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The effect of prenatal docosahexaenoic acid supplementation on breast milk fatty acid composition and morbidity and immune function in Mexican infants: a double-blind randomized, controlled trial

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Doctor of Philosophy

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B.S., American University, Washington DC, 1993
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An Abstract of
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ABSTRACT

The effect of prenatal docosahexaenoic acid supplementation on breast milk fatty acid composition and morbidity and immune function in Mexican infants: a double-blind randomized, controlled trial

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Docosahexaenoic acid (DHA, 22:6n-3), an omega-3 polyunsaturated fatty acid (PUFA) found in high concentrations in brain and retinal cell membranes, accumulates rapidly in neural tissue during later gestation and infancy. DHA is also found in cells of the immune system and modulates immunity and inflammation through mechanisms including modulation of gene expression, alteration of cell membrane structure and function, and subsequent modification of cytokine and eicosanoid profiles and alteration of lipid raft structures. DHA is preferentially transferred across the placenta and is present in breast milk, and availability of DHA to the developing fetus and its concentration in breast milk is primarily dependent upon maternal diet. Pre-formed DHA is found in high concentrations in oily coldwater fish such as salmon and herring, and dietary intake of DHA-rich foods in many populations of pregnant and lactating women is inadequate. Infants are born with immature immune systems and are therefore more susceptible to infectious disease, a leading cause of morbidity and mortality in children the developing world. The substantial impact of nutritional status on infectious disease is well known and is especially important in children.

Given the import role of DHA in child development and immune function, we investigated the effect of DHA in pregnancy on breast milk PUFA concentrations, infant morbidity and infant response to hepatitis B and tetanus vaccination. In a double-blind randomized, controlled trial in Mexico, 1040 pregnant women were supplemented daily with 400 mg algae-derived DHA or placebo from 18-22 weeks' gestation through parturition. We found that DHA supplementation improved breast milk DHA concentration at 1 month post-partum and overall, reduced the duration of illness symptoms in infants. Additionally, infants in the DHA group had lower mean concentrations of anti-hepatitis B IgG antibody at 3 months of age, after vaccination at birth and at 2 months.

Our findings contribute to the mounting evidence that perinatal n-3 PUFA nutriture, including *in utero* exposure and exposure through breast milk, may influence the development and maturation of fetal and infant immune response. Additionally, we report novel findings about the influence of DHA in pregnancy alone on breast milk DHA concentrations.

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Abbreviations used

AA:	arachidonic acid (20:4n-6)
ALA:	α-linolenic acid (18:3n-3)
CI:	confidence intervals
DHA:	docosahexaenoic acid (22:6n-3)
EFA:	essential fatty acids
EPA:	eicosapentaenoic acid (20:5n-3)
GM:	geometric mean
IFN:	interferon
Ig:	immunoglobulin
IL:	interleukin
LA:	linoleic acid (18:2n-6)
LCPUFAs:	long-chain polyunsaturated fatty acids
PG:	prostaglandin
PUFAs:	polyunsaturated fatty acids
SES:	socioeconomic status
TGF:	transforming growth factor
Th1:	T-helper cell type1
Th2:	T-helper cell type 2

CHAPTER 1: INTRODUCTION

Worldwide, infection is the leading cause of mortality in children <12 months of age (1). Immune responsiveness to agents that cause infections such as diarrheal diseases, acute respiratory infections, measles and malaria is essential for child health and survival. While infections are the leading cause of infant death in developing countries, more than half of all deaths among children <5 years of age are associated with malnutrition (1). Undernourished children are more susceptible to infection and suffer a higher burden of morbidity and mortality. This vicious nutrition-infection cycle can negatively affect both nutritional status and immune function. Malnutrition impairs immune function, causing an increased susceptibility to infection; in turn, infection can negatively alter nutritional status (2). Infection can cause anorexia, malabsorption of nutrients, changes in intestinal integrity and diarrhea, all of which negatively influence nutritional status (2). Infection can also deplete specific nutrients, such as vitamin A, iron and zinc, and deficiencies in these micronutrients can further impair immune function (3, 4). Supplementation with micronutrients such as vitamin A and zinc has been shown to influence morbidity and mortality in children. For example in children, these micronutrients have been shown to decrease child mortality (vitamin A), enhance response to hepatitis B, tetanus, diphtheria and measles vaccination (vitamin A), decrease the incidence and severity of diarrheal illness (zinc and vitamin A), decrease the severity of morbidity from measles (vitamin A) and decrease the risk of fever and clinical malaria episodes (vitamin A + zinc, vitamin A) (5-14). The well known cycle of infection and malnutrition is an important determinant of child health and survival in many developing

countries, and a child's ability to effectively combat infection can be influenced by *in utero* and early childhood nutritional exposures.

Early life nutrition, including *in utero* nutriture, can influence child health and development. Numerous studies examining the influence of maternal nutritional status during pregnancy on immune function in the child have provided evidence of *in utero* "fetal programming" of immune function (11, 15, 16). Tielsch *et al.* showed that children born to vitamin A deficient women, as evidenced by night blindness during pregnancy, suffered an increased risk of diarrhea, acute respiratory infection and dysentery compared to children born to women without night blindness (11). In a study of multiple-micronutrient supplementation in pregnancy conducted in Nepal, preterm infants of mothers supplemented with vitamin A and folic acid, iron, or folic acid + zinc experienced a decreased risk of mortality in the first three months of life (17). Because the immune system begins to develop *in utero* and continues to develop rapidly in the first year of life, infants are a vulnerable population born with immature immune systems and are unable to mount immune responses comparable to adults, rendering them especially susceptible to infection (18). Long chain polyunsaturated fatty acids (LCPUFAs), which modulate immune function, may influence the development of the fetal and infant immune system.

LCPUFAs, particularly docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), are integral for fetal neural and retinal development, accrete extensively in these tissues in the last trimester of pregnancy and are preferentially transported across the placenta to the developing fetus (19-24). LCPUFAs serve as structural constituents of cell membranes and act as signaling molecules, thereby

modulating immune function and inflammation. Specifically, LCPUFAs modulate immune function and inflammation through modification of the LCPUFA content of cell membranes and subsequent alteration in eicosanoid synthesis and lipid raft structure, alteration of cell membrane fluidity, regulation of nuclear transcription factors, modification of cytokine production and modification of cell adhesion molecule expression (25-33).

Although many studies in humans and animals have indicated that LCPUFAs modulate immune function, few studies have specifically addressed the independent effect of DHA on immune function. In addition, while numerous studies have reported the effect of n-3 PUFAs supplementation on immune function in adults, few studies have evaluated the specific effect of DHA on infant immune response and morbidity (34-39). One study in which infants were assigned to receive either regular formula or formula supplemented with DHA and AA showed that the infants consuming the EFA-supplemented formula had a decreased incidence of bronchiolitis/bronchitis (36). A double-blind study in which children aged 36-49 months at risk of recurrent respiratory infections were given dietary EFA supplementation reported a reduction in the number of infective episodes, days with fever and days of school missed (35). These and other trials indicate that n-3 PUFA supplementation in infancy or childhood might influence immune function; however, the effect of DHA nutriture in pregnancy on child immune function is yet unknown. This relationship is important because DHA intake is often inadequate in many populations of pregnant women.

Dietary intake of n-6 PUFAs such as AA in populations worldwide is generally adequate while dietary intake of n-3 PUFAs such as DHA is often insufficient, especially

in pregnant women whose stores are mobilized for fetal growth (40, 41). DHA is an n-3 LCPUFA found primarily in coldwater fatty fish such as salmon, herring and tuna, and is present in low concentrations in other foods such as eggs and organ meats. Many populations in both developed and developing countries consume low amounts of n-3 PUFAs and are exposed to possible adverse effects during early human development (40). Intake of n-3 LCPUFAs and DHA+ eicosapentaenoic acid (EPA, 20:5n-3) in the U.S. is 1.6 g/d and 0.1-0.2 g/d, respectively, and the ratio of dietary n-6:n-3 PUFAs is approximately 9.8:1 (42). Populations living in coastal countries such as Japan and Norway, where fish is widely consumed, have higher dietary intake of n-3 PUFAs and correspondingly high concentrations of DHA in their breast milk (40, 43). Although there is no official dietary recommendation from the Institute of Medicine for EPA and DHA intake in the United States, several groups suggest DHA intake of at least 200-220 mg/d (up to 1 g/d) for pregnant and lactating women, and 2.7 g/d of n-3 PUFAs, and the suggested n-6:n-3 ratio is approximately 2-5:1 (41, 42). Sufficient n-3 PUFA intake is especially important in pregnancy because maternal dietary intake directly influences infant status (24).

Maternal DHA nutriture in pregnancy and breast milk DHA levels correspond positively with infant DHA status (44, 45). LCPUFAs are present in breast milk and their concentration depends upon maternal stores, dietary intake and synthesis in the mammary glands; however, breast milk n-3 PUFA content is influenced most by dietary intake (46). Further, fish oil supplementation in pregnancy and/or lactation improves maternal and infant DHA status (24, 46-49). DHA concentration in breast milk has been shown to decrease between lactation weeks 6 and 30, and supplementation of lactating

women improves DHA breast milk concentrations; however, the effect of supplementation in pregnancy alone on breast milk n-3 PUFA profiles is less clear (50-52). Since the developing fetus and breastfed infant rely on maternal supply of DHA, adequacy of DHA in the maternal diet during pregnancy and lactation is critical.

The central hypotheses of this dissertation, that DHA supplementation during pregnancy will improve breast milk DHA concentrations and modulate infant immune function, will be tested in the context of an ongoing double-blinded randomized, placebo-controlled trial in Cuernavaca, Mexico in which pregnant women were supplemented daily with 400 mg DHA or placebo from 18-22 weeks' gestation through parturition. This dissertation explores the effect of prenatal DHA supplementation on infant morbidity and immune response and on breast milk fatty acid concentrations at 1 month post-partum, which could influence infant immune function. Specifically, this study was conducted to test the following hypotheses:

1) Women who receive DHA supplements from 18-22 weeks' gestation through delivery will have higher DHA concentrations in breast milk at 1 month post-partum than women who receive placebo;
2) Infants born to women who receive DHA supplements from 18-22 weeks' gestation through delivery will experience less severe and fewer episodes of illness than infants born to women who receive placebo;
3) Infants born to women who receive DHA supplements from 18-22 weeks' gestation through delivery will mount a different response to childhood hepatitis B and tetanus vaccination at 3 months than infants born to women who receive placebo.

