified according to both genotype for SNP rs174575 and rs174561, as well as daily DHA intake group (low=0-299mg, medium=300-599mg, high=>600mg per day).[a]

		MM				Mm				mm[d]		
Fatty Acid	**SNP**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**
Myristic 14:0	rs174575	0.58±0.18[b]	0.67±0.37	0.52±0.20	0.185	0.64±0.13	0.60±0.27	0.54±0.11	0.475	0.27±0	0.73±0.12	0.88±0.39
	rs174561	0.60±0.19	0.70±0.39	0.54±0.17	0.143	0.59±0.12	0.60±0.27	0.52±0.16	0.621	0.47±0.28	0.65±0.15	1.16±0
Palmitic 16:0	rs174575	25.15±2.28	25.08±2.36	23.45±1.39	0.019[c]	24.70±1.62	24.91±1.66	24.63±1.75	0.902	22.09±0	25.38±0.63	24.75±4.08
	rs174561	25.53±2.28	25.17±2.38	24.01±1.78	0.072	23.82±1.06	24.96±1.88	23.58±1.35	0.052	23.91±2.58	24.86±1.46	27.64±0
Stearic 18:0	rs174575	14.69±1.52	14.62±1.90	14.46±1.26	0.907	14.61±1.40	14.82±1.51	14.73±1.57	0.952	13.22±0	13.84±0.82	13.26±2.97
	rs174561	14.77±1.63	14.70±1.95	14.66±1.30	0.980	14.40±1.16	14.59±1.62	14.47±1.53	0.951	14.16±1.32	14.28±1.08	11.15±0
Oleic 18:1n9	rs174575	14.73±1.80	15.80±2.05	16.85±1.76	0.005[c]	16.01±1.17	15.28±1.86	15.50±1.37	0.571	17.31±0	15.70±0.89	17.93±2.74
	rs174561	14.68±1.85	16.20±2.14	16.31±1.81	0.022[c]	16.26±0.59	14.92±1.62	16.25±1.54	0.021[c]	15.40±2.70	15.87±0.62	19.87±0
Vaccenic 18:1n7	rs174575	1.44±0.22	1.67±0.73	1.72±0.74	0.358	1.74±0.53	1.63±0.21	1.54±0.12	0.297	1.62±0	1.54±0.18	1.55±0.16
	rs174561	1.43±0.20	1.80±0.74	1.56±0.31	0.057	1.76±0.54	1.49±0.39	1.81±0.88	0.280	1.57±0.08	1.50±0.18	1.67±0
Linoleic 18:2	rs174575	16.88±3.16	17.03±3.30	16.57±2.86	0.873	16.05±1.19	16.53±2.70	16.97±2.26	0.670	21.60±0	17.74±1.70	18.24±0.31
	rs174561	16.57±3.16	16.42±3.46	16.72±2.76	0.949	16.86±1.81	17.21±2.58	16.91±2.29	0.912	18.66±4.16	18.61±1.72	18.02±0
ALA 18:3	rs174575	0.34±0.18	0.36±0.25	0.36±0.24	0.969	0.36±0.18	0.42±0.21	0.33±0.22	0.507	0±0	0.45±0.08	0.51±0.22
	rs174561	0.30±0.19	0.32±0.24	0.37±0.23	0.648	0.44±0.12	0.43±0.22	0.31±0.23	0.255	0.22±0.31	0.48±0.12	0.66±0
DGLA 20:3	rs174575	2.23±0.52	2.45±0.57	2.07±0.47	0.040[c]	2.92±0.58	2.74±0.64	2.33±0.54	0.072	2.39±0	2.67±0.48	2.34±0.22
	rs174561	2.22±0.51	2.31±0.52	2.09±0.54	0.361	2.89±0.66	2.77±0.57	2.35±0.38	0.065	2.45±0.08	2.89±0.57	2.50±0
AA 20:4	rs174575	14.69±1.58	14.02±2.27	13.89±1.55	0.392	14.61±1.33	15.08±1.48	14.64±1.45	0.648	14.02±0	13.25±1.75	11.01±2.02
	rs174561	15.02±1.52	14.23±2.40	14.21±1.35	0.330	14.16±1.16	14.61±1.64	14.01±1.93	0.575	13.36±0.95	12.82±1.71	9.58±0
EPA 20:5	rs174575	0.62±0.31	0.67±0.69	0.59±0.37	0.875	0.40±0.47	0.48±0.39	0.47±0.42	0.888	0.40±0	0.71±0.32	0.58±0.17
	rs174561	0.55±0.35	0.63±0.67	0.53±0.35	0.760	0.46±0.37	0.60±0.54	0.55±0.46	0.781	0.83±0.60	0.63±0.50	0.69±0
DPA 22:5n3	rs174575	2.41±0.83	2.13±0.47	237±0.91	0.290	2.23±0.63	2.09±0.51	2.19±0.66	0.837	1.75±0	2.36±0.48	1.84±0.12
	rs174561	2.40±0.84	2.11±0.53	2.33±0.80	0.357	2.22±0.68	2.15±0.44	2.18±0.83	0.957	2.24±0.70	2.21±0.38	1.75±0
DHA 22:6	rs174575	6.20±1.40	5.48±1.16	7.13±1.84	0.0006[c]	5.39±1.75	5.43±1.29	6.15±1.42	0.356	5.32±0	5.63±1.63	6.54±1.73
	rs174561	5.89±1.29	5.37±1.07	6.67±1.56	0.004[c]	6.14±2.12	5.68±1.38	6.95±2.06	0.152	5.37±0.08	5.19±1.23	5.31±0

[a] The numbers of women with erythrocyte data in the low DHA intake group, medium DHA intake group, and high DHA intake group for SNP rs174575 were as follows: MM, 18, 35, and 19, respectively; Mm, 8, 15, and 13, respectively; mm, 1, 4, and 2, respectively. The numbers of women with erythrocyte data in the low DHA intake group, medium DHA intake group, and high DHA intake group for SNP rs174561 were as follows: MM, 17, 27, and 22, respectively; Mm, 8, 22, and 11, respectively; mm, 2, 5, and 1, respectively. SNP, single nucleotide polymorphism; MM, homozygous for major alelle; Mm, heterozygous; mm, homozygous for minor allele. ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between daily DHA intake group for each genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

[d] Due to low number of subjects homozygous for the minor alleles, ANOVA could not be run due to only one subject in the low DHA intake group for SNP rs174575 and only one subject in the high DHA intake group for SNP rs174561.

Table 5.9

Fatty acid composition (percentage of total fatty acids identified) of maternal baseline plasma phospholipid samples classified according to both genotype for SNP rs174575 and rs174561, as well as daily DHA intake group (low=0-299mg, medium=300-599mg, high=>600mg per day).[a]

		MM				Mm				mm[d]		
Fatty Acid	**SNP**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**
Myristic 14:0	rs174575	0.08±0.16[b]	0.07±0.17	0.06±0.13	0.884	0.05±0.15	0.13±0.23	0.07±0.16	0.549	0±0	0±0	0±0
	rs174561	0.07±0.16	0.11±0.21	0.07±0.16	0.770	0.03±0.08	0.08±0.17	0.03±0.10	0.546	0.21±0.29	0±0	0±0
Palmitic 16:0	rs174575	31.48±1.24	31.82±2.98	33.10±6.08	0.374	31.31±2.27	34.29±10.92	30.56±1.60	0.373	32.22±0	32.61±2.39	35.34±4.19
	rs174561	31.46±1.18	31.60±1.41	32.51±5.77	0.570	31.60±2.28	33.36±9.09	31.75±2.83	0.748	30.87±1.91	34.26±7.25	32.37±0
Palmitoleic 16:1	rs174575	0.12±0.24	0.11±0.26	0.05±0.15	0.568	0.05±0.15	0.17±0.32	0.07±0.17	0.434	0±0	0±0	0.32±0.45
	rs174561	0.10±0.23	0.16±0.32	0.06±0.16	0.415	0.07±0.18	0.10±0.22	0.04±0.14	0.711	0.22±0.31	0±0	0.63±0
Stearic 18:0	rs174575	12.91±1.10	12.33±1.53	13.21±3.17	0.285	12.17±1.10	13.50±3.74	12.37±1.05	0.389	12.36±0	11.92±0.65	15.05±5.29
	rs174561	12.88±1.02	12.16±0.89	12.95±2.94	0.266	12.32±1.38	12.98±3.16	13.24±2.25	0.757	12.25±0.15	13.61±3.51	11.31±0
Oleic 18:1n9	rs174575	9.56±0.81	9.74±1.09	10.46±1.29	0.028[e]	10.21±0.89	8.73±2.70	9.60±1.22	0.208	11.21±0	11.10±0.86	9.21±0.98
	rs174561	9.61±0.84	9.81±1.05	10.25±1.38	0.185	10.09±0.95	9.12±2.36	9.67±1.17	0.426	10.43±1.10	10.17±1.73	9.91±0
Vaccenic 18:1n7	rs174575	1.49±0.20	1.57±0.31	1.54±0.42	0.712	1.58±0.19	1.48±0.43	1.58±0.10	0.605	1.68±0	1.64±0.11	1.34±0.15
	rs174561	1.46±0.20	1.55±0.34	1.56±0.39	0.580	1.61±0.17	1.53±0.38	1.51±0.15	0.740	1.69±0.01	1.60±0.10	1.45±0
Linoleic 18:2	rs174575	23.36±2.18	23.10±2.99	22.25±3.83	0.501	23.02±1.59	22.00±6.43	22.84±1.77	0.828	22.35±0	23.97±1.77	21.11±4.60
	rs174561	23.25±1.78	22.66±2.29	22.18±3.59	0.474	23.01±2.50	23.05±5.64	22.69±2.28	0.975	23.86±2.14	23.14±5.05	24.36±0
ALA 18:3	rs174575	0.21±0.18	0.27±0.20	0.18±0.23	0.298	0.26±0.13	0.17±0.17	0.18±0.14	0.407	0±0	0.39±0.08	0.25±0.35
	rs174561	0.22±0.18	0.23±0.19	0.19±0.22	0.723	0.22±0.15	0.26±0.19	0.16±0.14	0.273	0.19±0.27	0.30±0.19	0.50±0
DGLA 20:3	rs174575	3.70±0.67	3.80±0.78	3.17±1.06	0.034[c]	4.58±0.83	3.75±1.16	3.69±0.80	0.106	3.83±0	4.17±0.80	3.74±1.53
	rs174561	3.78±0.70	3.86±0.72	3.25±1.02	0.033[c]	4.45±0.93	3.73±1.07	3.58±0.89	0.157	3.60±0.32	3.91±1.02	4.83±0
AA 20:4	rs174575	10.89±1.49	11.14±2.27	9.76±2.94	0.109	11.01±1.79	10.25±3.17	11.62±1.48	0.328	11.12±0	8.15±1.23	7.44±0.34
	rs174561	11.34±1.35	11.76±1.94	10.62±3.01	0.214	10.18±1.79	9.97±2.81	10.00±1.64	0.977	10.51±0.86	7.85±1.61	7.68±0
EPA 20:5	rs174575	0.52±0.35	0.52±0.44	0.51±0.52	0.993	0.45±0.50	0.31±0.28	1.01±0.91	0.016[c]	0±0	0.61±0.44	0.49±0.70
	rs174561	0.47±0.32	0.50±0.38	0.64±0.71	0.479	0.48±0.50	0.43±0.44	0.79±0.82	0.233	0.58±0.81	0.51±0.52	0.98±0
DPA 22:5n3	rs174575	0.55±0.38	0.64±0.34	0.40±0.46	0.092	0.59±0.41	0.47±0.35	0.65±0.36	0.405	0±0	0.69±0.48	0.48±0.67
	rs174561	0.54±0.39	0.64±0.36	0.43±0.42	0.162	0.56±0.38	0.51±0.34	0.60±0.47	0.819	0.53±0.74	0.71±0.41	0.95±0
DHA 22:6	rs174575	5.10±1.25	4.88±1.45	5.32±1.65	0.570	4.72±1.55	4.74±1.58	5.74±1.47	0.185	5.23±0	4.76±0.90	5.23±0.29
	rs174561	4.80±0.98	4.97±1.13	5.26±1.64	0.528	5.38±1.96	4.88±1.52	5.94±1.28	0.190	5.07±0.22	3.92±2.37	5.03±0

[a] The numbers of women with plasma data in the low DHA intake group, medium DHA intake group, and high DHA intake group for SNP rs174575 were as follows: MM, 10, 25, and 14, respectively; Mm, 6, 13 and 10, respectively; mm, 1, 2, and 2, respectively. The numbers of women with plasma data in the low DHA intake group, medium DHA intake group, and high DHA intake group for SNP rs174561 were as follows: MM, 10, 22, and 16, respectively; Mm, 5, 16 and 9, respectively; mm, 2, 2, and 1, respectively. SNP, single nucleotide polymorphism; MM, homozygous for major alelle; Mm, heterozygous; mm, homozygous for minor allele. ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between daily DHA intake group for each genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

[d] Due to low number of subjects homozygous for the minor alleles, ANOVA could not be run due to only one subject in the low DHA intake group for SNP rs174575 and only one subject in the high DHA intake group for SNP rs174561.