This study will provide novel insight into the possible effects of DHA supplementation during pregnancy on infant morbidity and immune function. This is a particularly relevant area of investigation because: 1) many populations in both developed and developing countries consume inadequate amounts dietary of n-3 PUFAs, 2) the consumption of fish oil or DHA/EPA-enriched oils as supplements during pregnancy is gaining popularity in many developed countries and 3) maturation of immune response is an important factor for infant health (53). The present study will also provide new information about the influence of DHA in pregnancy on breast milk n-3 PUFA profiles, which is important because breast milk contains many immunomodulatory compounds that might be influenced by DHA and because breast milk is the only source of DHA for the breastfed infant (54).

The following dissertation chapters include an overview of PUFAs including structure and function, dietary intake and recommendations, and PUFAs in pregnancy and lactation (Chapter 2), a review of literature regarding DHA and immune function (Chapter 3) and a detailed description of the study methodology including a description of the study population and setting, laboratory methods and statistical methods (Chapter 4). Chapters 5-7 include reports describing the influence of prenatal DHA supplementation on breast milk DHA concentrations (Chapter 5), infant morbidity (Chapter 6) and infant immune response to hepatitis B and tetanus vaccinations (Chapter 7). Finally, a summary of findings, overall conclusions and implications of our findings are presented in Chapter 8. We expect that findings from this study will contribute new knowledge regarding the influence of prenatal DHA supplementation on infant immune

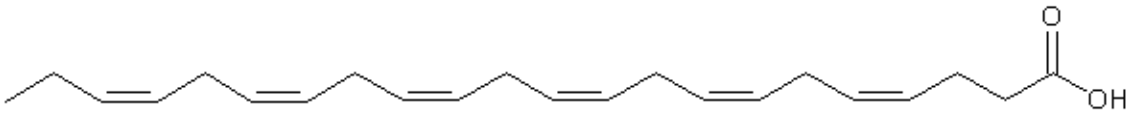
function and morbidity, and the influence of prenatal DHA intake on breast milk fatty acid composition.

CHAPTER 2: LITERATURE REVIEW
OVERVIEW OF n-3 POLYUNSATURATED FATTY ACIDS

Essentiality, structure and metabolism of DHA

Essential fatty acids (EFAs) cannot be synthesized *de novo* or inter-converted by the human body due to the lack of necessary enzymes and must therefore be obtained through the diet (24). The two18-carbon EFAs α-linolenic acid (ALA,18:3n-3) and linoleic acid (LA, 18:2n-6) are termed n-3 and n-6 polyunsaturated fatty acids (PUFAs) because of the position of the first double bond in their hydrocarbon chain. n-3 PUFAs are named as such because the first double bond occurs at the third carbon of the hydrocarbon chain; likewise, the first double bond of n-6 PUFAs is positioned at the 6th carbon **(Figure 1)**.

Figure 1: Docosahexaenoic acid structure



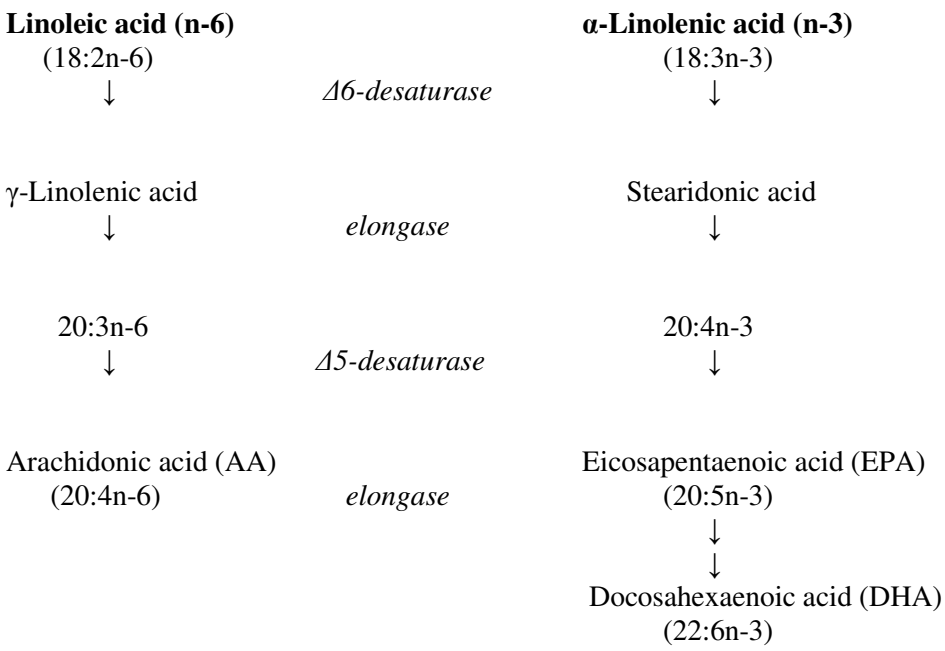
The LCPUFAs docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) can be synthesized from the parent EFA precursor ALA through a series of desaturation and elongation reactions that occur primarily in the endoplasmic reticulum and peroxisomes of the liver, though efficiency of conversion is limited **(Figure 2)** (55-59). One study, in concurrence with several other studies, reported that

<0.1% of dietary ALA is converted to DHA in humans suggesting that DHA status is almost entirely dependent upon dietary supply of pre-formed DHA (55, 60). Studies have suggested that conversion of ALA to DHA may be more efficient in women than in men due to the influence of hormones on the conversion processes (55, 61). A recent review (2009) including 21 trials supported previous findings that conversion of ALA to DHA is limited in humans (59). Overall, studies have shown that dietary supplementation of adults with ALA improves plasma EPA status but has little influence on DHA status. Two studies that demonstrated improvement in plasma DHA status with ALA supplementation decreased intake of dietary LA as part of the intervention; hence, improvement in plasma DHA was also likely due to a concomitant decrease in n-6 PUFA consumption (59, 62, 63). Studies indicate that infants convert ALA to DHA more efficiently than adults at an average of 1%, as evidenced by trials of ALA-supplementation of infant formula (59, 64, 65). DHA can be retro-converted to EPA at a rate in humans ranging from 1-12%, depending on dietary DHA consumption (56). Arachidonic acid (AA, 20:4n-6) and other n-6 LCPUFAs can be synthesized from LA using the same Δ6 and Δ5 desaturase and elongase enzymes needed to convert ALA to DHA; hence, the ratio of n-6 to n-3 PUFAs in the diet is important because of competition by substrates for the shared enzymes (28, 66, 67). The ratio of n-6 to n-3 PUFAs is also significant due to competition for incorporation into tissue lipids.

DHA and other fatty acids are incorporated into membrane phospholipids, utilized for β-oxidation and are stored in adipose tissue, though DHA accounts for only 0.1% of fatty acids stored in adipose tissue (55). Because adipose stores of DHA are low and

conversion of ALA to DHA is limited, adequate dietary intake of pre-formed DHA is essential.

Figure 2. Metabolism of LCPUFAs



Dietary sources and recommended intake of DHA

Pre-formed DHA is found in high concentrations in algae and coldwater oily fish such as herring, salmon and sardines, and in lower concentrations in eggs and meats including lamb and chicken (40). Fish do not synthesize DHA from shorter chain precursor fatty acids rather, their flesh contains DHA because they consume algae. DHA's precursor EFA ALA is found in high concentrations in flax and its oil (linseed

oil), canola oil and walnuts, whereas commonly consumed vegetable oils such as corn and soybean oil serve as rich sources of AA's precursor LA.

Intake of n-6 PUFAs in the United States and other countries has increased over the last decade, resulting in a high ratio of dietary n-6:n-3 PUFAs (40, 59). This increased consumption of n-6 PUFAs may be due, in part, to a decrease in saturated fat intake and replacement with n-6 PUFA-rich vegetable oils such as soybean, sunflower and safflower. Intake of n-3 PUFAs in the United States is an estimated 1.6 g/d (100-200 g EPA+DHA and 1.4 g ALA) and the n-6:n-3 PUFA ratio is approximately 10:1, reflecting high consumption of n-6 rich foods such as corn oil, soybean oil and poultry (42). A study of pregnant Canadian women reported a mean intake of 160 mg DHA/d and 78 mg EPA/d (68). Worldwide estimates of dietary DHA intake indicate that consumption is low in many regions including parts of Eastern and Central Europe and the United States, whereas DHA intake is high in countries such as Iceland, Japan, Malaysia and Norway, where seafood is widely consumed (40).

The Institute of Medicine (IOM) of the National Academies established Adequate Intake (AI) values for n-3 PUFAs in 2002; however, no RDA has been set due to insufficient evidence to establish Estimated Average Requirements (EAR) (69). The IOM recommends an n-3 PUFA intake of 1.1 g/d for women and 1.6 g/d for men, with no recommendation for individual n-3 LCPUFAs such as DHA and EPA. In a recent consensus statement by the European Commission several expert groups established a recommended intake of at least 200 mg DHA/d for women of childbearing age and pregnant and lactating women (41). The American Heart Association recommends an intake of 1 g/d of DHA+EPA in persons with existing coronary heart disease, and 400-

500 g/d, or 2 fish meals per week, for unaffected persons (69). The worldwide consensus recommendation of intake of at least 200 mg DHA/d for women of childbearing age and pregnant and lactating women is currently not achieved in most countries.

Overview of n-3 PUFA functions

LCPUFAs, particularly DHA and AA, are integral for fetal neural and retinal development and accrete extensively in these tissues in the last trimester of pregnancy (21-23). LCPUFAs serve as structural and functional constituents of cell membranes and act as signaling molecules, thereby influencing numerous biological processes. Deficiency of certain LCPUFAs can result in symptoms such as severe dermatitis and growth failure (70). n-3 LCPUFAs have been shown to influence health outcomes including duration of gestation, cognitive development in children, post-partum depression, neurologic degeneration and certain chronic inflammatory diseases such as cardiovascular disease, asthma and rheumatoid arthritis, and immune function (24, 71-74). The role of DHA in immune function is detailed in Chapter 3.

n-3 PUFAs in pregnancy

LCPUFAs, particularly DHA and AA, are essential for fetal vascularization and neural tissue growth and are transported across the placenta to the developing fetus (20, 23). Studies have demonstrated that DHA is preferentially transferred across the placenta above all other LCPUFAs, and that this materno-fetal transfer is mediated by fatty acid transport proteins (FATP-4, FATP-1) and placental lipases (19, 75). The fetus has limited ability to synthesize DHA and therefore its supply is almost entirely dependent upon maternal transfer (24).

Studies have shown an initial increase in total maternal n-3 PUFA concentration in blood during pregnancy, likely due to an increased mobilization of maternal stores, and a subsequent decrease in maternal n-3 PUFA status due to rapid fetal accretion suggesting that pregnancy leads to a depletion of maternal n-3 PUFAs (44, 76). Evidence indicates that multigravidas women suffer an increase in deterioration of LCPUFA status compared to primgravidas women (23). Al and colleagues monitored pregnant women longitudinally from 10 weeks gestation through delivery and found that the women's DHA status decreased after 18 weeks gestation and continued to decline through delivery (44). Recently, Bonham *et al.* found in a study of pregnant women in the Seychelles that even in a population where fish consumption is high, maternal serum DHA levels fell significantly from 28 weeks' gestation through delivery (51).

Numerous studies have demonstrated that supplementing pregnant women with fish oil, DHA or DHA-enriched foods improves both maternal and neonatal fatty acid status (47, 48, 66, 77-85) . Van Houwelingen *et al.* supplemented pregnant women with fish oil (2.3 g n-3 PUFA/d) from gestation week 30 through delivery and reported that the women supplemented with fish oil had significantly higher plasma phospholipid DHA concentrations, as well as correspondingly higher DHA concentrations in both umbilical wall phospholipids and umbilical cord plasma phospholipids, compared to placebo (77). Similar results were seen when Connor *et al.* supplemented pregnant women from the 26^{th} through the 35^{th} week of gestation with sardines and fish oil (2.6 g n-3 PUFAs and 1.1 g DHA/d) (78). Both maternal and newborn erythrocyte and plasma DHA concentrations were significantly higher at delivery in the supplemented group. In a study conducted by Velzing-Aarts *et al.*, women between 18 and 23 weeks' gestation

were supplemented through delivery with either low-dose fish oil (336 mg n-3 PUFAs, providing 123 mg DHA/d), a milk-based supplement (528 mg n-3 PUFAs, providing 185 mg DHA/d), or high dose fish oil (1008 mg n-3 PUFAs, providing 369 mg DHA/d) (79). This study showed that cord vessel n-3 PUFA concentrations, particularly DHA, were significantly higher in the two higher dose groups, again illustrating improvement in cord vessel n-3 PUFA concentrations through supplementation during pregnancy. In another supplementation trial of pregnant women, Otto *et al.* supplemented women16-20 weeks' gestation with 570 mg DHA and 260 mg AA or placebo (85). After 4 weeks of supplementation, mothers in the DHA + AA-supplemented group had significantly higher plasma and erythrocyte concentrations of both DHA and AA.

Montgomery *et al.*, in a double-blind randomized, controlled trial supplemented women from 15 weeks' gestation through delivery with either fish oil (200 mg DHA/d) or placebo (80). This study showed that DHA supplementation in pregnancy improved maternal erythrocyte and plasma phospholipids DHA status; however, this study did not find associations between DHA supplementation in pregnancy and cord blood DHA concentrations. In a randomized, double-blinded trial conducted by Helland and colleagues, women in gestation week 18 were supplemented with cod liver oil (1183 mg DHA and 803 mg EPA/d) or corn oil through 3 months post-partum (83). At both 4 weeks and 3 months post-partum, plasma phospholipid DHA concentrations were significantly higher in infants born to women in the cod liver oil group. Additionally, umbilical cord plasma phospholipid DHA and total n-3 PUFA concentrations were significantly higher in the cod liver oil-supplemented group. This study illustrates a clear positive association between maternal DHA intake in pregnancy and lactation and infant

status. Smuts *et al.* supplemented women from 24-28 weeks' gestation through delivery with DHA-enriched eggs or regular eggs and reported significantly higher DHA concentrations in infants whose mothers consumed DHA-enriched eggs (84). Dunstan *et al.* supplemented atopic pregnant women with 4 g fish oil/d from 20 weeks' gestation through delivery and found significantly higher n-3 PUFA levels in infants whose mothers received fish oil (47, 81). In a study conducted by de Groot and colleagues, pregnant women were supplemented from 14 weeks' gestation through delivery with ALA- + LA- or LA-enriched margarine (82). This study did not reveal an association between ALA- and LA-enriched margarine intake and maternal and infant DHA status.

More recently, a multi-center trial in Europe (NUHEAL) showed that fish oil supplementation (500 mg DHA + 150 mg EPA/d) from 22 weeks' gestation through delivery increased maternal and cord blood DHA concentrations (48). Finally, Bergmann *et al.* (2008) reported that supplementing women with 200 mg DHA/d from gestation week 21 through mid-lactation resulted in higher red blood cell DHA concentration in both the mother and her infant (49).

n-3 PUFAs in lactation

LCPUFAs are present in breast milk and their variable concentration depends upon maternal stores, dietary intake and synthesis in the mammary glands; however, synthesis of DHA in the mammary gland is likely minimal (46, 72, 86, 87). Because breast milk n-3 PUFA concentrations vary with diet, PUFA content of breast milk differs widely among populations. Populations living in coastal countries such as Japan and Norway, where fish is widely consumed, have higher dietary intake of n-3 PUFAs and

correspondingly high concentrations of DHA in their breast milk compared to countries such as the United States and countries in the United Kingdom (40, 43, 88).

DHA concentration in breast milk has been shown to decrease between lactation weeks 6 and 30, and numerous studies have assessed the impact of maternal supplementation with n-3 PUFAs during pregnancy and/or lactation on breast milk fatty acid composition (50-52). In general, these studies have shown that maternal supplementation with fish oil or DHA, particularly during lactation, results in an increased n-3 PUFA concentration in breast milk and a concomitant improvement in infant DHA nutriture.

A recent study conducted in Australia by Dunstan *et al.* (2007) showed that supplementation of pregnant atopic women with 2.2 g DHA and 1.1 g EPA/d from 20 weeks' gestation through parturition resulted in higher breast milk DHA and EPA concentrations at 3 days and 6 weeks post-partum (46). By 6 months post-partum, there was no difference in breast milk PUFA composition between intervention and control groups. In another recent study conducted by Bergmann *et al.* German women were supplemented with either a multi-vitamin, a multi-vitamin plus fructo-oligosaccharides (prebiotics), or a multi-vitamin plus fructo-oligosaccharides plus 200 mg DHA daily from 21 through 36 weeks' gestation, and then from the second week after delivery through 3 months post-partum (49). This study showed that the concentration of DHA in breast milk was twice as high in the DHA-supplemented women at 3 months post-partum, compared to the other two treatment groups. Another study by Dunstan *et al.* showed that supplementing pregnant women daily with fish oil from 20 weeks' gestation through delivery resulted in significantly higher breast milk DHA and EPA concentrations at 3

days post-partum, compared to the control group (89). Helland *et al.* supplemented pregnant women with cod liver oil or corn oil from 17-19 weeks' gestation through 3 months post-partum and found higher concentrations of DHA in breast milk in the fish oil group, compared to the control group (90). Several studies that provided n-3 PUFA supplementation during lactation alone reported higher breast milk DHA concentrations in the supplemented groups.

Jensen and colleagues supplemented 3 groups of lactating women from post-partum weeks 2 through 8 with either DHA derived from algae, high-DHA eggs, or marine oil (45). The three groups of supplemented women had higher breast milk DHA concentrations than the control group, and a dose-response was observed. The investigators noted a strong correlation between maternal plasma erythrocyte DHA concentration and breast milk DHA concentration. In a study by Hawkes *et al.* lactating women who were supplemented with two different doses of tuna oil had significantly higher DHA concentrations in their breast milk, compared to controls (91, 92). Similarly, Helland *et al.* demonstrated that short-term supplementation with cod liver oil during lactation resulted in significantly higher DHA concentrations in breast milk, compared to placebo (93). Boris and colleagues supplemented pregnant Danish women with fish oil from the 30th week of pregnancy through 30 days post-partum, or through delivery (94). Women who consumed fish oil through 30 days post-partum had significantly higher breast milk DHA concentrations at 4, 16 and 30 days post-partum, compared to controls and to women who ceased supplementation at delivery. Lauritzen and colleagues found that supplementing Danish women with fish oil within one week of delivery through 4 months post-partum resulted in higher breast milk DHA concentration, compared to

placebo (95, 96). Additionally, infants of fish oil-supplemented mothers had higher DHA erythrocyte concentrations. These studies demonstrate that maternal intake of fish oil or DHA in pregnancy and/or lactation results in improvement in maternal, breast milk and infant n-3 PUFA status.

Chapter summary

In summary, many populations of pregnant and lactating women consume sub-optimal amounts (<200 mg/d) of dietary DHA and are therefore potentially predisposed to adverse health outcomes. The aforementioned studies demonstrate that maternal dietary intake of DHA, most often in the form of fish oil, in pregnancy and/or lactation results in improved maternal and infant n-3 LCPUFA nutriture. Breast milk n-3 PUFA status improves with fish oil supplementation during lactation, but no studies have described the influence of DHA in pregnancy alone on breast milk n-3 PUFA profiles. Overall, study show that fish oil or DHA supplementation (≥200 mg DHA/d), rather than ALA, from mid-pregnancy through lactation confers the most benefit to both the mother and her breastfed infant. Since the developing fetus and breastfed infant rely on maternal supply of DHA, adequacy of DHA in the maternal diet during pregnancy and lactation is critical.

CHAPTER 3: LITERATURE REVIEW, POLYUNSATURATED FATTY ACIDS AND IMMUNE FUNCTION

Overview of infant immune function

Infants are born with immature immune systems and are unable to mount immune responses comparable to adults, rendering them especially susceptible to infection (18). However, although both innate and acquired immunity are not fully developed in infants, infants indeed have partial functional capacity to respond to immunologic challenges (97). The acquired immune system begins to develop and become functional *in utero*, as evidenced by the fetus' ability to produce immunoglobulin M (IgM) in response to maternal tetanus toxoid vaccination (although no class switching to IgG occurs), and continues to mature during the first two years of life (98, 99). Additionally, fetuses have been shown to produce mature $CD4^+$ and $CD8^+$ T cells at 20 weeks' gestation, indicating *in utero* development of cell-mediated immune response (99). At birth, infants can produce immunoglobulins and exhibit cytotoxic effector function; however, response is reduced (98). Infants can mount a primary IgG response to certain vaccinations starting at 2-3 months of age; however, antibody response can be weak and fleeting (100). Th1-type response in infants is limited due to their Th2 polarization at birth, and childhood vaccines such as hepatitis B elicit Th2-driven responses, as evidenced by high concentrations of Th2-specific cytokines (54, 101, 102).