Table 5.10

Fatty acid composition (percentage of total fatty acids identified) of maternal 2 month breastmilk samples classified according to both genotype for SNP rs174575 and rs174561, as well as daily DHA intake group (low=0-299mg, medium=300-599mg, high=>600mg per day).[a]

		MM				Mm				mm[d]		
Fatty Acid	**SNP**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**	**p**	**Low**	**Medium**	**High**
Myristic 14:0	rs174575	4.37±1.09[b]	5.60±2.17	5.14±1.76	0.552	4.73±1.12	5.09±2.50	4.50±1.49	0.812	3.49±0	8.11±1.23	5.14±0.10
	rs174561	4.45±1.14	5.07±2.08	4.90±1.75	0.664	4.67±1.17	5.30±2.70	4.93±1.42	0.874	3.89±0.57	6.08±1.64	5.07±0
Palmitic 16:0	rs174575	18.61±3.93	16.80±4.44	19.67±4.66	0.105	16.44±4.16	18.51±5.59	20.54±6.49	0.284	26.96±0	20.77±3.09	16.96±2.51
	rs174561	17.33±4.68	17.33±4.39	19.57±5.61	0.276	18.19±3.50	17.58±5.54	20.84±4.45	0.233	22.84±5.82	19.56±4.80	15.19±0
Palmitoleic 16:1	rs174575	2.51±0.51	3.11±0.83	2.29±0.81	0.003[c]	2.83±0.68	2.98±0.94	2.80±1.32	0.902	1.74±0	3.00±0.37	2.13±0.57
	rs174561	2.54±0.61	3.11±0.85	2.47±1.14	0.065	2.62±0.37	3.02±0.89	2.43±0.80	0.186	2.79±1.48	2.86±0.56	2.54±0
Stearic 18:0	rs174575	6.55±2.65	5.22±1.50	7.29±2.25	0.043[c]	5.78±2.50	6.00±2.26	7.28±3.96	0.485	9.33±0	6.06±1.54	5.85±0.82
	rs174561	6.22±2.72	5.37±1.57	6.96±3.02	0.109	6.45±2.64	5.59±2.12	7.96±2.60	0.061	7.20±3.02	6.51±0.91	5.27±0
Oleic 18:1n9	rs174575	38.46±4.12	38.51±2.73	38.92±4.42	0.920	41.12±2.59	38.86±4.63	37.84±4.46	0.288	38.11±0	37.53±4.96	41.96±4.01
	rs174561	39.14±4.56	38.62±2.88	37.89±4.61	0.683	40.01±2.46	38.51±4.00	40.03±3.25	0.492	39.53±2.01	38.78±6.73	44.79±0
Vaccenic 18:1n7	rs174575	1.81±0.28	1.82±0.25	1.73±0.22	0.473	1.53±0.71	1.85±0.23	1.99±0.36	0.087	1.63±0	1.81±0.15	1.66±0.02
	rs174561	1.80±0.30	1.81±0.21	1.85±0.35	0.868	1.46±0.74	1.85±0.27	1.78±0.11	0.095	1.87±0.34	1.90±0.28	1.68±0
Linoleic 18:2	rs174575	23.31±5.42	24.87±6.16	20.61±5.45	0.064	23.72±4.83	23.05±5.41	21.11±8.26	0.654	16.59±0	18.84±0.41	22.91±0.18
	rs174561	24.13±5.80	24.33±5.95	21.97±6.57	0.416	22.91±4.13	23.93±6.17	18.32±5.56	0.068	18.43±2.60	20.83±2.39	22.78±0
ALA 18:3	rs174575	2.26±0.92	2.41±0.79	1.98±0.89	0.261	2.12±1.06	1.90±0.60	1.92±0.86	0.827	1.43±0	1.93±0.36	2.12±0.43
	rs174561	2.33±0.93	2.25±0.63	2.12±0.93	0.777	2.01±1.12	2.20±0.93	1.62±0.50	0.284	1.76±0.45	2.07±0.17	1.82±0
DGLA 20:3	rs174575	0.68±0.71	0.43±0.27	0.38±0.18	0.095	0.41±0.31	0.46±0.29	0.45±0.20	0.929	0±0	0.46±0.10	0.20±0.28
	rs174561	0.64±0.74	0.47±0.25	0.40±0.20	0.245	0.47±0.27	0.43±0.28	0.42±0.14	0.936	0.27±0.39	0.27±0.38	0±0
AA 20:4	rs174575	0.76±0.38	1.01±0.53	0.78±0.47	0.190	1.17±0.35	0.85±0.45	0.77±0.34	0.117	0.72±0	0.64±0.05	0.68±0.28
	rs174561	0.84±0.43	0.97±0.54	0.83±0.46	0.616	1.05±0.43	0.96±0.47	0.63±0.24	0.099	0.88±0.22	0.58±0.13	0.88±0
EPA 20:5	rs174575	0.09±0.14	0.10±0.23	0.15±0.16	0.605	0.06±0.16	0.02±0.06	0.16±0.19	0.060	0±0	0.15±0.21	0±0
	rs174561	0.09±0.14	0.05±0.09	0.12±0.16	0.257	0±0	0.09±0.27	0.22±0.18	0.172	0.21±0.30	0.15±0.21	0±0
DPA 22:5n3	rs174575	0.18±0.22	0.05±0.09	0.14±0.20	0.055	0±0	0.06±0.11	0.12±0.18	0.167	0±0	0±0	0±0
	rs174561	0.18±0.22	0.05±0.09	0.13±0.19	0.093	0±0	0.06±0.10	0.13±0.20	0.174	0±0	0±0	0±0
DHA 22:6	rs174575	0.41±0.48	0.60±0.46	0.86±0.36	0.030[c]	0.10±0.26	0.32±0.27	0.52±0.47	0.056	0±0	0.70±0.21	0.40±0.57
	rs174561	0.31±0.45	0.53±0.44	0.74±0.46	0.040[c]	0.17±0.41	0.50±0.40	0.70±0.36	0.048[c]	0.34±0.48	0.42±0.60	0.0±0

[a] The numbers of women with 2 month breastmilk data in the low DHA intake group, medium DHA intake group, and high DHA intake group for SNP rs174575 were as follows: MM, 10, 25, and 14, respectively; Mm, 6, 13 and 10, respectively; mm, 1, 2, and 2, respectively. The numbers of women with 2 month breastmilk data in the low DHA intake group, medium DHA intake group, and high DHA intake group for SNP rs174561 were as follows: MM, 10, 22, and 16, respectively; Mm, 5, 16 and 9, respectively; mm, 2, 2, and 1, respectively. SNP, single nucleotide polymorphism; MM, homozygous for major alelle; Mm, heterozygous; mm, homozygous for minor allele. ALA, α-linolenic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences of fatty acid proportions between daily DHA intake group for each genotype were assessed by using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

[d] Due to low number of subjects homozygous for the minor alleles, ANOVA could not be run due to only one subject in the low DHA intake group for SNP rs174575 and only one subject in the high DHA intake group for SNP rs174561.

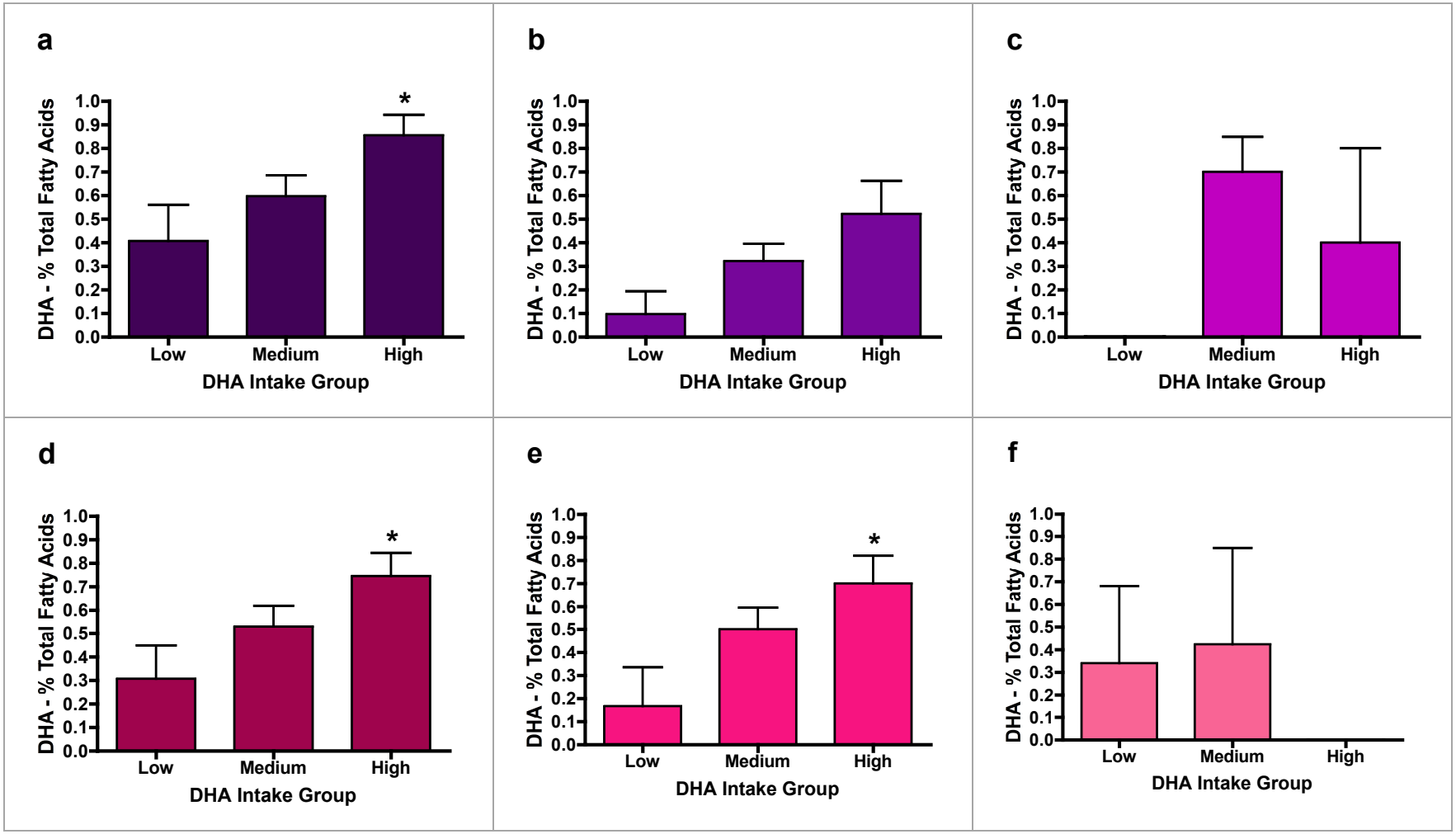

Figure 5.3. Maternal 2 month breastmilk docosahexaenoic acid (DHA) classified by daily DHA intake group as well as by genotype for SNP rs174575 and rs174561: graph **a**, homozygous for the major allele SNP rs174575; graph **b**, heterozygous SNP rs174575; graph **c**, homozygous for the minor allele SNP rs174575; graph **d**, homozygous for the major allele SNP rs174561; graph **e**, heterozygous SNP rs174561; graph **f**, homozygous for the minor allele SNP rs174561. SNP, single nucleotide polymorphism; low intake group, 0-299 mg DHA per day; medium intake group, 300-599 mg DHA per day; high intake group, >600 mg DHA per day. * Significantly different from low DHA intake group (graphs **a**, **d** and **e**), $p<0.05$ ANOVA, Tukey's post hoc test.