Passive immune protection is provided to the infant via breast milk and maternal transfer of immunoglobulins *in utero*. Breast milk is comprised of numerous immune-modulating compounds including cytokines, secretory IgA (sIgA), IgG, IgM, PUFAs, neutrophils and B and T lymphocytes (54). The sIgA in breast milk is transferred to the

infant's gut, where it helps protect against enteric pathogens such as *E. coli, Shigella, Vibrio cholera* and *Campylobacter* (54, 103). Maternal IgG is transferred across the placenta to the fetus *in utero,* and infants are born with IgG levels similar to their mothers.

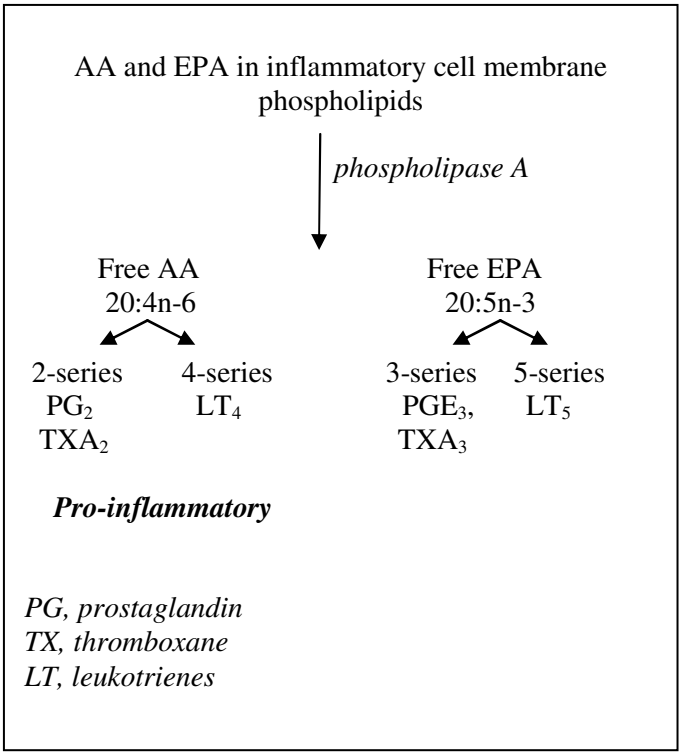
LCPUFAs and immune function: mechanisms of action

Inflammatory cells of the immune system contain membrane-bound LCPUFAs in the form of phospholipids, which consist of a glycerol backbone and long chain fatty acids whose amount and composition varies by phospholipid class and cell type (29, 30). n-3 PUFAs influence inflammation and immune function via modification of the fatty acid content of inflammatory cell membrane phospholipids, regulation of transcription factors, and other possible mechanisms (25-30, 99). n-3 PUFAs are thought to modulate immune function through the following specific proposed mechanisms: 1) modification of the AA content of inflammatory cell membranes and subsequent alteration in eicosanoid synthesis, 2) alteration of inflammatory cell membrane structure and function and 3) regulation of nuclear transcription factors, resulting in modification of cytokine and resolvin production, cell adhesion molecule expression and lipid raft structure (27, 28, 30, 33, 104).

Studies have shown that supplementation with n-3 PUFAs results in partial replacement of AA by EPA in inflammatory cell membranes (i.e. macrophages and monocytes). AA and EPA serve as substrates for the synthesis of eicosanoids such as prostaglandins, leukotrienes, and thromboxanes, which modulate inflammation and immune response (**Figure 3)**. A decrease in membrane AA content would therefore result in a concomitant decrease in production of pro-inflammatory AA-derived

eicosanoids. AA-derived eicosanoids such as the 2-series prostaglandins modulate inflammation and immune response by influencing lymphocyte proliferation, natural killer cell activity, cytokine production, leukocyte activation and IgE production (27, 33).

Figure 3. Synthesis of eicosanoids from AA and EPA



Adapted from Calder 2001, 2003 (29, 30); Gottrand 2008 (33)

Modification of inflammatory cell membrane LCPUFA content influences membrane fluidity by increasing the desaturation of the membrane and therefore increasing its rigidity (105). This change in membrane fluidity and composition can result in alterations in lipid raft structure, cell signal transduction, receptor expression and intercellular interaction, which could affect immune response (30). Specifically,

membrane-bound n-3 PUFAs can modify lipid raft structure and subsequently inhibit T-cell response (33). n-3 PUFAs have also been shown to modulate gene expression by regulation of transcription factors such as nuclear factor-κB (NF-κB) and peroxisome proliferator-activated receptors α and γ (PPAR) (32). Transcription factors such as NF-κB can regulate genes involved in cytokine production (i.e. TNF-α, IL-6) and cell adhesion molecule expression (i.e. VCAM-1, ICAM-1). Hence, n-3 PUFAs can modulate cytokine production and cell adhesion molecule expression via their influence on nuclear transcription factors. Alteration of cytokine production and cell adhesion molecule expression can influence inflammatory and immune responses involving T and B cells, monocytes, macrophages and endothelial cells (33).

T-helper cell types 1 and 2 (Th1 and Th2, respectively) produce different types cytokines involved in inflammatory and immune responses (26, 103, 106). Th1 cells drive cell-mediated immune response and produce cytokines such as IL-2 and IFN-γ, whereas Th2 cells generally produce more pro-inflammatory cytokines such as IL-13, IL-10, IL-4 and IL-5, and induce humoral response (29, 107, 108). Th1- and Th2-type immune responses are regulated and balanced, and cytokines can determine their differentiation. An imbalance in immune response, such as a sustained increase in Th2-type response, can result in atopic disorders such as asthma. Studies have revealed the possibility that LCPUFAs modulate Th1/ Th2 balance via modification of cytokine profiles, hence illustrating another possible link between LCPUFAs and immune function (106).

n-3 PUFAs and immune function: trials in animals

n-3 PUFA intake in pregnancy and offspring immune function

A number of experimental studies have demonstrated a relationship between maternal PUFA intake during pregnancy and offspring immune function. Calder *et al.* demonstrated that offspring immune function might be influenced *in utero* by maternal diet during pregnancy in a study in which pregnant rats were fed diets of differing levels of protein and types of fat (109, 110). Several marker of immune function including natural killer cell activity, thymocyte proliferation, and spleen lymphocyte numbers differed among the experimental groups, illustrating an impact of maternal diet during pregnancy on immune function in offspring. In an unpublished study by Frokiaer, recently reported by Calder *et al.* (2006), mouse dams were fed fish oil or a saturated fat throughout pregnancy and lactation and offspring immune function was assessed (99). Pups of dams fed fish oil had higher IFN-γ production and lower IL-5 (pro-inflammatory cytokine) production compared to pups of dams fed saturated fats. Berger *et al.* demonstrated in a murine model that maternal PUFA intake during pregnancy and lactation might affect immune function in offspring by feeding mouse dams one of 4 different types of oils (fish oil, olive oil, linseed oil, or safflower oil) during pregnancy and lactation and evaluating immune function of their offspring (111). The offspring of the dams fed fish oil showed a decrease in natural killer T-cell activity and had smaller thymuses and spleens than those of dams fed other oils. In a similar study by Rayon *et al.*, pregnant mouse dams were fed corn oil or fish oil during pregnancy. Neonate immune function was assessed by challenging the pups with live Group B *Streptococcus* (112). Pups of the dams fed fish oil had a lower mortality rate than those of dams fed

corn oil, again suggesting that fatty acid composition of maternal prenatal diet might influence neonatal immune function. Additionally, this study suggests a potential positive relationship between fish oil consumption during pregnancy and infant immune response. More recently, Lauridsen *et al.* supplemented pregnant sows with either fish oil, rapeseed oil or sunflower oil and found that piglets of sows in the fish oil group had lower levels of the pro-inflammatory eicosanoids prostaglandin E2 (PGE_2) and thromboxane B2 compared to the sunflower seed oil group (113). These and other studies illustrate that maternal diet during fetal development can affect immune cell function in offspring; however, these studies were conducted in animals and cannot be directly compared with humans.

n-3 PUFA intake and response to immunologic challenge

Because n-3 PUFAs have been shown to modulate cell-mediated immune response, numerous animal models have been used to evaluate the impact of n-3 PUFA supplementation on host infectious disease resistance (114). These studies have shown mixed results; some reporting beneficial effects of n-3 PUFAs on host survival, others reporting adverse effects, and others reporting no effect. Olive *et al.* reported that rats fed fish oil, compared with rats fed control diets, mounted a similar intestinal immune response to infection with *Trichinella spiralis* (115). A study by Rayon *et al.* showed that pups of mouse dams that were fed fish oil during pregnancy had a lower mortality rate after infection with group B streptococcal pathogen, compared to controls (112). Alternatively, Paul *et al.* reported that weanling guinea pigs fed n-3 PUFAs, compared with guinea pigs fed n-6 PUFAs and control chow, suffered the most severe progression of disease when infected with *M tuberculosis* (116). Puertollano *et al.* fed mice different

sources of fats, including fish oil, for 4 weeks and infected them with *Listeria monocytocgenes* (117). Mice fed fish oil had lower body and spleen weights 96 hours after infection and had higher spleen levels of viable bacteria, suggesting the fish oil group could not mount as effective immune response as the other groups.

Recently, Beli *et al.* (2008) found that mice supplemented with fish oil experienced delayed clearance of reovirus compared to placebo, as evidenced by higher levels of viral shedding in the feces (118). In another study (2007), neonatal piglets were weaned at 2 d, given DHA+AA-enriched formula, standard formula or sow's milk for 30 d, and immunized with the influenza virus vaccine at 9 d (119). $CD4^+$ and $CD8^+$ T cell *ex vivo* proliferation was lower in piglets receiving the DHA+AA-enriched formula and IL-10 expression was up-regulated, compared to piglets that received control formula. Similarly, Byleveld *et al.* found that mice supplemented with fish oil and subsequently immunized with the influenza virus experienced delayed virus clearance and decreased production of IFN-γ and antibodies (120). Pierre *et al.* (2007) infected mice receiving either a control diet, an n-3 PUFA-enriched diet, or an n-6 PUFA-enriched diet with the bacteria Pseudomonas aeruginosa and reported longer survival time with chronic pneumonia in the n-3 PUFA-supplemented group (121). Similarly, mice were fed either control diet, EPA-enriched diet or AA-enriched diet and were subsequently infected with Pseudomonas aeruginosa in a study conducted by Auvin *et al.* (122). Higher levels of TNF-∞ and increased lung edema were found in mice consuming the EPA diet; however, survival was similar between groups. McFarland *et al.* (2008) infected guinea pigs consuming diets enriched with either n-3- or n-6 PUFAs with Mycobacterium tuberculosis and found reduced *in vitro* lymphocyte proliferation and reduced clearance

of infection in the guinea pigs consuming the n-3 PUFA-enriched diet (123). Numerous other studies have evaluated the influence of n-3 PUFA supplementation on infectious disease resistance in the host and have shown mixed results; however, many report a decrease of lymphocyte proliferation in n-3 PUFA-supplemented animals.

n-3 PUFAs and immune function: trials in adults

Numerous intervention trials have assessed the influence of dietary n-3 PUFAs on markers of immune function and inflammation and have, overall, supplemented healthy adults with fish oil (DHA+EPA). Although these studies report varying and sometimes conflicting results, there is clear evidence that n-3 PUFAs have some capacity to influence immune function (29).

In a study by Wallace *et al.*, healthy 18-39 year-olds were supplemented daily with α-linolenic acid (ALA) or various doses of fish oil for 12 weeks (124). The investigators found no difference in the production of cytokines such as TNF-α, IL-1β and Il-10, and no difference in lymphocyte proliferation between intervention groups; however, IL-6 production decreased significantly in participants supplemented with higher doses of fish oil. Kew *et al.* examined the influence of supplementation with different doses of ALA and DHA+EPA on adult immune function and found that supplementation with n-3 PUFAs did not alter immune markers including phagocytic activity, cytokine production, lymphocyte proliferation, and delayed-type hypersensitivity response (125). The effect of marine oil supplementation on immune function in elderly persons was examined by Bechoua *et al.* (126). Persons 70-83 years of age were supplemented with 150 mg DHA + 30mg EPA or placebo daily for 6 weeks. A marked decrease in lymphocyte proliferation was observed in the DHA + EPA group. These

important findings demonstrate that very low doses of marine oil can modulate immune function in the elderly, a population who likely suffer from a decline in immune function. Miles *et al.* showed that n-3 PUFA supplementation caused an increase in plasma IgG concentrations and a decrease in natural killer cell activity, but no difference in lymphocyte proliferation or delayed-type hypersensitivity response (127).

Kew *et al.* conducted a study in healthy adults evaluating the independent effects of DHA and EPA on immune function. Markers of immune function and inflammation were measured in three study groups (DHA-supplemented, EPA-supplemented, and placebo) (128). A decrease in T-lymphocyte activation was found in the DHA-supplemented group; however, no difference in cytokine production was found in either intervention group. Kelly *et al.* examined the effects of DHA on immune function and inflammatory mediators by supplementing healthy young men with 6 g DHA/d for 90 days (129, 130). DHA supplementation did not modify lymphocyte proliferation or delayed-type hypersensitivity; however, the DHA group experienced a decrease in NK cell activity and had lower concentration of inflammatory cytokines such as PGE_2 and LTB_4, compared to controls. DHA did not significantly influence IgG concentration in serum or antibody response to vaccination against influenza; however, the increase in antibody titers in the DHA group was close to significantly different (p=.059) than controls. Thies *et al.* compared the effects of ALA, γ-linolenic acid (GLA), AA, DHA, and fish oil on lymphocyte proliferation, NK cell activity, and number of circulating inflammatory cells in healthy 55-75 year-olds (131-133). The study revealed no effect of supplementation on inflammatory cell numbers (neutrophils, monocytes, and macrophages). The fish oil-supplemented group showed a decrease in NK cell activity,

while the fish oil and the GLA group showed a significant decrease in lymphocyte proliferation. Recently, Rees *et al.* reported modified neutrophil respiratory burst and prostaglandin production by mononuclear cells in men supplemented daily with EPA for 12 weeks (38).

Differences in the aforementioned study findings may be attributed to varying doses and forms of n-3 PUFAs used, differences in study subjects or insufficient study power (28). A limited number of studies have evaluated the independent effect of DHA on immune function. The above studies illustrate the variation in the reported effect of DHA supplementation on immune response and inflammation; however, evidence that n-3 PUFAs influence markers of inflammation and immune function is compelling. The below review summarizes the evidence from trials of n-3 PUFA supplementation and markers of immune function.

A recent review by Sijben and Calder (2007) summarized results from trials evaluating the influence of n-3 PUFAs on immune markers in humans, and provided overall conclusions based on available evidence (134). The authors focused on trials examining the following immune markers: *ex vivo* mitogen-induced T lymphocyte proliferation, natural killer (NK) cell activity, mitogen-induced cytokine production by lymphocytes and cytokine production by monocytes. Overall, evidence from 8 cohorts suggests n-3 PUFA supplementation decreases *ex vivo* lymphocyte proliferation in adults, especially in older subjects, and causes a decrease in inflammatory cytokine production by monocytes. Reports of the influence of n-3 PUFAs on cytokine production by lymphocytes and NK cell activity are inconsistent and, the authors report, inconclusive.

n-3 PUFAs and infection

Merchant *et al.* (2005) evaluated, using food frequency questionnaires, the influence of dietary n-6 and n-3 PUFAs on the incidence of pneumonia among males in the US Health Professionals Follow-up Study (n=38,378) (34). Study participants were followed for 10 years, and dietary intake was reported every 4 years. Higher self-reported dietary intake of n-3 and n-6 PUFAs reduced the risk of community-acquired pneumonia in this population by 31% for every 1 g/d increase in ALA intake; however, intake of EPA and DHA alone did not influence pneumonia risk.

n-3 PUFAs and immune function: trials in children

Several studies have examined the influence of n-3 PUFA supplementation on immune function or infection in children. A double-blind trial in which 5-7 year-olds were given a dietary supplement containing AA and DHA or placebo for 7 months showed alterations in immune cell phenotypes in the LCPUFA group, compared to placebo (135). In a study by Venuta *et al.*, 36-49 month-old children with a history of recurrent respiratory infections were supplemented with ALA (855 mg/d) and LA (596 mg/d) or placebo for two winter seasons (35). The EFA-supplemented children experienced significantly fewer infective episodes per month, fewer days with fever, and fewer days missed from school because of illness. Studies focusing on n-3 PUFA supplementation in asthmatic children have shown that fish oil supplementation can potentially decrease asthmatic symptoms such as wheezing (136-138).

n-3 PUFA-supplemented formula

Studies have examined the influence of formula with added n-3 PUFAs on infant immune function or infection in preterm infants. Field *et al.* provided preterm Canadian infants human milk (HM), control formula or formula containing LCPUFAs and reported that infants in the HM and LCPUFA groups had a higher proportion of antigen mature CD4+ cells and increased synthesis of IL-10, compared to infants in the control formula group (139). A recent meta-analysis of randomized controlled trials of PUFA supplementation of infant formula in preterm babies reported no difference in rates of sepsis or necrotizing enterocolitis between the LCPUFA and control groups (37). The authors of this meta-analysis noted that few trials reported other outcomes common to preterm infants such as bronchopulmonary dysplasia and therefore further studies are necessary.

Several trials of n-3 PUFA-supplemented formula have been conducted in term infants, including a trial in which infants were either breastfed or received one of three formulas (control formula, ARA+DHA formula or DHA-enriched formula) from birth through 1 year (140). The investigators reported no difference between groups in outcomes such as “had 3 or more prescriptions for antibiotics”, chronic otitis media, or hospitalization. One study in which 1342 infants were assigned to receive either regular formula or formula supplemented with DHA and AA showed that the infants consuming the EFA-supplemented formula had a decreased incidence of bronchiolitis/bronchitis at 5, 7 and 9 months of age (36). A recent randomized trial in which term infants were given either regular formula or formula supplemented with long chain PUFAs showed that the LCPUFAs influenced the presence and function of infant immune cells such as CD3+

and CD44++ (107). Finally, Danish infants were provided either formula or cow's milk alone or with fish oil (2 X 2 design) from 9-12 months of age (141). Increased *L. paracasei*-induced INF-γ was observed in the fish oil groups, suggesting that fish oil may influence the maturation of the infant immune system.

n-3 PUFA supplementation in pregnancy and/or lactation and infant immune function

Detailed in the discussion section of Chapter 7.

n-3 PUFA supplementation in lactation and breast milk immunoglobulins

Dietary n-3 PUFAs may influence immune-modulating components of breast milk, providing a mechanism through which immune response in the breast fed infant might be altered by maternal diet in pregnancy and/or lactation. A study by Dunstan *et al.* found that supplementation of atopic women with dietary fish oil (3.7 g DHA+EPA/d) from 20 weeks' gestation through delivery resulted in higher DHA and lower AA concentrations in breast milk at 3 days post-partum, compared to placebo (89). The investigators reported a positive correlation between maternal n-3 PUFA concentration and IgA, IL-10 and IL-6 concentrations in breast milk, suggesting a potential positive relationship between n-3 PUFAs and immune-modulators in breast milk. Conversely, a study by Hawkes and colleagues showed that supplementation of lactating women with tuna oil for 4 weeks improved n-3 PUFA status but did not change cytokine levels in breast milk (91).

in utero programming of immune function

Several studies have examined the potential influence of *in utero* programming on immune function. Specifically, studies have addressed the influence of maternal nutrition during pregnancy and early postnatal nutrition on immune function. Moore *et al.* conducted a study in rural Gambia examining the effect of season on child mortality (142, 143). The investigators studied detailed birth records of children born between 1949 and 1994 and found that children born during the "hungry season" (season in which pregnant women were most likely to be severely undernourished) suffered higher rates of intra-uterine growth retardation and of subsequent mortality in adolescence and older. The investigators suggested that *in utero* insults, such as maternal malnutrition, might influence the development of the fetal, and subsequent infant immune system. Moore *et al.* later conducted another study in the same area of rural Gambian and examined specific measures of immune function (antigen-specific antibody response to vaccination) (143). Findings from this later study showed no association between immune response, measured by response to vaccination, and season of birth and birth weight. McDade *et al.* addressed the possible influence of prenatal undernutrition on immune-competence in adolescence (16). The investigators recruited a sub-sample of 14-15 year old subjects from an ongoing longitudinal study in the Philippines, in which birth weight and gestational age had been recorded (for determining intra-uterine growth retardation). Unlike the Moore *et al.* study, when challenged with vaccination, adolescents who had been prenatally undernourished had a lower probability of mounting an adequate immune response compared with adolescents who were adequately nourished during fetal development. These results indicate a potential relationship between *in utero* nutritional

insults and subsequent immune dysfunction. In the same study in the Philippines, McDade and colleagues also examined the influence of prenatal undernutrition, resulting in infants born small for gestational age, on adolescent thymic function (144). Adolescents who suffered prenatal undernutrition had significantly lower thymopoietin production, again illustrating the potential influence of intra-uterine insults on immune system development. McDade *et al.* studied an additional cohort within this population-based longitudinal study in the Philippines to determine, partly, the influence of prenatal growth on total IgE production in adolescence (145). The investigators found that under certain conditions (a dirty household), prenatal undernutrition was associated with increased IgE production in adolescence. Overall, studies examining *in utero* programming of immune function have shown varied results; however, studies report compelling evidence that prenatal environments can predict immune function.