Table 5.11

Neurocognitive development measured by the Mental Development Index (MDI) of the Bayley Scale of Infant Development (BSID) III of infants classified according to maternal genotype for SNP rs174575 and rs174561 at 12 months of age.[a]

	SNP	MM	Mm	mm	p
Cognitive	rs174575	108.7±12.0[b]	111.1±10.4	113.8±18.9	0.553
	rs174561	109.0±12.1	110.2±11.2	116.3±12.5	0.484
Language	rs174575	97.8±11.5	98.5±11.7	87.8±4.0	0.211
	rs174561	98.1±10.3	96.6±13.4	97.3±11.4	0.847
Social	rs174575	108.1±13.3	105.7±15.5	106.3±7.5	0.782
	rs174561	107.5±15.8	107.7±10.8	100.0±9.1	0.573
General Adaptation	rs174575	106.7±12.1	107.6±12.1	112.0±12.1	0.762
	rs174561	106.6±12.2	107.4±11.8	115.0±8.7	0.510

[a] The numbers of infants with BSID-III MDI testing for SNP rs174575 were as follows: MM 51, Mm 28, and mm 4. The numbers of infants with BSID-III MDI testing for SNP rs174561 were as follows: MM 49, Mm 30, and mm 4. SNP, single nucleotide polymorphism; MM, homozygous for major allele; Mm, heterozygous; mm, homozygous for minor allele. Differences of BSID-III MDI subtest scores between intake groups were assessed by using ANOVA.

[b] Mean ± SD (all such values).

CHAPTER 6

DISCUSSION

Docosahexaenoic acid nutrition is of interest in pregnancy nutrition because of recent evidence that increased intakes can improve pregnancy outcomes, increase infant growth, and enhance neurocognitive development of the offspring. DHA is present in high concentrations in the brain and neural tissues and accumulates during the third trimester of gestation through the first 2 years of life.[2] Laboratory experiments and animal studies have shown that DHA influences inflammatory processes related to preterm birth, as well as neurogenesis, synaptogenesis and neurochemistry, enhancing cognitive processes throughout the brain.[41,57,253,254] Randomized controlled trials (RCT) in humans have demonstrated numerous benefits of maternal DHA intake during pregnancy, such as increased gestational age, reduced risk of preterm birth, and improved infant growth and cognitive development.[9,10,16,20,127,155] However, results remain mixed and inconclusive.

The Omega Smart Baby Project was a double-blinded, randomized, placebo controlled trial that was designed to determine the relationship between maternal DHA intake during pregnancy and lactation and infant neurocognitive development. The study supplement increased maternal DHA intake during the last trimester of pregnancy through the first 3 months of breastfeeding. Daily maternal DHA intake positively correlated with maternal erythrocyte and breastmilk DHA proportions, and 2 month breastmilk DHA positively correlated with infant cognitive test scores at 12 months of age. This study was different from other RCT in that it

supplemented DHA during both pregnancy and early lactation and accounted for all sources of DHA in the maternal diet, including food and self-supplementation.

Prenatally, it is important for the fetus to receive adequate DHA for optimal growth and development. The fetus is supplied with DHA from the maternal circulation, which is directly influenced by maternal DHA synthesis and stores as well as maternal nutrition.[4] Observational studies in several large birth cohorts have shown an association between increased fish and seafood intake during pregnancy and improved infant cognitive development.[11-13,150] Although results vary, RCT have provided evidence of increased DHA intake during pregnancy with enhanced neurologic development and improved cognitive measurement scores.[9,16,20,124,127,156] However, the majority of these studies do not continue DHA supplementation after birth, reducing potential benefits DHA may have on brain development postnatally in early infancy.

Like human adults, infants have a limited ability to synthesize DHA from n-3 fatty acid precursor alpha-linolenic acid (ALA), and, therefore, must obtain preformed DHA from breastmilk or enriched infant formulas for optimal development.[7] RCT involving infant DHA supplementation postnatally have shown a positive association between increased DHA intake and enhanced neurocognitive development.[22,23,161-164] Yet, results from postnatal RCT have not established an indisputable benefit to postnatal DHA supplementation, and many trials show the most beneficial results in preterm infants. Additionally, postnatal DHA supplementation trials often fail to supplement prenatally, excluding a time of immense brain DHA accumulation and growth.

A limited number of RCT have supplemented with DHA and/or accounted for DHA intake during both pregnancy and lactation, with mixed results.[10,15,20,159] Campoy et al.

supplemented women with 500 mg of DHA per day during pregnancy, and postnatally, encouraged breastfeeding or matched infant formula DHA based on study randomization for the first 6 months of life. Cognitive testing of the children at 6.5 years of age found no significant improvement in cognitive function based on maternal DHA intake.[20] Helland et al. found infants born to women supplemented with 1.18 g DHA cod liver oil per day from 18 weeks of gestation through 3 months after delivery scored higher on the Mental Processing Composite of the Kaufman Assessment Battery for Children (K-ABC) at 4 years of age when compared to corn oil controls.[10] Yet, no significant differences occurred between the two groups on the Fagan Test of Infant Intelligence (FTII) at 6 or 9 months of age or on the K-ABC at 7 years of age.[14,15] In the current study, infants born to mothers consuming 600 mg of DHA per day or greater exhibited significantly improved scores on the cognitive scale of the Bayley Scales of Infant Development III (BSID-III) Mental Development Index (MDI) at 12 months of age. It is possible that the positive association between maternal DHA intake and improved infant cognitive test scores was due to the prolonged supplementation period during a critical time of brain development in the last trimester of pregnancy through the first 3 months of life.

An emerging body of evidence points to differences in the response to DHA supplementation during development between the genders. Evidence is particularly compelling in preterm infants in which boys showed slower and poorer neurodevelopment compared to girls in early childhood. It is also suggested that due to potential estrogen effects, females may synthesize DHA more efficiently from precursor fatty acids than males.[7] Several RCT have failed to determine a significant association between DHA supplementation and neurocognitive development, yet when analyzed based upon gender, found significant differences, with girls significantly benefiting developmentally from supplementation.[23,70] In the DINO trial of early

preterm infants, no significant results were seen between treatment groups, however, a significant interaction between higher postnatal DHA supplementation and gender was found. Higher DHA significantly improved the cognitive development of girls and reduced the risk of both mild (developmental quotient (DQ) <85) and severe (DQ<70) cognitive delay at 18 months of age, while no effect of higher DHA was noted for boys.[23] In the current study, a differing gender response to high DHA supplementation was seen on neurocognitive development. Female infants born to mothers consuming ≥600 mg of DHA per day scored significantly higher on the 12 months cognitive BSID-III MDI subtest compared to female infants born to mothers with <300 mg of DHA intake per day, yet, no significant difference was seen between intake groups in male infants. Female infants appear to be more responsive to early DHA supplementation and further studies are necessary to validate these results and possibly define additional sub-groups of children that can benefit from DHA supplementation. No significant differences in 4 month BSID-III testing were found between any group, and no significant differences were seen between treatment groups on the 12 month BSID-III testing in this study.

Several observational and prospective studies have positively associated increased fish and seafood intake with longer gestation durations, decreased preterm birth incidence and increased infant birth weights.[114,115,118,120,135] RCT have attempted to validate the effect DHA supplementation has on increasing gestational length and decreasing preterm births with variable results.[15,123-127,132,133,138] An Australian study of pregnant women found a daily supplementation of 800 mg of DHA per day was associated with a reduction in preterm births, an increase in gestational length and a significant reduction in the risk of having a low birth weight baby, compared to women supplemented with vegetable oil.[127] The current study showed a significant increase of 4.5 days in gestational length in the DHA treatment group compared to the placebo

group. Women consuming >300 mg DHA per day were >4 times more likely not to have a preterm birth (<37 weeks) with a significantly decreased incidence of preterm birth of 8% (n=7) compared to 22% (n=6) in women consuming <300 mg of DHA per day. However, no significant effects of DHA supplementation were associated with infant birth weights. The reasons and mechanisms behind the influence of DHA on gestational length and preterm birth are currently unknown. It is possible that DHA may affect and prevent several causes of preterm birth, such as inflammatory processes or vascular tone, or possible further unknown causes, subsequently increasing gestational length and infant birth weight.

Unlike some previous studies linking increased DHA intake during pregnancy with increased infant birth weight, in the present study there was no significant increase in the birth weight of infants in any DHA intake or treatment group. The same was true for infant birth length and head circumference, with no significant differences between any of the study groups. Additionally, no statistically significant differences were determined between any of the 2 month measurements based upon treatment or daily DHA intake. However, it is interesting to note that increases in the proportion of DHA in 2 month breastmilk fatty acids correlated with decreases in infant 2 month weights, showing a negative association between DHA consumption and weight gain in early infancy. It is possible that as breastmilk DHA increases and the infant receives more DHA, adipose deposition could be affected, which may have an effect later in childhood.[212,213] Pedersen et al. showed a significant inverse association between breastmilk DHA and child BMI from 2 to 7 years of age in the Copenhagen Prospective Study on Asthma in Childhood birth cohort.[214] Additionally, Donahue et al. determined higher maternal DHA+EPA intake during pregnancy and higher DHA+EPA umbilical cord plasma phospholipids were associated with lower adiposity in children at 3 years of age.[213] The negative correlation shown

here is worth following up in future studies involving this same cohort of infants to determine if DHA intake during pregnancy and lactation has any affect on childhood body composition.

Breastfeeding has been associated with improved infant visual and neurocognitive development, improved behavior and decreased risk for infection. Enhanced neurocognitive development has been directly associated with the concentrations of DHA in breastmilk.[164,190,196] Due to limited endogenous synthesis of DHA, postnatally, the infant must receive preformed DHA from breastmilk or enriched infant formulas.[7] Average concentrations of DHA in breastmilk range from 0.17% to 1.0% of total fatty acids and are directly effected by maternal DHA intake.[189,190] Breastmilk DHA content has been shown to increase by 76% and infant plasma phospholipid DHA by 35% when the mother is exclusively breastfeeding and supplemented with 200 mg of DHA per day.[164] In the current study, successfully increasing the dietary intake of DHA resulted in increased breastmilk DHA, showing a positive association between daily DHA intake and 2 month breastmilk DHA proportions.