Specific micronutrients in pregnancy have also been shown to influence immune function. For example, Tielsch *et al.* showed that children born to vitamin A deficient women, as evidenced by night blindness during pregnancy, suffered an increased risk of diarrhea, acute respiratory infection and dysentery compared to children born to women without night blindness (11). In a study of multiple-micronutrient supplementation in pregnancy conducted in Nepal, preterm infants of mothers supplemented with vitamin A and folic acid, iron, or folic acid + zinc experienced a decreased risk of mortality in the first three months of life (17).

Chapter summary

In summary, the immune system begins to develop *in utero* and immune response, albeit sometimes weak and fleeting, is active in newborns. The *in utero* environment,

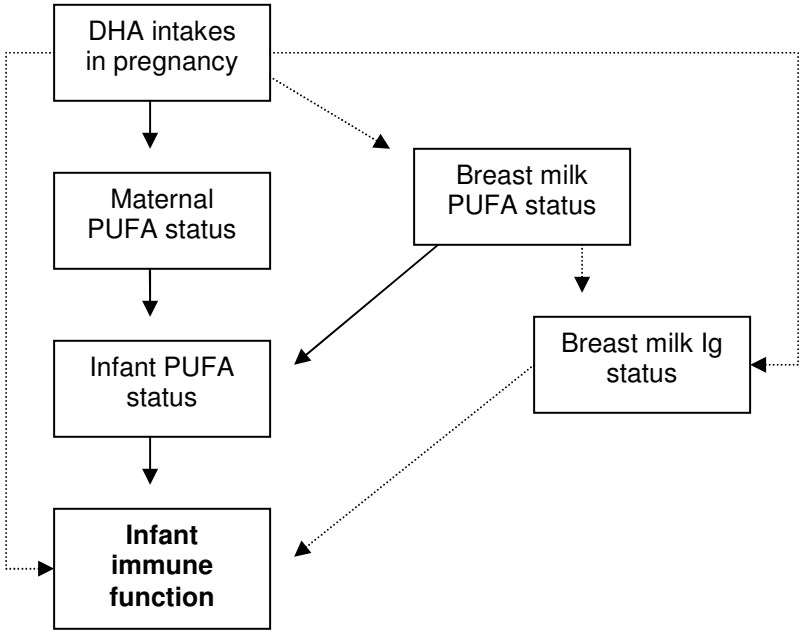
including nutritional exposures such as n-3 PUFAs, may influence the development of immune function. The n-3 PUFA DHA is found in cells of the immune system and modulates immunity and inflammation through various mechanisms including incorporation of DHA into cell membrane phospholipids and modification of membrane structure and function, subsequent alteration in eicosanoid synthesis, cell adhesion molecule expression, cytokine production and lipid raft structure, and modulation of factors involved in gene expression. Evidence from studies in humans and animals illustrates a link between n-3 PUFA nutriture and immune function; however, the influence of DHA in pregnancy on immune response to vaccination is yet unknown.

Conceptual framework describing the potential relationship between DHA in pregnancy and infant immune function

The following conceptual framework describes the pathways by which dietary intake of DHA during pregnancy can influence immune function during early infancy **(Figure 4)**. Maternal DHA intake during pregnancy and/or lactation influences maternal and infant DHA nutriture, as detailed in the above literature review. Furthermore, numerous studies have demonstrated a positive relationship between maternal fish oil supplementation during pregnancy and lactation or during lactation alone and breast milk DHA status. Few studies have examined the effect of fish oil in pregnancy alone on breast milk PUFA status. The relationships that are less clear, which we will examine in our study, are those between maternal DHA intake in pregnancy and 1) breast milk DHA concentration and 2) infant immune function, measured by infant morbidity and response

to vaccination. Solid lines indicate relationships that are well-established, while dashed lines indicate relationships that are not yet fully understood.

Figure 4: Conceptual framework describing the potential influence of in utero exposure to DHA on infant immune function



CHAPTER 4: METHODS

This chapter describes the research objectives and hypotheses, study setting, design and participants, and data collection. Additionally, laboratory and statistical methods are detailed.

Objectives and hypotheses

The primary objectives of this study are to evaluate the effect of maternal DHA supplementation during pregnancy on 1) breast milk fatty acid concentrations, 2) infant morbidity and 3) infant immune function.

The **specific hypotheses** are:

1. Women who receive DHA supplements from 18-22 weeks gestation through delivery will have higher breast milk DHA concentrations at 1 month post-partum than women who receive placebo.
2. Infants born to women who receive DHA supplements from 18-22 weeks gestation through delivery will experience less severe and fewer episodes of illness than infants born to women who receive placebo.
3. Infants born to women who receive DHA supplements from 18-22 weeks gestation through delivery will mount a stronger response to childhood hepatitis B and tetanus vaccination than infants born to women who receive placebo, specifically,
    a. Higher plasma anti-hepatitis B IgM and IgG antibody concentrations at 3 months of age after vaccination at birth and at 2 months of age

b. Higher plasma anti-tetanus IgM and IgG antibody concentrations at 3 months of age after vaccination at 2 months of age

Study setting and population and ethical considerations

This study is a collaborative effort of Emory University's Hubert Department of Global Health in Atlanta, Georgia, and the Instituto Nacional de Salud Pública (INSP) and Mexican Institute of Social Security (Instituto Mexicano del Seguro Social, IMSS) General Hospital I in Cuernavaca, Mexico. This ongoing trial is being conducted in Cuernavaca, Mexico in the state of Morelos, which is located in central Mexico approximately 1 hour south of Mexico City. This study was conducted in the context of an ongoing randomized, double-blinded, placebo-controlled trial, whose main outcome is child growth and development.

Study participants were recruited at the IMSS General Hospital I, a large hospital located in Cuernavaca, Mexico, and 3 small health clinics within the IMSS system in Cuernavaca during routine prenatal care visits between February 2005 and February 2007. The IMSS health care system provides employed persons access to medical care. Generally, the women who utilize hospital are of medium-to-low socioeconomic status and either they and/or their husbands are employed. In most cases, the IMSS hospital patient pays one third of the health care costs and their employer and the federal government pay the remaining two-thirds of the costs.

Routine prenatal care was provided by physicians, and women attended prenatal checkups beginning with the confirmation of pregnancy monthly during the 1st and 2nd trimester, every 2 weeks during the third trimester, and weekly during the last month of

pregnancy. All pregnant women that attend the IMSS hospital are routinely provided tetanus toxoid vaccination and iron-folate supplementation (5 mg/d of folic acid and 100 mg of iron fumarate or sulfate) during pregnancy. For the first year of life, infants are scheduled for monthly well baby visits at the hospital, where they receive routine immunizations and growth and development monitoring. Coverage rates for routine vaccinations are very high (>95%) and almost all mothers initiate breast feeding.

Women were considered for inclusion in the study if they were in gestation weeks 18-22, planned to predominantly breastfeed for at least 3 months, were 18-35 years old, planned to deliver at the IMSS General Hospital in Cuernavaca and planned to live in the area for 2 years after delivery. Women were excluded if any of the following criteria were present: 1) high risk pregnancy, 2) lipid metabolism or absorption disorders, 3) regular intake of fish oil or DHA supplements or 4) chronic use of certain medications (i.e. medications for epilepsy).

Written informed consent was obtained from each participant after a thorough explanation of the study details, and participants were free to withdraw from the study at any time. The study protocol and all informed consent documents were approved by Emory University's Human Investigations Board and by the Instituto Nacional de Salud Pública (INSP) and the IMSS General Hospital I's Human Subjects Boards. The welfare of the subjects was monitored by an external Data Safety Monitoring Committee. This study is an ongoing collaborative project between the INSP in Cuernavaca, Mexico and Emory University, and is a registered clinical trial (registered at INSP in Mexico: #CI-011, and at clinicaltrials.gov: NCT00646360).

Study design

Women were assigned to receive either 400 mg DHA or placebo daily, from gestation weeks 18-22 through parturition. The DHA capsules contained 200 mg DHA each derived from an algal source (Martek Biosciences Corporation, Columbia, MD). The placebo capsules contained olive oil, and were similar in appearance and taste to the DHA capsules. Fieldworkers visited the women's homes weekly to deliver a new bottle of 14 capsules, and compliance was monitored by counting any remaining pills and through interviews with participants.

All participants and members of the study team were blinded to the treatment scheme throughout the intervention period of the study. The blinding code was placed in sealed envelopes at the beginning of the study, and these envelopes were held by investigators at Emory who were not involved in the study. Data were un-blinded for the analytical study team after the last baby in the study was born and had completed 6 months of age, at which time participants were no longer taking supplements.

All eligible women were randomized to either the treatment or the control group using a computer-generated list created by the study biostatistician at Emory University. Women were randomized to either the treatment or the control group using the block randomization method to randomly create balanced replication of 4 treatments (2 colors for DHA and 2 for control) using a block size of 8. The list was generated for a sample size of 1104. Success of randomization was assessed by comparison of a variety of baseline maternal characteristics in the two treatment groups.