In addition to increased breastmilk DHA, this study showed high maternal daily DHA intake ≥600 mg resulted in significant decreases in 2 month breastmilk arachidonic acid (AA), as well as desaturase substrates linoleic acid (LA) and ALA, compared to women consuming <300 mg DHA per day. AA is an omega-6 (n-6) fatty acid essential for growth and development and studies have shown significantly increasing DHA in the maternal diet could result in decreased AA proportions.[95,96] The decrease in AA in breastmilk of women with high daily DHA intake could be due in part to decreased enzyme transcription and/or activity in the endogenous synthesis of LCPUFA. Increased dietary DHA has been shown to alter gene expression in n-6 and n-3 synthesis pathways, possibly affecting the feedback regulation of the synthesis of LCPUFA.[41] Since the synthesis pathways of both n-6 and n-3 fatty acids compete for the same

desaturase enzymes at multiple steps, it is possible that altering the enzyme activity through feedback mechanisms or precursor ratios may affect downstream fatty acid proportions unless supplemented from the diet.

Although limited, breastmilk DHA can be endogenously synthesized from precursor fatty acids by elongation and desaturation, in which the rate-limiting step in humans appears to be the Δ6-desaturase enzyme.[39] Both Δ5- and Δ6-desaturases necessary for LCPUFA synthesis are present in the lactating mammary gland, however, decreased desaturase expression and/or activity could be due to numerous common single nucleotide polymorphisms (SNP).[40,246] The Δ5- and Δ6-desaturase enzymes involved in LCPUFA synthesis are encoded by fatty acid desaturase (FADS)1 and FADS2, respectively, which are arranged in a head-to-head orientation on chromosome 11q, with a third desaturase gene, FADS3, which function is currently unknown.[24] Recent studies have demonstrated that SNP in the FADS1 FADS2 gene cluster contribute to variability of DHA in erythrocyte and plasma phospholipids, as well as breastmilk, indicating a possible link between SNP and DHA enrichment of breastmilk.[18,19,245] DHA proportions in erythrocyte, plasma and breastmilk were lower in women homozygous for the minor alleles of 2 SNP recently studied, FADS2 rs174575 and FADS1 rs174561, compared to women homozygous for the major allele.[18] This study showed a significant reduction in 2 month breastmilk DHA in women heterozygous for the rs174575 SNP, but not for those women homozygous for the minor allele, when compared to women homozygous for the major allele. Additionally, no significant differences in breastmilk fatty acids based on SNP were found regarding SNP rs174561. It is possible that significant results in carriers of homozygous minor alleles were not seen due to the low number of subjects and the high variability of breastmilk DHA proportions.

It would be expected that decreased breastmilk DHA in women carriers of SNP within the FADS1 FADS2 gene cluster could be compensated with increased dietary DHA intake, however, this appears to not be the case. In the current study, increased daily DHA intake did not significantly increase 2 month breastmilk DHA in women heterozygous or homozygous for the minor allele for SNP rs174575, yet, women homozygous for the major allele did see significantly increased breastmilk DHA with high daily DHA intake compared to low daily intake. Similarly, Moltó-Puigmartí et al. found proportions of DHA in breastmilk only increased with increased fish and fish-oil intake in the major allele carriers for SNP rs174575 and rs174561 and not in minor allele carriers.[18] If synthesis and transfer of DHA into breastmilk is impaired by SNP within the FADS1 FADS2 gene cluster, it may not be beneficial to increase maternal DHA intake for the purposes of increasing DHA transfer to the infant, and thus, alternative DHA supplementation for the infant may need to be investigated.

SNP within the FADS1 FADS2 gene cluster may affect a reduction in breastmilk DHA due to decreased Δ5- and Δ6-desaturase transcription and/or activity, while also possibly affecting transfer and incorporation of DHA into breastmilk from dietary and maternal sources. The reduction in DHA transferred into breastmilk in women carriers of FADS SNP could be due to alterations involving fatty acid transport proteins (FATP), fatty acid binding proteins (FABP) or modifications of the incorporation of DHA in phospholipid classes or chylomicrons.[60,61,65,66,69,72,248,255] A polymorphism within the FABP2 gene was recently found to be associated with impaired Δ6-desaturase activation and resulted in decreased plasma AA. Although dietary increases in DHA did not result in increased breastmilk DHA in women with SNP within the FADS1 FADS2 gene cluster, it is not possible for the current study to infer the

mechanisms involved in the affect genetic variations may have on DHA transfer. Future, more detailed studies are necessary to determine how FADS SNP are associated with breastmilk DHA.

Previous research has shown increased DHA intake during lactation, increased breastmilk DHA, and higher DHA status results in improved neurocognitive development in young children and enhanced IQ in later childhood, however results remain mixed.[10,16,17,20,22,127,155,160-165,168,169] Several recent studies have reported a possible association between FADS SNP and child IQ.[25,245,252] A recent study of large birth cohorts reported children breastfed that were major allele carriers for SNP rs174575 had a significant increase in IQ compared to carriers of the same allele that were not breastfed.[26] Additionally, the study found that children who were minor allele carriers did not have any IQ advantage to being breastfed, suggesting the SNP somehow influences the positive benefits breastfeeding has on neurocognitive development. It is believed to be unlikely that the breastfeeding effects on IQ are caused directly by genetic variation, but rather the SNP may influence the availability of DHA to the child through breastmilk. However, a study of nearly 6000 children found no significant differences between breastfeeding and IQ based upon genetic variations, indicating the influence of genotype on IQ remains inconclusive.[27] In the current study, a significant interaction was found between SNP rs174575 genotype and daily DHA intake on infant cognitive test scores. While SNP alone did not have any significant effect on infant scores on the BSID-III MDI cognitive subtest at 12 months of age, SNP appeared to interact with daily DHA intake by possibly affecting the availability of DHA to the infant through reduced breastmilk concentrations, subsequently impacting infant neurocognitive development.

Many recent RCT and meta-analyses have reported inconclusive results regarding an association between DHA and neurocognitive development.[20,127,155,160,168-170] Differences in RCT

are possibly due to several differences in study designs. RCT supplement with a wide range of DHA, from 200 mg to 2.2 g of DHA per day, and the length and timing of supplementation varies largely in cognitive studies. Trials most often supplement during pregnancy only, not taking into account the importance of brain development and increased dietary DHA need in early infancy after birth. The most convincing RCT supplement DHA during the last trimester of pregnancy through the first few months of life and benefit preterm infants the greatest. For these reasons, the current study chose to supplement women during the last trimester of pregnancy through the first 3 months of lactation with a dose of DHA based on previous research studies and above the daily DHA intake recommended by the European Influence of Dietary Fatty Acids on the Pathophysiology of Intrauterine Foetal Growth and Neonatal Development (PERILIP) and other groups.[38,243]

Inconclusive results from DHA RCT may also be due to the disparity between cognitive tests used and the age at which the tests are administered. Current assessments of early cognitive function, such as the BSID and Fagan Test of Infant Intelligence (FTII), may not be sensitive enough to detect the specific effects DHA supplementation may have on certain aspects of cognitive development at certain ages. Several recent RCT have found no effects of maternal DHA supplementation on infant neurocognitive development during the first few months of life, yet, found significant improvements in cognitive scores once the children reached 12 months of age into preschool ages.[10,15,21,164,166] Standardized infant development tests, such as the BSID, use a variety of tasks to assess cognitive function and may provide a better estimate of later cognitive ability. The current study used the BSID because the test is widely accepted and commonly used throughout cognitive studies, and when administered in late infancy has been shown to correlate with IQ scores at 4 years of age.[229] Additionally, the current study did not find

an association between daily maternal DHA intake and infant neurocognitive development early in infancy at 4 months of age, but found a significant association at 12 months of age on the BSID-III MDI cognitive subtest. However, more specific tests of attention and information processing have recently been shown to possibly be better measures of infant cognitive function and predictors of later childhood IQ than the BSID, all of which were determined after the current study was designed and completed, but are important to consider in future cognitive studies.[232,233]

Additionally, the majority of RCT were performed in developed countries in presumably well-nourished women. Failure to determine a significant association between DHA and infant neurocognitive development in such trials could be explained by a sufficient baseline of DHA and not accounting for all sources of DHA in the maternal diet. By accounting for all sources of DHA, this study was able to estimate total daily DHA intake and determined a significant dosing effect of DHA on infant growth and cognitive development. In this study, DHA supplementation was not associated with infant neurocognitive development based on treatment, most likely due to a 56% higher DHA intake, through food and self-supplementation, in the placebo control group compared to the DHA treatment group. Several previous RCT have shown no significant results based upon DHA treatment, yet did show a significant association between DHA status and infant neurocognitive development.[20-23,156,157] Recent systematic reviews and meta-analyses have noted children with low DHA status benefit the most from increased DHA during gestation and lactation.[167,168] Additionally, genetics may also play a large role on DHA status and the benefits of DHA supplementation on infant neurocognitive development. SNP within the FADS1 FADS2 gene cluster and other currently unknown genetic differences may dramatically affect DHA synthesis, transfer and utilization involved in neurocognitive development.

A possible limitation to this study is a completion rate of 80% in the DHA treatment group with only a 64% completion rate in the placebo control group. The reasons for the differences between completion rates are unknown, however, total numbers per treatment group were greater than the 32 infants determined necessary by a pilot study to detect a significant difference between groups. Despite the disparity in completion rates between groups, the adherence of women that completed the study was excellent, with greater than 90% of the women taking all of the supplied study supplements. Study compliance was measured by end of study pill counts and not by a physiologic measurement, which could present another possible study limitation. Yet, estimated baseline daily DHA intake positively correlated with baseline maternal erythrocyte DHA ($r=0.24$, $p=0.011$) and at 2 months, daily DHA intake estimated from the 2 month food frequency questionnaire (FFQ) positively correlated with breastmilk DHA ($r=0.21$, $p=0.045$). Postpartum and infant blood samples would have allowed for an objective mean of measuring study compliance and also provided information on infant DHA status.

Another potential limitation to this study was the homogeneity of the study population which resulted in a limited number of minor allele carriers for each of the SNP investigated. Unfortunately, this study had less than the anticipated 10 homozygous minor allele carriers, based upon previously described minor allele frequency of ≥18% , and even fewer minor allele carriers that completed the study.[18] Additionally, only two SNP were analyzed in the current study, thus further limiting potential genetic interaction with fatty acid composition and infant neurocognitive development. The SNP studied were chosen based upon previous research indicating possible SNP effects on breastmilk DHA, yet more SNP could have provided a clearer picture to effect SNP within the FADS1 FADS2 gene cluster have on DHA proportions. The

study also lacked infant genetic information, which could have shown further important influences of DHA on neurocognitive development.

The study had several strengths in design and execution, most notably that all sources of DHA were accounted for in the maternal diets, including DHA from voluntary supplementation and food. The current study collected data from each subject regarding prenatal vitamins, DHA or fish oil supplements, as well as food sources of DHA through a FFQ administered at baseline and 2 months postpartum and a 7-day food record. No effects were determined between DHA treatment group and infant neurocognitive development, most likely due to the high level of self supplementation in the placebo control group. By accounting for all sources of DHA, this study was able to effectively determine a significant association between high daily maternal DHA intake and improved cognitive development scores on the BSID-III at 12 months of age. Additionally, DHA was supplemented in this study for a longer period of time than most RCT, through important times of extensive infant brain growth and development. The study utilized the BSID-III as a general measure of overall cognitive performance and administered the test at two time points in infant development. By testing at one year of age, it was possible to successfully determine a significant association between early maternal DHA intake and cognitive development. If BSID-III testing was only performed early in infancy, results would not have shown any positive association of maternal DHA intake and the specific function of DHA on cognitive development may have been missed. Additionally, this study controlled for potential confounders, including gestational age, maternal IQ and the home environment, all of which could have dramatically impacted the effects DHA had on neurocognitive development.

Future DHA supplementation studies should focus on supplementing DHA for a longer length of time during critical times of infant neurological growth and development in a large

study population. A larger and more heterogeneous population would allow for more minor allele SNP to be detected and possibly allow for the determination of the impact SNP may have on DHA treatment. The current study could analyze additional SNP from stored purified DNA to determine if other SNP within or outside of the FADS1 FADS2 gene cluster have a more significant effect on DHA proportions and neurocognitive development. Additionally, cord blood and infant blood would be of interest in future studies to provide infant DHA status measurements and infant genetic information. Mechanism of action studies would be beneficial, focusing on effects of DHA in neurological development, preterm births, infant anthropometric measurements. Studies focusing on mechanisms behind SNP interactions with DHA and neurological development are also of great interest to determine not only desaturase activity effects but also SNP effects on DHA transfer into breastmilk.

It would be of great interest to perform IQ and cognitive testing on the children within this study at a later age to determine the effect maternal DHA supplementation may have on childhood cognitive ability. This would also provide information on whether or not the positive association seen in this study between maternal DHA supplementation and improved infant cognitive scores on the BSID-III at 12 months of age continues into preschool, school or even adolescent ages. Additionally, it would be of great interest to investigate any affect DHA may have on BMI, adipocity and childhood obesity. Future studies could investigate an association between increased maternal DHA intake and infant adipocity using infant Peapod or DEXA scans and follow the children into early adolescence. Furthermore, childhood adipocity, BMI and obesity could be analyzed in children from the current study through future research.

In conclusion, supplementing pregnant and lactating women with DHA could safely enhance the neurocognitive development of infants as well as possibly increase gestational

length and reduce preterm birth risk. Additionally, SNP within the FADS1 FADS2 gene cluster significantly alter erythrocyte and breastmilk DHA, subsequently impacting the DHA received by the infant through breastmilk and possibly influence infant neurocognitive development. Total DHA intake and maternal genetics should be taken into account in future RCT to properly assess the effects DHA supplementation has on infant outcomes. The current study provides information on optimal DHA intake and may also help to impact future national standards and recommendations for DHA supplementation during pregnancy and lactation. While further research is necessary, DHA supplementation during pregnancy and lactation could improve the health of the infant by improving neurocognitive development and increasing gestational length.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL LETTER

COPY

Colorado State University

Notice of Approval for Human Research

Office of Regulatory Compliance
Office of Vice President for Research
Fort Collins, CO 80523-2011
(970) 491-1553
FAX: (970) 491-2293

Principal Investigator: Mary Harris, FSHN, 1571

Title: Relationship between Infant Growth & Neurocognitive Development and Maternal Intake of n-3 Docosahexaenoic Acid (DHA)

Protocol #: 05-352H **Funding Source:** USDA NRI

Number of Participants/Records: The original 200 women and their babies

Committee Action: **Approval Date:** March 23, 2007 **Expires:** March 23, 2008

HRC Administrator: Janell Meldrem Janell Meldrem

Consent Process:
The above-referenced project was approved by the Human Research Committee with the condition that the attached consent form is signed by the subjects and each subject is given a copy of the form. *NO changes may be made to this document without first obtaining the approval of the Committee.*

Amendment approved: To add an IQ test and environmental questions.

Investigator Responsibilities:

- It is the PI's responsibility to obtain this consent form from all subjects.
- It is the responsibility of the PI to immediately inform the Committee of any serious complications, unexpected risks, or injuries resulting from this research.
- It is also the PI's responsibility to notify the Committee of any changes in experimental design, participant population, consent procedures or documents. This can be done with a memo describing the changes and submitting any altered documents.
- Students serving as Co-Principal Investigators must obtain PI approval for any changes prior to submitting the proposed changes to the HRC for review and approval.
- The PI is ultimately responsible for the conduct of the project.
- A status report of this project will be required within a 12-month period from the date of review. Renewal is the PI's responsibility, but as a courtesy, a reminder will be sent approximately two months before the protocol expires. The PI will be asked to report on the numbers of subjects who have participated this year and project-to-date, problems encountered, and provide a verifying copy of the consent form or cover letter used. The necessary continuation form (H-101) is available from the RCO web page www.research.colostate.edu/rcoweb/.
- Upon completion of the project, an H-101 should be submitted as a close-out report.
- If approval did not accompany a proposal when it was submitted to a sponsor, it is the PI's responsibility to provide the sponsor with the approval notice.
- **Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.**

This approval is issued under Colorado State University's OHRP Federal Wide Assurance 00000647.

Please direct any questions about the Committee's action on this project to me for routing to the Committee.

Attachment Date of Correspondence: 5/14/07

APPENDIX B

PARTICIPANT CONSENT FORM

Consent to Serve as a Subject in Human Research

Title of Project: Omega Smart Baby Project
(Study of the relationship between infant growth and neurocognitive development and maternal intake of omega-3 DHA)

Principal Investigator: Mary Harris, PhD, RD, BC-ADM
Co-Investigators: Susan Baker, EdD
Deana Davalos, PhD

WHO IS DOING THE STUDY? Colorado State University is conducting the study. The study is being funded by the USDA.

WHAT IS THE PURPOSE OF THIS STUDY?
The primary purpose of the study is to see if DHA taken during pregnancy and lactation improves your infant's performance on standard tests of infant growth (weight and length measurements) and mental development using a state-of-the-art electrophysiological measure of brain activity. You will be asked to fill out pencil and paper surveys of your infant's temperament and home environment. At various time points, we will ask you for a record of your infant's dietary intake.

WHY AM I BEING ASKED TO PARTICIPATE IN THIS RESEARCH? You are being asked to participate in a study in which you will take an additional supplement of an essential nutrient (an omega-3 fatty acid called DHA, or docosahexaenoic acid) from week 26 of pregnancy through the first 3 months of lactation. The supplement will be provided as an all natural, highly purified tuna oil capsule. You will be asked to commit to exclusive breastfeeding to feed your baby during the three months of life (exclusive breastfeeding means nothing except breast milk). If you need to use formula to supplement your breast milk, we ask that you use non-DHA containing formula. During the study, you may be receiving the tuna oil or you may receive the control treatment (Sunola oil). DHA and tuna oil have been used as a dietary supplement in many pregnancy trials and has been found to be safe for use by pregnant women. It is available on the market but the optimum dose is not yet determined, many women may need more than is currently present in the maternal vitamin supplement. You will continue to take your prescribed maternal vitamin and mineral supplement with or without small amounts of DHA that you and your physician agree upon.

WHAT WILL I BE ASKED TO DO? If you agree to be in the study, you will be regularly given a supply of study capsules which may contain tuna oil or placebo (Sunola) oil. We will ask you to take one capsule each day. We will ask you to provide an extra tube of blood during your test today for n-3 DHA analysis (this will amount to 10 milliliters, equivalent to about 2 teaspoonfuls). We will ask you to complete a food frequency questionnaire about your dietary protein intake today. Upon delivery of your baby, we will give you a card upon which to have your doctor record your infant's vital statistics (birth weight, length and head circumference and gestational age) and at your first postnatal visit. During your pregnancy throughout the time you breastfeed, we will provide a personal Breastfeeding Coach to assist you with any questions or concerns you might have while breastfeeding your baby. We will contact you periodically to see how you are doing and to obtain information about your baby's current diet. Your Breastfeeding Coach will give you her cell phone number so that you may contact her whenever the need or a question arises. When your baby is 4 and 12 months of age he/she will be tested for mental development using a measure of brain waves. The tests will be completed at CSU at no charge to you. These tests take about 45-90 minutes to administer. Most mothers and babies find it to be a fun experience. During these developmental tests we will ask you to fill out a questionnaire of your baby's temperament, the baby's environment (toys, siblings to play with etc), and a simple Maternal Health Measures survey. In addition, you will be asked to complete a brief cognitive measure assessing verbal and visual processing. These should not take more than 30 - 45 minutes to fill out. We will also ask you to give a small sample (10cc) of breast milk after 2 and 4 months of lactation so that we may analyze breast milk DHA content and to keep a diet record at this time. Milk samples will be obtained in about the middle of a feeding using your breast pump and your Breastfeeding Coach will be available to assist you with this collection if you wish.

WHERE WILL THE STUDY TAKE PLACE? Blood will be drawn at the Women's Clinic lab in Fort Collins. Infant and toddler testing will take place at Colorado State University.

HOW LONG WILL THE STUDY LAST? The study will last from week 24-26 of pregnancy through the first year of your baby's life.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS? It is not possible to identify all potential risks in a research procedure, but the researchers have taken

reasonable precautions to minimize any known and potential, but unknown, risks**.** There are no anticipated physical risks for participating in the brainwave measurement. Your infant will be sitting on your lap for the tests but it is possible that he/she could possibly become upset by the testing. We will stop the test immediately if such an event occurs. The risks of blood drawing are: a possible hematoma (bruise) at the site where blood was drawn and the remote risk of infection. There are no other known or anticipated risks associated with the study other than those addressed above.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? You will receive a free high-quality manual breast pump and the assistance of a personal Breastfeeding Counselor for participating in the study. You will receive a complete diet analysis during lactation. Your infant will receive tests of cognitive development and we will send the results to your baby's doctor if you wish us to. While there is no guarantee that your baby will receive any health benefits from participation in this study, your infant may perform better on the cognitive tests if you and your baby were in the DHA group.

WHAT WILL IT COST ME TO PARTICIPATE? There will be no costs to you.

DO I HAVE TO TAKE PART IN THE STUDY? / CAN MY TAKING PART IN THE STUDY END EARLY? Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled. If you decide that you cannot breastfeed exclusively as defined in the study, you will still remain in the study for the purpose of infant testing if you wish to continue.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private. We will not ask for access to your medical records, we will only ask that you have your baby's doctor record your baby's gestational age, birth weight, height and head circumference information.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the investigator, Dr. Mary Harris at 970-491-7462. If you have medical questions about the study we encourage you to discuss them with your obstetrician. If you have any questions about your rights as a volunteer in this research contact Janell Barker, Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 4 pages.

_____
Participant name (printed)

_____ _____
Participant Signature Date

_____ _____
Witness to signature (project staff) Date

APPENDIX C

NU-MEGA STUDY SUPPLEMENTS VERIFICATION REPORT

Nu-Mega Ingredients Pty Ltd
A.B.N: 98 102 460 739
31 Pinnacle Road
Altona North VIC 3025
Australia
PO Box 1111
Altona Gate, VIC 3025
Australia
Telephone: 61 3 8369 2100
Facsimile: 61 3 9369 8900

Nu-Mega Ingredients Pty Ltd

LABORATORY REPORT

Date: 23/04/2009
Product: Sunola Placebo Capsule
Batch Number: B 3438-2
Sample Identification: CCPTMO – Sunola Oil
Received: 22 April 2009
Lab Sample No: L-0739

Analysis	RESULTS	METHOD
Chemicals:		
p-Anisidine Value	1.1	TM04 In house Method
Peroxide Value mEq O_2/kg	0.3	TM03 In house Method
TOTOX Value (pAV + 2PV)	1.7	-
FA Profiles		
Oleic Acid (18:1w9) %	82	Nu-Mega CLOV 036
Linoleic Acid (LA) %	7.7	Nu-Mega CLOV 036
Alpha Linoleic Acid (ALA) %	0.3	Nu-Mega CLOV 036

Please Note: Results pertain to sample provided.