Data collection

Ascertainment of prenatal dietary intake

Using a validated food frequency questionnaire (FFQ), we assessed maternal dietary intake at 18-22 weeks' gestation. The 110 item FFQ included specific questions about consumption of sources of DHA such as fresh water fish, seafood, canned tuna and sardines, salmon, trout, cod liver oil, and dried fish called "charales" in the previous 3 months. A previous study reported that estimates of n-3 PUFA intake ascertained using this FFQ correlate reasonably well with erythrocyte membrane n-3 PUFA phospholipid concentration (146).

Ascertainment of infant morbidity information

Child morbidity information was captured using a 15-day recall questionnaire when mothers brought their infants to the hospital for study visits at 1, 3 and 6 months (see appendix). Mothers were asked questions about the occurrence of illness symptoms, duration of symptoms and whether they sought medical care for their infant's symptoms. For example, mothers were asked "In the past 15 days, has your child experienced fever"; mothers who responded affirmatively were then asked "on what date did the symptoms begin, and on what date did they end" and "did you seek medical care for this illness?"

Laboratory methods

Blood and breast milk sample collection

Breast milk samples were collected from all women who agreed to provide samples at the IMSS hospital during routine study visits at 1 month post-partum. The milk samples were taken during a morning feed between 8AM and 12PM, and mothers were asked to feed their infant before coming to the hospital so that the milk sample was not from the first feed of the day. Initially, 5 ml of foremilk was hand-expressed before infants were allowed to suckle the nipple for 10 minutes; afterwards an expression of 5 ml of hindmilk was collected into the same 14 ml plastic test tube and was thoroughly mixed with the initial 5 ml foremilk sample. The samples were then aliquoted into multiple cryovials and frozen in a nitrogen environment -70° C.

Seven ml of maternal blood was obtained by venipuncture by trained technicians at the IMSS hospital at 18-22 weeks' gestation, delivery and 1 month post-partum. A 5 ml venous blood sample was obtained from infants at 3 months of age. All samples were collected into tubes containing EDTA. Plasma and erythrocytes were separated by centrifugation at 800 G for 10 minutes at room temperature. Plasma was then aliquoted into multiple cryovials, and were immediately frozen in a nitrogen environment and stored at -70° C.

Determination of PUFA concentrations

All plasma phospholipid fatty acid concentration determinations were carried out at INSP in Cuernavaca, Mexico. The total fat from milk and plasma was extracted with a chloroform-methanol mixture (2:1). The yield was determined by gravimetry following a

modification of the method described by Folch and was expressed as g/100 g of serum or milk (147). Fatty acids were derivatized using borum trifluride and were then extracted with pure hexane. The extracts were injected into a gas chromatograph Hewlett Packard (USA) Mod. 5890 Series II, using a 100 m length x 0.25 mm internal diameter Supelco SP 2560 (USA) column. The chromatographic peaks were identified using true reference standards for 37 fatty acids, (Supelco, St. Louis, MO, USA). The fatty acid content is expressed as percentage of total fat.

Determination of immunoglobulin concentrations

Antigen-specific plasma immunoglobulin concentrations are appropriate functional markers for measuring immune response to vaccination (109, 130). We measured anti-hepatitis B and anti-tetanus IgM and IgG antibody concentrations in infant plasma at 3 months of age. In accordance with the childhood vaccine schedule in Mexico, infants received hepatitis B vaccination at birth, and hepatitis B and tetanus vaccinations, among others, at 2 months of age. We determined hepatitis B- and tetanus-specific antibody concentrations in plasma at 3 months of age.

Anti-hepatitis B and anti-tetanus IgM and IgG antibody concentrations were measured by a flow cytometry system (Luminex technology) at the Centers for Disease Control and Prevention in Atlanta, Georgia. Luminex technology is a microsphere-based flow cytometry system that can quantify up to 100 analytes, such as immunoglobulins, in a single sample of <100 microliters (148-150). Each microsphere is manufactured with a unique proportion of red and orange fluorescent dye, and can be coupled with a different biological probe such as a capture antibody or an antigen. Biotinylated detection antibodies bind the analyte and fluoresce at a wavelength unique to that bead. The

microsphere is excited by a laser and emits a distinct fluorescent intensity which is captured by a detector. The amount of detection antibody that binds to each bead is proportional to the amount of analyte that is bound to the capture antibody. Results are compared to standards to quantify the amount of analyte in each sample.

Laboratory protocol for determination of immunoglobulin concentrations

Sample preparation

Samples underwent one freeze-thaw cycle to accomplish the aliquoting. Three aliquots were obtained from each original sample of approximately 200 µl. One aliquot of 50 µl was used for the current study and two additional aliquots, consisting of 100 µl each were stored in a -20°F freezer for future analysis.

Plate preparation

Ninety-six well plates were prepared by adding 100 µl of blocking buffer to each well and agitating them on speed 4 for twenty minutes. Plates then underwent a single wash with washing buffer (150 µl PBS + Tween 20 (.05%)) and were placed on a vacuum to remove the liquid. Forty µl of prepared beads that had binding regions for both hepatitis B and tetanus were then added to the plate.

For the current study, a 1:50 dilution was used. Five µl of sample was added to 245 µl of dilution buffer (0.01 M PBS+0.5% BSA) (BSA= bovine serum albumin, PBS = phosphate buffered saline). After mixing the samples on speed 6, 10 µl of dilution buffer was added to the beaded prepared plates. Forty two samples, run in duplicate, with three internal standards were run per plate **(Figure 5)**. Sample A was an external plasma sample and samples B1 and B2 were derived from pooled infant plasma. B1was neat

infant plasma and served as the 'high' standard and B2 underwent a 1:50 dilution and served as the 'low' standard.

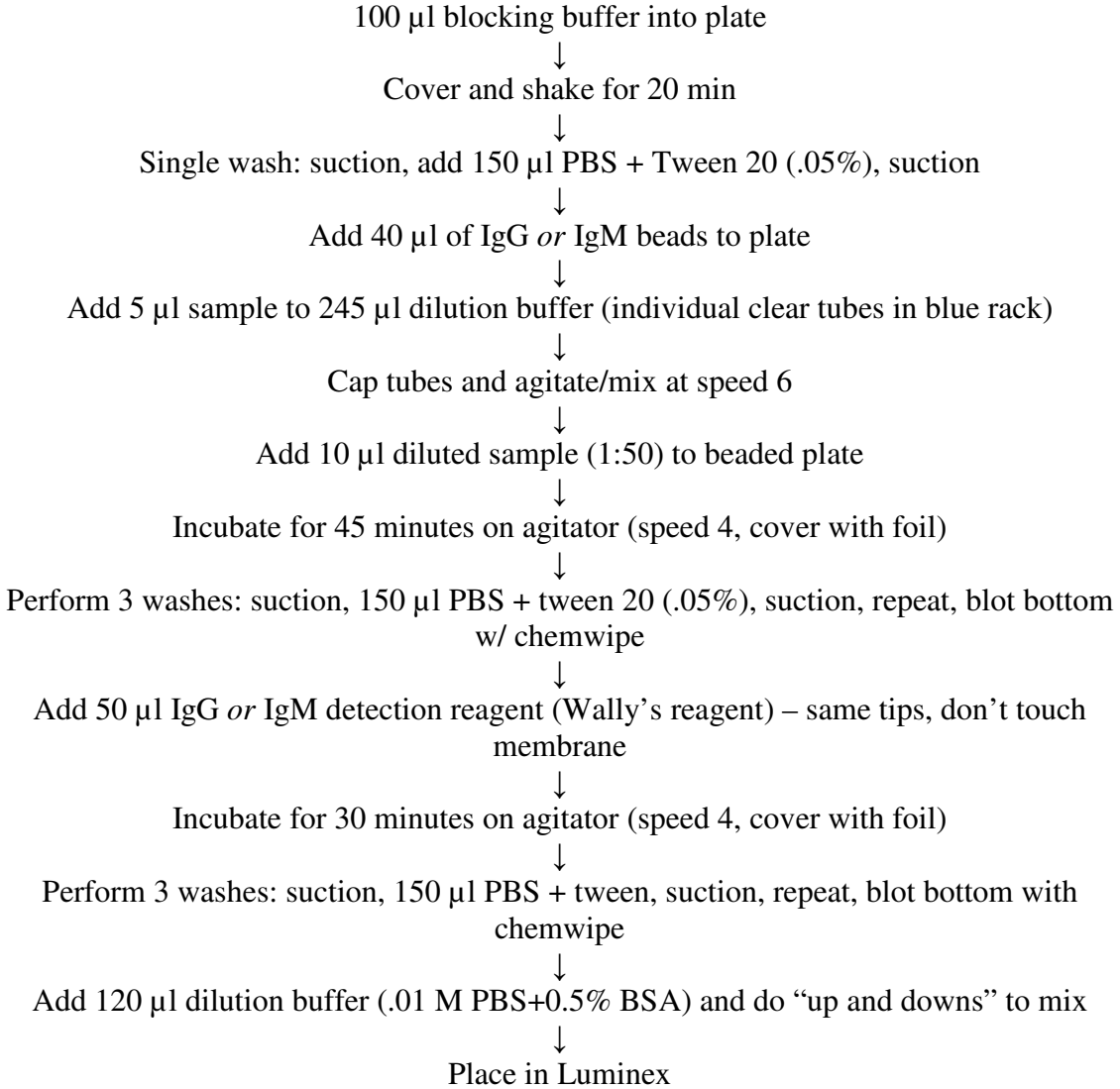
Plates were then incubated on the agitator at speed 4, covered with foil, for 45 minutes. After incubation, plates were washed and vacuum suctioned three times with the wash buffer (150 µl PBS + Tween 20 (.05%)). The detection reagent was then added. For the IgG assay, the detection reagent was Wally's reagent, (from Quantaplex – 1:5 of diluted solution) and for the IgM assay, Donkey Antihuman reagent was used (1:100 of stock solution). Plates were then covered with foil and incubated for a further 30 minutes at speed 4 on the agitator. After the second incubation, plates underwent three washes with the wash buffer. One hundred and twenty µl of dilution buffer was then added to each plate to prepare the sample for reading on the Luminex. Plates were then placed in Luminex to read. A flow chart outlining abbreviated methods is shown below **(Figure 6)**.