Micro Analysis	BN: B1753-2
E.Coli (37°C / 48 hrs) in 1g	ND
Standard Plate Count (30°C / 72 hrs) CFU/g	<10
Enterobacteriaceae (37°C / 24hr) CFU/g	<0.3
Yeast and Mould (25°C / 5 days) CFU/g	<10
Listeria spp (30°C / 24 hrs) in 10g	ND
Salmonella spp (37°C / 18hrs) in 10g	ND

Analyst: Cindy Koentjoro & Stenny Adi

Date: 30/04/2009

Nu-Mega Ingredients Pty Ltd
A.B.N: 98 102 460 739
31 Pinnacle Road
Altona North VIC 3025
Australia
PO Box 1111
Altona Gate, VIC 3025
Australia
Telephone: 61 3 8369 2100
Facsimile: 61 3 9369 8900

Nu-Mega Ingredients Pty Ltd

Laboratory Report

Date: 28 November 2008

Product: Tuna Capsules

Batch Number: B1752-2

Manufacturing Date: October 2008

Sample Identification: CCPTMO01

Lab Sample No: L-0480

Analysis	BN: B1752-2
Oxidative:	
Peroxide Value (meq O_2/Kg)	2.6
p-Anisidine Value	7.7
TOTOX Value (p-Anisidine Value + 2xPV)	12.9
FA Profiles:	
Docosahexaenoic Acid (DHA) %	26.0
Eicosapentaenoic Acid (EPA) %	5.8
Linoleic Acid (LA) %	2.3
Arachidonic Acid (AA) %	1.8
Gamma Linolenic Acid (GLA) %	0.3
Alpha Linoleic Acid (ALA) %	0.4

Micro Analysis	**BN: B1752-2**
E.Coli (37°C / 48 hrs) in 1g	ND
Standard Plate Count (30°C / 72 hrs) CFU/g	~10
Enterobacteriaceae (37°C / 24hr) CFU/g	<0.3
Yeast and Mould (25°C / 5 days) CFU/g	~10
Listeria spp (30°C / 24 hrs) in 10g	ND
Salmonella spp (37°C / 18hrs) in 10g	ND

Please Note: Results pertain to sample provided. Micro Analysis were performed externally.

Analyst... Stenny Adi / Maria Cary

APPENDIX D

FOOD FREQUENCY QUESTIONNAIRE

Food Frequency Questionnaire **Study Number _____**

___Salmon/Trout
___times per day, week or month
(circle one)

___White tuna
(also called albacore tuna)
___times per day, week or month
(circle one)

___Light tuna
___times per day, week or month
(circle one)

___Sardines/
Herring/Anchovies
___times per day, week or month
(circle one)

___Pork/Beef/Lamb
___times per day, week or month
(circle one)

___Chicken/Turkey
___times per day, week or month
(circle one)

___Eggs
___times per day, week or month
(circle one)

___Goldcircle or Store
brand Omega-3 eggs
___times per day, week or month
(circle one)

___Milk
___times per day, week or month
(circle one)
With DHA???? yes/

___Cheese
___times per day, week or month
(circle one)

___Cod liver oil
or other fish oils
___times per day, week or month
(circle one)

___DHA
(docosahexanoic acid) or Omega 3
fatty acid or fish oil suppolement

Brand _____
Amount of DHA/EPA_____

Thank you for your time and assistance with this study. Your input will help other women in the future. The Omega-3 Smart Baby Project Follow-Up Team!

APPENDIX E

7-DAY FOOD RECORD

Omega Smart Baby Project Food Journal

1. **Please write down everything you eat or drink for a 7-day period.**

2. **The attached sheets will give you some directions on how to fill out a food journal so that we can provide you with an accurate analysis of your diet.**

3. **You do not need to have a special form to record your information on, use the sheets attached or whatever is convenient for you.**

4. **If the food you eat is a mixed dish, please provide the recipe and the yield (or the package label if purchased ready to eat).**

Thank you for your time!!

Food Record – Day 1 **Date:_____**

Day of the week MEAL was eaten (please circle): Sun Mon Tues Wed Thurs Fri Sat

Directions: Record all foods and beverages (except water) that you ate on the day circled above. Write the time (column 1), food eaten (column 2), and how much you ate (column 3).
Please keep a record for 7 days. Use a separate sheet for each day.

Time of Day	List and describe each food/drink eaten	Brand Name of food (if any)	How much did you eat?

Food Record – Day 2 **Date:_____**

<u>Day of the week MEAL was eaten (please circle):</u> **Sun Mon Tues Wed Thurs Fri Sat**

Time of Day	List and describe each food/drink eaten	Brand Name of food (if any)	How much did you eat?

Food Record – Day 3 **Date:_____**

<u>Day of the week MEAL was eaten (please circle):</u> **Sun Mon Tues Wed Thurs Fri Sat**

Time of Day	List and describe each food/drink eaten	Brand name of food (if any)	How much did you eat?

Food Record – Day 4 **Date:_____**

<u>Day of the week MEAL was eaten (please circle):</u> **Sun Mon Tues Wed Thurs Fri Sat**

Time of Day	List and describe each food/drink eaten	Brand name of food (if any)	How much did you eat?

Food Record – Day 5 **Date:_____**

<u>Day of the week MEAL was eaten (please circle):</u> **Sun Mon Tues Wed Thurs Fri Sat**

Time of Day	List and describe each food/drink eaten	Brand name of food (if any)	How much did you eat?

Food Record – Day 6 **Date:_____**

<u>Day of the week MEAL was eaten (please circle):</u> **Sun Mon Tues Wed Thurs Fri Sat**

Time of Day	List and describe each food/drink eaten	Brand name of food (if any)	How much did you eat?

Food Record – Day 7 **Date:_____**

<u>Day of the week MEAL was eaten (please circle):</u> **Sun Mon Tues Wed Thurs Fri Sat**

Time of Day	List and describe each food/drink eaten	Brand name of food (if any)	How much did you eat?

APPENDIX F

SOCIODEMOGRAPHIC INFORMATION SHEET

Subject Information and Follow-Up Form Study Number _____

Name: _____

How long do you plan to breastfeed your baby? _____

Preferred Phone Number: _____

Date of Birth: _____

Date of Last Menstrual Period: _____

Expected Due Date: _____

Height: _____

Weight before you became pregnant:_____

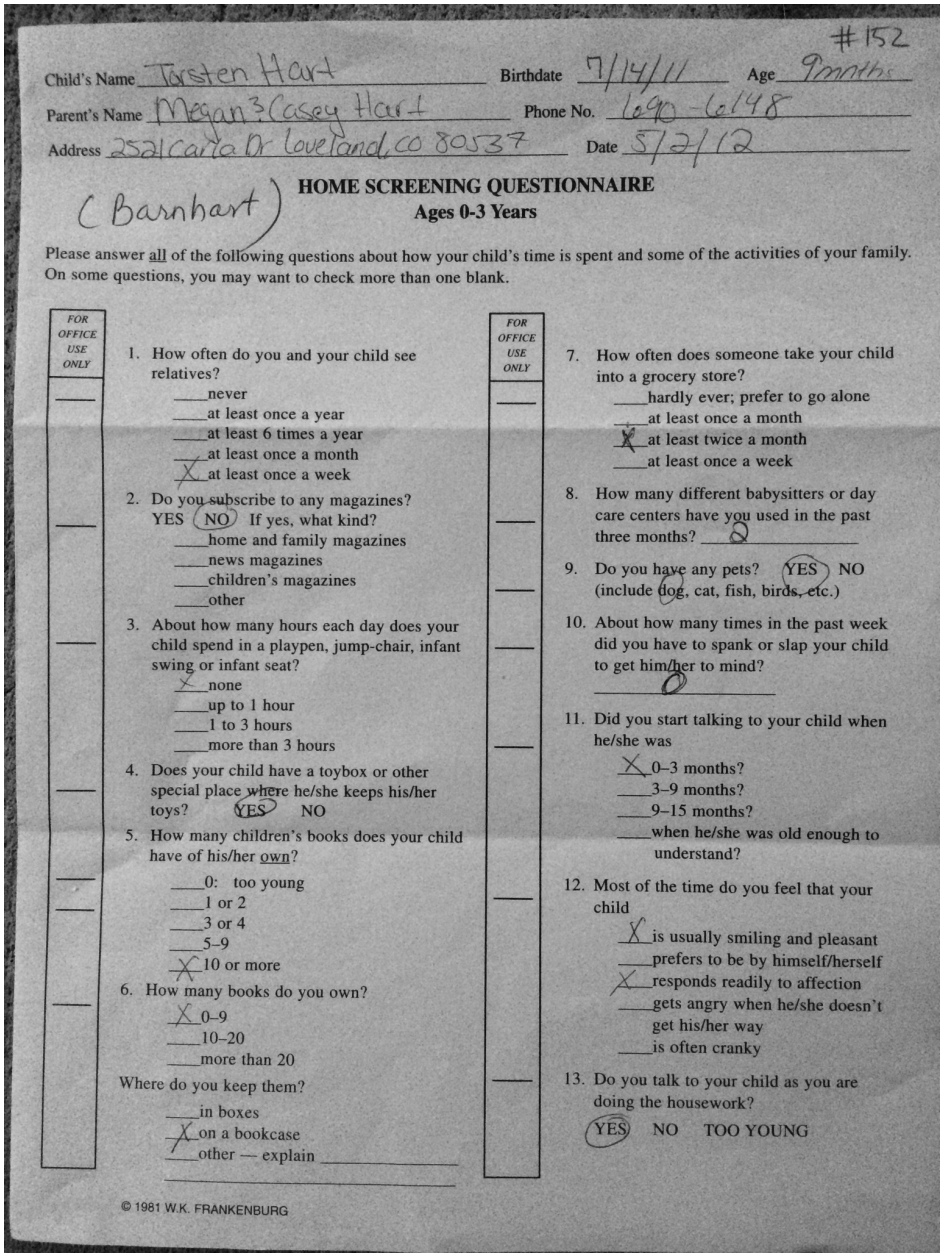
Brand of prenatal vitamin you are taking: _____

Do you take other nutritional supplements:_____
If yes, Which ones?_____

Ethnicity:
Non-hispanic White _____
Hispanic/Latino _____
Black _____
Asian/Pacific Islander_____
Other_____

APPENDIX G

HOME SCREENING QUESTIONNAIRE



#152

Child's Name Torsten Hart Birthdate 7/14/11 Age 9months

Parent's Name Megan & Casey Hart Phone No. 690-6148

Address 2521 Carla Dr Loveland, CO 80537 Date 5/2/12

(Barnhart)

HOME SCREENING QUESTIONNAIRE
Ages 0-3 Years

Please answer all of the following questions about how your child's time is spent and some of the activities of your family. On some questions, you may want to check more than one blank.

FOR OFFICE USE ONLY

1. How often do you and your child see relatives?
   ___never
   ___at least once a year
   ___at least 6 times a year
   ___at least once a month
   X at least once a week

2. Do you subscribe to any magazines?
   YES (NO) If yes, what kind?
   ___home and family magazines
   ___news magazines
   ___children's magazines
   ___other

3. About how many hours each day does your child spend in a playpen, jump-chair, infant swing or infant seat?
   X none
   ___up to 1 hour
   ___1 to 3 hours
   ___more than 3 hours

4. Does your child have a toybox or other special place where he/she keeps his/her toys? (YES) NO

5. How many children's books does your child have of his/her own?
   ___0: too young
   ___1 or 2
   ___3 or 4
   ___5–9
   X 10 or more

6. How many books do you own?
   X 0–9
   ___10–20
   ___more than 20

   Where do you keep them?
   ___in boxes
   X on a bookcase
   ___other — explain _____

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7. How often does someone take your child into a grocery store?
   ___hardly ever; prefer to go alone
   ___at least once a month
   X at least twice a month
   ___at least once a week

8. How many different babysitters or day care centers have you used in the past three months? 2

9. Do you have any pets? (YES) NO
   (include dog, cat, fish, birds, etc.)

10. About how many times in the past week did you have to spank or slap your child to get him/her to mind?
    0

11. Did you start talking to your child when he/she was
    X 0–3 months?
    ___3–9 months?
    ___9–15 months?
    ___when he/she was old enough to understand?

12. Most of the time do you feel that your child
    X is usually smiling and pleasant
    ___prefers to be by himself/herself
    X responds readily to affection
    ___gets angry when he/she doesn't get his/her way
    ___is often cranky

13. Do you talk to your child as you are doing the housework?
    (YES) NO TOO YOUNG

Subject sample Home Screening Questionnaire (HSQ)

APPENDIX H

INFANT TEMPERAMENT QUESTIONNAIRE

6 month

Bates Lab
Dept. of Psychology
Indiana University
Bloomington, IN 47405

INFANT QUESTIONNAIRE

Part I.

Your baby's name _____

Your baby's birth date _____

Your baby's sex _____

Birth weight _____

Length at birth _____

Present weight (if known) _____

Your name _____

Address _____

_____

Phone No. _____

Today's date _____

Part II. On the following questions please circle the number that is most typical of your baby. "About average" means how you think the typical baby would be scored.

1. How easy or difficult is it for you to calm or soothe your baby when he/she is upset?

   1 2 3 4 5 6 7

   very easy about average difficult

2. How easy or difficult is it for you to predict when your baby will go to sleep and wake up?

   1 2 3 4 5 6 7

   very easy about average difficult

3. How easy or difficult is it for you to predict when your baby will become hungry?

1	2	3	4	5	6	7
very easy			about average			difficult

4. How easy or difficult is it for you to know what's bothering your baby when he/she cries or fusses?

1	2	3	4	5	6	7
very easy			about average			difficult

5. How many times per day, on the average, does your baby get fussy and irritable—for either short or long periods of time?

1	2	3	4	5	6	7
never	1-2 times per day	3-4 times per day	5-6 times per day	7-9 times per day	10-14 times per day	more than 15

6. How much does your baby cry and fuss in general?

1	2	3	4	5	6	7
very little; much less than the average baby			average amount; about as much as the average baby			a lot; much more than the average baby

7. How did your baby respond to his/her first bath?

1	2	3	4	5	6	7
very well-- baby loved it			neither liked nor disliked it			terribly-- didn't like it

8. How did your baby respond to his/her first solid food?

1	2	3	4	5	6	7
very favorably-- like it immediately			neither liked nor disliked it			very negatively— did not like it at all

9. How does your baby typically respond to a new person?

1	2	3	4	5	6	7
almost always responds favorably		responds favorably about half the time				almost always responds negatively at first

10. How does your baby typically respond to being in a new place?

1	2	3	4	5	6	7
almost always responds favorably		responds favorably about half the time				almost always responds negatively at first

11. How well does your baby adapt to things (such as in items 7-10) eventually?

1	2	3	4	5	6	7
very well, always likes it eventually		ends up liking it about half the time				almost always dislikes it in the end

12. How easily does your infant get upset?

1	2	3	4	5	6	7
very hard to upset-- even by things that upset most babies		about average				very easily upset by things that wouldn't bother other babies

13. When your baby gets upset (e.g., before feeding, during diapering, etc.), how vigorously or loudly does he/she cry and fuss?

1	2	3	4	5	6	7
very mild intensity or loudness		moderate intensity or loudness				very loud or intense, really cuts loose

14. How does your baby react when you are dressing him/her?

1	2	3	4	5	6	7
very well-- likes it		about average—doesn't mind it				doesn't like it at all

15. How active is your baby in general?

1	2	3	4	5	6	7
very calm and quiet			average			very active and vigorous

16. How much does your baby smile and make happy sounds?

1	2	3	4	5	6	7
a great deal, much more than most infants			an average amount			very little, much less than most infants

17. What kind of mood is your baby generally in?

1	2	3	4	5	6	7
very happy and cheerful			neither serious nor cheerful			serious

18. How much does your baby enjoy playing little games with you?

1	2	3	4	5	6	7
a great deal, really loves it			about average			very little, doesn't like it very much

19. How much does your baby want to be held?

1	2	3	4	5	6	7
wants to be free most of the time			sometimes wants to be held, sometimes not			a great deal-- wants to be held almost all the time

20. How does your baby respond to disruptions and changes in everyday routine, such as when you go to church or a meeting, on trips, etc.?

1	2	3	4	5	6	7
very favorably, doesn't get upset			about average			very unfavorably, gets quite upset

21. How easy is it for you to predict when your baby will need a diaper change?

1	2	3	4	5	6	7
very easy			about average			very difficult

22. How changeable is your baby's mood?

1	2	3	4	5	6	7
changes seldom, and changes slowly when he/she does change			about average			changes often and rapidly

23. How excited does your baby become when people play with or talk to him/her?

1	2	3	4	5	6	7
very excited			about average			not at all

24. Please rate the overall degree of difficulty your baby would present for the average mother.

1	2	3	4	5	6	7
super easy			ordinary, some problems			highly difficult to deal with

APPENDIX I

NON-SIGNIFICANT RESULT TABLES

Table I.1

Fatty acid composition (percentage of total fatty acids identified) of maternal baseline erythrocyte, baseline plasma phospholipids and 2 month breastmilk samples classified according to treatment and Pearson's correlation coefficients between erythrocyte and breastmilk fatty acids as well as plasma phospholipids and breast milk fatty acids.[a]

	Erythrocyte			Plasma			2 mo BM		
Fatty Acid	**Placebo**	**DHA**	**P**	**Placebo**	**DHA**	**P**	**Placebo**	**DHA**	**P**
Myristic 14:0	0.59±0.21	0.62±0.31	0.528	0.04±0.13	0.10±0.19	0.072	4.52±1.39	5.27±2.14	0.062
Palmitic 16:0	24.73±1.98	24.67±2.08	0.888	32.13±3.87	32.29±5.96	0.868	17.43±4.11	19.14±5.40	0.103
Palmitoleic 16:1				0.07±0.19	0.13±0.26	0.210	2.77±0.87	2.77±0.93	0.973
Stearic 18:0	14.41±1.54	14.76±1.61	0.235	12.66±2.04	12.82±2.40	0.696	5.91±2.30	6.50±2.54	0.263
Oleic 18:1n9	15.71±2.06	15.79±1.70	0.822	9.92±0.98	9.64±1.80	0.309	40.23±3.67	37.80±3.59	0.002[c]
Vaccenic 18:1n7	1.65±0.61	1.59±0.45	0.562	1.53±0.30	1.55±0.31	0.696	1.79±0.39	1.81±0.26	0.832
Linoleic 18:2	16.92±2.56	16.85±3.04	0.891	22.94±2.89	22.70±3.88	0.702	23.23±5.05	22.48±6.61	0.557
ALA 18:3	0.37±0.22	0.35±0.22	0.576	0.24±0.19	0.21±0.19	0.445	2.17±0.83	2.09±0.81	0.610
DGLA 20:3	2.39±0.57	2.43±0.60	0.676	3.81±0.98	3.66±0.87	0.376	0.46±0.44	0.43±0.23	0.625
AA 20:4	14.22±1.78	14.35±1.91	0.710	10.41±2.18	10.83±2.56	0.353	0.88±0.43	0.88±0.47	0.965
EPA 20:5	0.56±0.39	0.60±0.57	0.649	0.51±0.44	0.56±0.59	0.604	0.08±0.14	0.11±0.20	0.478
DPA 22:5n3	2.23±0.74	2.21±0.57	0.845	0.56±0.39	0.55±0.39	0.896	0.10±0.16	0.08±0.14	0.611
DHA 22:6	6.17±1.47	5.75±1.55	0.137	5.17±1.36	4.97±1.53	0.455	0.40±0.45	0.62±0.43	0.017[c]

[a] The numbers of women with plasma and RBC data in the low DHA intake group, medium DHA intake group, and high DHA intake group were as follows: 55 and 60, respectively; 39 and 53, respectively, for women also with 2 month breast milk samples. ALA, α-linoleic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid, 2 mo BM, 2 month breastmilk samples. Differences between fatty acid proportions between intake groups were assessed using ANOVA.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table I.2

Fatty acid composition (percentage of total fatty acids identified) of maternal baseline erythrocyte samples classified according to both genotype for SNP rs174575 and rs174561, as well as treatment type.[a]

		MM			Mm			mm		
Fatty Acid	**SNP**	**Placebo**	**DHA**	**p**	**Placebo**	**DHA**	**p**	**Placebo**	**DHA**	**p**
Myristic 14:0	rs174575	0.56±0.17[b]	0.66±1.49	0.180	0.63±0.23	0.56±0.17	0.300	0.77±0.45	0.66±0.06	0.641
	rs174561	0.59±0.17	0.64±0.36	0.533	0.56±0.23	0.59±0.22	0.676	0.69±0.34	0.64±0.08	0.811
Palmitic 16:0	rs174575	24.71±2.06	24.62±2.40	0.863	24.67±1.81	24.83±1.55	0.786	25.26±2.86	24.33±1.70	0.608
	rs174561	25.06±2.04	24.72±2.39	0.547	24.20±1.82	24.51±1.65	0.568	24.80±2.41	25.25±0.71	0.769
Stearic 18:0	rs174575	14.48±1.60	14.71±1.69	0.544	14.55±1.17	14.88±1.66	0.512	12.34±1.07	14.52±0.58	0.017[c]
	rs174561	14.57±1.64	14.81±1.67	0.553	14.36±1.29	14.66±1.65	0.516	13.31±1.55	14.78±0.77	0.184
Oleic 18:1n9	rs174575	15.56±2.20	16.05±1.87	0.313	15.75±1.66	15.36±1.47	0.471	17.73±1.96	15.69±0.89	0.118
	rs174561	15.52±2.38	16.12±1.72	0.235	15.87±1.45	15.25±1.67	0.217	16.54±2.32	15.78±0.85	0.616
Vaccenic 18:1n7	rs174575	1.64±0.71	1.62±0.58	0.925	1.71±0.42	1.56±0.13	0.130	1.63±0.03	1.50±0.18	0.270
	rs174561	1.56±0.57	1.68±0.50	0.378	1.82±0.73	1.47±0.37	0.057	1.60±0.06	1.44±0.22	0.150
Linoleic 18:2	rs174575	16.93±2.88	16.81±3.38	0.869	16.27±1.36	16.81±2.73	0.483	19.76±1.79	17.44±1.30	0.101
	rs174561	16.42±2.74	16.67±3.44	0.745	17.07±2.11	17.06±2.55	0.986	19.19±2.37	17.48±0.85	0.286
ALA 18:3	rs174575	0.35±0.21	0.36±0.25	0.972	0.43±0.19	0.33±0.20	0.117	0.40±0.35	0.40±0.05	0.990
	rs174561	0.35±0.21	0.32±0.23	0.625	0.41±0.21	0.39±0.22	0.775	0.46±0.27	0.40±0.06	0.753
DGLA 20:3	rs174575	2.20±0.48	2.40±0.60	0.128	2.86±0.57	2.46±0.62	0.058	2.47±0.07	2.59±0.54	0.728
	rs174561	2.16±0.49	2.26±0.55	0.426	2.76±0.57	2.61±0.57	0.404	2.46±0.06	3.18±0.59	0.028[c]
AA 20:4	rs174575	14.30±1.87	14.00±2.02	0.511	14.39±1.18	15.12±1.52	0.130	11.84±2.22	13.38±1.64	0.337
	rs174561	14.59±1.89	14.29±1.91	0.526	14.16±1.17	14.53±1.96	0.474	11.91±1.65	13.62±1.84	0.222
EPA 20:5	rs174575	0.57±0.37	0.70±0.66	0.308	0.52±0.47	0.41±0.37	0.457	0.58±0.16	0.66±0.34	0.738
	rs174561	0.54±0.32	0.61±0.62	0.609	0.50±0.47	0.61±0.49	0.477	0.85±0.38	0.41±0.49	0.206
DPA 22:5n3	rs174575	2.33±0.82	2.19±0.58	0.404	2.08±0.55	2.21±0.61	0.522	1.87±0.21	2.32±0.52	0.227
	rs174561	2.37±0.88	2.17±0.54	0.257	2.06±0.52	2.27±0.65	0.282	2.11±0.41	2.24±0.54	0.703
DHA 22:6	rs174575	6.34±1.49	5.85±1.63	0.188	5.97±1.56	5.48±1.37	0.327	5.34±0.04	6.23±1.91	0.467
	rs174561	6.25±1.49	5.67±1.29	0.095	6.23±163	6.00±1.92	0.678	5.53±0.39	4.78±1.49	0.301

[a] The numbers of women with baseline erythrocyte data in the placebo treatment group and DHA treatment group for SNP rs174575 were as follows: MM, 36 and 36, respectively; Mm, 15 and 21, respectively; mm, 3 and 4, respectively. The numbers of women with baseline erythrocyte data in the placebo treatment group and DHA treatment group for SNP rs174575 were as follows: MM, 30 and 36, respectively; Mm, 19 and 22, respectively; mm, 5 and 3, respectively. MM, homozygous for major alelle; Mm, heterozygous; mm, homozygous for minor allele. ALA, α-linoleic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences between fatty acid proportions between treatment groups were assessed by two-tailed *t* test.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table I.3

Fatty acid composition (percentage of total fatty acids identified) of maternal baseline plasma phospholipid samples classified according to both genotype for SNP rs174575 and rs174561, as well as treatment type.[a]

		MM			Mm			mm		
Fatty Acid	**SNP**	**Placebo**	**DHA**	**P**	**Placebo**	**DHA**	**P**	**Placebo**	**DHA**	**P**
Myristic 14:0	rs174575	0.05±0.14[b]	0.09±0.17	0.358	0.03±0.11	0.14±0.22	0.093	0±0	0±0	
	rs174561	0.04±0.13	0.12±0.21	0.061	0.04±0.12	0.07±0.15	0.465	0.08±0.18	0±0	
Palmitic 16:0	rs174575	32.41±4.54	31.73±2.89	0.451	31.38±2.04	32.93±9.39	0.534	32.54±0.42	33.93±3.76	0.560
	rs174561	32.46±4.91	31.37±1.40	0.207	31.82±2.02	33.25±9.22	0.513	31.33±1.68	36.27±9.33	0.269
Palmitoleic 16:1	rs174575	0.08±0.20	0.12±0.26	0.451	0.03±0.11	0.16±0.29	0.102	0.21±0.36	0±0	
	rs174561	0.06±0.17	0.16±0.30	0.109	0.06±0.18	0.09±0.21	0.567	0.21±0.30	0±0	
Stearic 18:0	rs174575	12.91±2.37	12.50±1.62	0.390	12.19±1.05	13.23±3.21	0.235	11.95±0.56	13.58±3.54	0.476
	rs174561	13.06±2.51	12.24±1.00	0.075	12.17±1.19	13.56±3.33	0.092	12.11±0.48	14.43±4.70	0.291
Oleic 18:1n9	rs174575	9.92±0.96	9.86±1.29	0.823	9.82±1.10	9.05±2.41	0.254	10.38±0.72	10.72±1.55	0.741
	rs174561	9.93±1.02	9.89±1.24	0.873	9.85±1.01	9.12±2.39	0.222	10.08±0.66	10.41±2.39	0.772
Vaccenic 18:1n7	rs174575	1.50±0.34	1.58±0.29	0.313	1.58±0.17	1.50±0.36	0.445	1.58±0.12	1.55±0.24	0.852
	rs174561	1.48±0.37	1.58±0.28	0.221	1.58±0.15	1.51±0.38	0.410	1.63±0.11	1.57±0.12	0.498
Linoleic 18:2	rs174575	22.86±3.39	23.03±2.72	0.821	23.11±1.63	22.11±5.46	0.499	23.09±1.11	22.79±3.62	0.896
	rs174561	22.46±3.32	22.81±2.05	0.594	23.31±2.13	22.63±5.69	0.627	24.49±2.23	21.78±6.11	0.386
ALA 18:3	rs174575	0.23±0.20	0.22±0.21	0.849	0.23±0.14	0.17±0.16	0.251	0.31±0.27	0.28±0.20	0.876
	rs174561	0.23±0.20	0.19±0.19	0.395	0.22±0.15	0.23±0.19	0.758	0.33±0.20	0.25±0.23	0.607
DGLA 20:3	rs174575	3.55±0.93	3.66±0.80	0.579	4.33±0.95	3.62±0.97	0.036[c]	4.30±0.50	3.77±1.09	0.474
	rs174561	3.58±1.00	3.68±0.73	0.639	4.19±0.89	3.53±1.06	0.039[c]	3.74±0.91	4.29±0.84	0.427
AA 20:4	rs174575	10.36±2.45	11.06±2.23	0.211	10.77±1.47	11.02±2.93	0.764	9.21±1.75	7.74±1.20	0.241
	rs174561	10.78±2.58	11.68±1.90	0.106	10.17±1.47	9.89±2.89	0.713	9.15±1.38	7.41±2.09	0.201
EPA 20:5	rs174575	0.53±0.45	0.50±0.42	0.795	0.46±0.42	0.69±0.82	0.314	0.57±0.51	0.42±0.51	0.717
	rs174561	0.50±0.37	0.57±0.59	0.562	0.46±0.52	0.60±0.63	0.432	0.81±0.49	0.21±0.37	0.119
DPA 22:5n3	rs174575	0.54±0.40	0.57±0.39	0.640	0.59±0.34	0.54±0.39	0.713	0.68±0.59	0.42±0.48	0.549
	rs174561	0.54±0.39	0.55±0.40	0.964	0.53±0.36	0.55±0.40	0.843	0.77±0.45	0.56±0.48	0.545
DHA 22:6	rs174575	5.03±1.34	5.07±1.57	0.905	5.49±1.52	4.81±1.58	0.203	5.18±0.13	4.80±0.93	0.528
	rs174561	4.87±1.30	5.15±1.28	0.384	5.62±1.52	4.96±1.61	0.189	5.26±0.37	2.83±2.58	0.071

[a] The numbers of women with baseline plasma data in the placebo treatment group and DHA treatment group for SNP rs174575 were as follows: MM, 36 and 36, respectively; Mm, 15 and 21, respectively; mm, 3 and 4, respectively. The numbers of women with baseline plasma data in the placebo treatment group and DHA treatment group for SNP rs174575 were as follows: MM, 30 and 36, respectively; Mm, 19 and 22, respectively; mm, 5 and 3, respectively. MM, homozygous for major alelle; Mm, heterozygous; mm, homozygous for minor allele. ALA, α-linoleic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences between fatty acid proportions between treatment groups were assessed by two-tailed *t* test.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).

Table I.4

Fatty acid composition (percentage of total fatty acids identified) of maternal 2 month breastmilk samples classified according to both genotype for SNP rs174575 and rs174561, as well as treatment type.[a]

		MM			Mm			mm		
Fatty Acid	**SNP**	**Placebo**	**DHA**	**P**	**Placebo**	**DHA**	**P**	**Placebo**	**DHA**	**P**[3]
Myristic 14:0	rs174575	4.62±1.61[b]	5.21±2.04	0.258	4.39±1.02	5.10±2.32	0.315	4.28±1.12	7.14±1.89	0.158
	rs174561	4.65±1.55	5.03±1.93	0.451	4.37±1.36	5.60±2.50	0.106	4.44±0.72	7.24±0	
Palmitic 16:0	rs174575	17.60±3.84	18.40±5.02	0.526	16.57±4.03	20.25±6.26	0.072	21.07±8.32	20.09±2.48	0.850
	rs174561	16.90±4.34	18.97±5.23	0.142	17.67±3.53	19.26±5.89	0.379	19.26±5.34	22.95±0	
Palmitoleic 16:1	rs174575	2.63±0.75	2.81±0.92	0.436	3.12±1.02	2.72±1.00	0.273	2.14±0.56	2.58±0.78	0.551
	rs174561	2.60±0.73	2.85±1.09	0.374	3.05±1.04	2.59±0.56	0.112	2.64±0.87	3.26±0	
Stearic 18:0	rs174575	5.84±2.13	6.36±2.22	0.394	5.82±2.64	6.78±3.20	0.380	7.30±4.14	6.18±1.11	0.564
	rs174561	5.70±2.26	6.41±2.63	0.317	6.09±2.55	6.62±2.50	0.555	6.38±2.00	7.15±0	
Oleic 18:1n9	rs174575	39.71±4.03	37.86±2.99	0.055	40.96±2.91	37.67±4.59	0.030[c]	41.45±4.73	38.06±3.62	0.424
	rs174561	39.95±4.58	37.54±3.18	0.027[c]	40.16±2.15	38.48±4.24	0.184	41.85±2.96	34.03±0	
Vaccenic 18:1n7	rs174575	1.84±0.26	1.76±0.24	0.259	1.73±0.59	1.89±0.30	0.326	1.65±0.03	1.75±0.14	0.415
	rs174561	1.81±0.26	1.83±0.30	0.795	1.75±0.57	1.77±0.17	0.848	1.88±0.26	1.70±0	
Linoleic 18:2	rs174575	23.50±5.24	22.97±6.58	0.754	23.31±4.94	22.00±7.20	0.574	19.69±4.38	20.24±2.44	0.862
	rs174561	23.95±5.73	23.04±6.46	0.606	22.97±4.42	21.65±7.08	0.544	20.54±2.86	19.13±0	
ALA 18:3	rs174575	2.28±0.84	2.21±0.87	0.769	2.07±0.87	1.87±0.74	0.490	1.63±0.27	2.10±0.38	0.234
	rs174561	2.36±0.89	2.13±0.75	0.330	2.02±0.82	2.00±0.95	0.948	1.82±0.28	2.19±0	
DGLA 20:3	rs174575	0.52±0.50	0.41±0.24	0.289	0.43±0.29	0.45±0.24	0.810	0±0	0.44±0.08	
	rs174561	0.53±0.54	0.44±0.23	0.369	0.46±0.25	0.41±0.24	0.583	0.14±0.27	0.53±0	
AA 20:4	rs174575	0.76±0.44	0.99±0.51	0.088	1.10±0.37	0.75±0.38	0.015[c]	0.80±0.11	0.59±0.10	0.102
	rs174561	0.85±0.47	0.91±0.50	0.651	0.94±0.43	0.84±0.43	0.485	0.78±0.23	0.67±0	
EPA 20:5	rs174575	0.08±0.11	0.13±0.23	0.362	0.09±0.19	0.07±0.12	0.697	0±0	0.10±0.17	
	rs174561	0.09±0.12	0.09±0.14	0.938	0.07±0.16	0.14±0.27	0.371	0.11±0.21	0.30±0	
DPA 22:5n3	rs174575	0.12±0.17	0.09±0.16	0.536	0.07±0.15	0.07±0.13	0.949	0±0	0±0	
	rs174561	0.11±0.18	0.10±0.16	0.834	0.10±0.16	0.04±0.11	0.227	0±0	0±0	
DHA 22:6	rs174575	0.47±0.47	0.78±0.41	0.012[c]	0.33±0.42	0.35±0.35	0.925	0±0	0.73±0.16	
	rs174561	0.47±0.50	0.63±0.44	0.246	0.35±0.39	0.60±0.42	0.091	0.17±0.34	0.85±0	

[a] The numbers of women with 2 month breastmilk sample data in the placebo treatment group and DHA treatment group for SNP rs174575 were as follows: MM, 23 and 32, respectively; Mm, 14 and 18, respectively; mm, 2 and 3, respectively. The numbers of women with 2 month breastmilk sample data in the placebo treatment group and DHA treatment group for SNP rs174575 were as follows: MM, 20 and 34, respectively; Mm, 15 and 18, respectively; mm, 4 and 1, respectively. MM, homozygous for major alelle; Mm, heterozygous; mm, homozygous for minor allele. ALA, α-linoleic; DGLA, dihomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid. Differences between fatty acid proportions between treatment groups were assessed by two-tailed *t* test.

[b] Mean ± SD (all such values).

[c] Significant difference ($p<0.05$).