Figure 5: Plate layout for determinations of antibody concentrations

	A	B	C	D	E	F	G	H	I	J	K	L
1	Blank	Blank	Blank	Blank	Blank	Blank	A	A	B1	B1	B2	B2
2												
3												
4												
5												
6												
7												
8												

Column B is a duplicate of column A
Column D is a duplicate of column C, etc.

Figure 6: Flow chart instructions for Luminex (for determination antibody concentrations)



Recruitment of study subjects and power calculations

No changes were made to the recruitment of study subjects for the present study because the parent study was already underway. The power calculations of the parent study dictated that a minimum of 676 mother-child pairs would complete the original study and therefore at least 994 pregnancies would be recruited (approximately 50-70 per month) assuming a 15% loss to follow up during pregnancy and a further 20% loss in the first 18 months of life. The original study estimates were conservative and included losses due to deaths and migrations out of the study area. All efforts were made to keep losses to a minimum.

Assuming 994 pregnancies would be recruited for the original study, and assuming a 15% loss to follow-up during pregnancy and a 5% loss during the first 3 months of the infants' lives, the present study would ideally have access to 803 mother-child pairs. Of those, we had planned to have access to at least 232 mother-child pairs who would be eligible for inclusion in the infant immunoglobulin analysis in at 3 months of age (116 in the DHA group and 116 in the placebo group).

Power calculations

Since the parent study design was fixed, we had planned to have a minimum sample of 116 mother-child pairs per group (control and treatment) for comparing key outcomes in the present study. Assuming a two-tailed test and confidence level < 0.05, we will have $\geq$ 80% power with this sample size to detect effect sizes of 0.4 (151). Such effect sizes have been characterized as small to moderate and represents small but important differences for the immunological outcomes that were examined in the present

study (151). For example, an effect size of 0.4 is a difference of 40 mg/dl in mean IgG in infants at 3 months of age between groups assuming the S.D. is 100 mg/dl.

Statistical methods

We conducted all statistical analyses using SAS version 9.2, Cary, North Carolina. Statistical significance was defined as a p-value < 0.05. For each of the following reports, continuous baseline characteristics, characteristics at birth and outcome variables were tested for normality using histograms, box-and-whisker plots and the Kolmogorov–Smirnov test for normality, and by examining normal probability and quantile-quantile (Q-Q) plots. We performed no data imputations and missing values were not included in analyses. For all analyses, baseline demographic characteristics of our sample did not differ significantly from characteristics of the study sample, so we assumed that no selection bias occurred. Non-parametric analytical methods such as Poisson regression modeling, Wilcoxon rank sum tests and Spearman correlations were used for non-normally distributed variables. Group differences of continuous baseline variables were calculated using T-Tests, Wilcoxon rank sum tests or generalized linear models, as appropriate. Categorical baseline characteristics were tested using Chi-square tests. We identified potential effect modifiers based on *a priori* knowledge and tested for interaction in models accordingly for each analysis. For example, studies have shown that factors such as parity, maternal age, SES and infant sex can influence immunoglobulin levels and we therefore included these variables in interaction terms (152, 153). We found no significant interactions and therefore did not include interaction terms in our models.

For all analysis, baseline characteristics of the two treatment groups were comparable; hence, upon initial analysis, we did not include covariates aside from "treatment group" in models. Because this was a randomized, controlled clinical trial in which treatment groups were similar at baseline we calculated group differences using T-Tests, Wilcoxon rank sum tests and unadjusted models.

Breast milk analysis: statistical methodology

Four hundred and fifty participants provided breast milk at 1 month post-partum. Of the 450 samples, a random sub-sample of 240 samples was selected for PUFA analysis, and 174 were analyzed due to logistical reasons (budget and personnel constraints). Baseline demographics and infant outcomes such as birth weight and gestational age at delivery were similar between the sub-sample and our study sample, so we assume that the selection process did not introduce selection bias in our analyses. Extreme high and low outliers in the main outcomes variables (breast milk PUFAs) were deleted using the following criteria for problematic outliers: quartile 3 + (3*inter-quartile range) and quartile 1 – (3*inter-quartile range) for group comparisons of PUFA concentrations in breast milk. Between-group differences in breast milk fatty acid concentrations were evaluated using either Wilcoxon rank sum tests or T-Tests, as appropriate. We computed Spearman correlation coefficients to describe the relationship between breast milk fatty acids and dietary intake and plasma DHA, EPA, AA levels. The effect of treatment group on breast milk fatty acids concentrations was also examined using general linear models. We conducted statistical analyses using TTEST, NPAR1WAY, GLM and CORR procedures.

Morbidity analysis: statistical methodology

We conducted the analysis according to the intention-to-treat principle, and analyzed the entire study population with available data at 1, 3 and 6 months post-partum. We created a variable of aggregate symptoms including phlegm, nasal congestion, nasal secretion, or cough, which we classified as an upper respiratory tract infection (henceforth denoted as "cold") (154). Mothers were asked at the 1 and 3 month study visits if they breast fed their child (yes/no); hence, breast feeding information is available through 3 months post-partum.

Differences between reported illness symptoms in the previous 15 days, care seeking, and severity of diarrhea were tested using logistic regression, Chi-square and Fisher's exact tests, as appropriate. Duration of child illness was assessed using, because it was count data (number of days ill), unadjusted Poisson regression models that included only children who experienced that particular illness symptom being tested.

Response to hepatitis B and tetanus vaccination analysis

We analyzed all available 3-month infant plasma samples (n=592). Immunoglobulin concentrations were natural log-transformed to approximate normal distributions, and concentrations were expressed as geometric means (GM) with 95% confidence intervals (CI). The effect of treatment on immunoglobulin concentration was assessed using unadjusted generalized linear models, and estimates were exponentiated because of the initial log-transformation of the values. We computed Spearman correlation coefficients to describe the relationship between immunoglobulin concentrations at 3 months and select characteristics such as SES, parity, gestational age,

birth weight and maternal age. We conducted statistical analyses using TTEST, NPAR1WAY, GLM and CORR procedures.

Description of my specific contributions to the study:

Laboratory: I conceptualized the methods used to measure immune function in the infants, identified a laboratory at CDC willing to perform the immunoglobulin analyses and helped in the CDC laboratory with the immunoglobulin determinations using Luminex technology.

Data management and analysis: I helped maintain the study's adverse events database and my duties included receiving, reviewing, entering and analyzing data sent by Mexico. I assisted in the preparation of progress reports sent to NIH and the March of Dimes by creating tables and summarizing data.

Grant writing: I was the primary author of the supplemental NIH grant that funded this dissertation research. I also assisted in writing other grants related to the DHA project by performing literature reviews and statistical analyses and by summarizing preliminary findings.

Additional study activities: I assisted in the development of study protocols and questionnaires including the child health diary, the 15-day morbidity recall and the child health history. I also took several trips to the study site in Mexico to assist with study oversight, particularly, collection, organization and storage of maternal and infant blood samples.

CHAPTER 5

DOCOSAHEXAENOIC ACID SUPPLEMENTATION FROM MID-PREGNANCY THROUGH PARTURITION INFLUENCED BREAST MILK FATTY ACID CONCENTRATIONS AT 1 MONTH POSTPARTUM IN A DOUBLE-BLIND RANDOMIZED, CONTROLLED TRIAL IN MEXICO

Docosahexaenoic acid supplementation from mid-pregnancy through parturition influenced breast milk fatty acid concentrations at 1 month post-partum in a double-blind randomized, controlled trial in Mexico

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Keywords

n-3 fatty acids, docosahexaenoic acid, DHA, breast milk, pregnancy

This trial has been registered at clinicaltrials.gov (identifier NCT00646360).

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ABSTRACT

Objectives: Omega-3 polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA, 22:6*n*-3), are essential for neural development and accumulate extensively in the fetal and infant brain. The concentration of omega-3 PUFAs in breast milk influences the PUFA status of the breastfed infant. DHA concentration in breast milk has been shown to increase with maternal supplementation with fish oil during pregnancy and/or lactation, but the benefits of prenatal supplementation with DHA alone are unknown. We investigated the effect of prenatal DHA supplementation on PUFA concentrations in breast milk at 1 month post-partum. **Methods:** In a double-blind randomized, controlled trial in Mexico, 1040 pregnant women were supplemented daily with 400 mg algae-derived DHA or placebo from 18-22 weeks' gestation through parturition. Fatty acid concentrations (% of total fatty acids) were determined in breast milk obtained from 174 women at 1 month post-partum using gas liquid chromatography. **Results:** Women in the DHA group had higher mean breast milk DHA and alpha-linolenic acid (ALA, 18:3*n*-3) concentrations, expressed as % of total fatty acids, compared to the placebo group at 1 month post-partum. Mean breast milk DHA concentrations in the DHA and placebo groups were 0.20 (SD=0.06) and 0.17 (SD=0.07), respectively (Δ=0.02, 95% CI=-0.04, -0.004), and mean ALA concentrations were 1.38 (SD=0.47) and 1.24 (SD=0.46), respectively. Concentrations of other PUFAs such as eicosapentaenoic acid (EPA) and arachidonic acid (AA) did not differ between groups ($p>0.05$). Maternal plasma DHA concentrations correlated positively with breast milk DHA at 1 month post-partum in both the placebo and DHA groups ($r=0.4$, $p<0.01$ for both treatment groups).

Conclusions: Prenatal DHA supplementation from 18-22 weeks' gestation through

parturition increased concentrations of DHA and ALA in breast milk at 1 month post-partum, providing a mechanism through which breastfed infants could benefit.

INTRODUCTION

Long chain polyunsaturated fatty acids (LCPUFAs) such as docosahexaenoic acid (DHA, 22:6*n*-3) and arachidonic acid (AA, 20:4*n*-6) are important components of brain and retinal cell membrane lipids and accumulate extensively in the fetal and infant brain during the last trimester of pregnancy and infancy (1-3). LCPUFAs are preferentially transported across the placenta to the developing fetus, and *in vivo* studies conducted in humans have shown preferential placental transfer of DHA, specifically (4). LCPUFAs are present in breast milk and their variable concentration depends upon maternal stores, dietary intake and synthesis in the mammary glands; however, synthesis of DHA in the mammary gland is likely minimal (1, 5, 6). DHA concentration in breast milk has been shown to decrease between lactation weeks 6 and 30, and supplementation of lactating women improves DHA breast milk concentrations (7-9). Since the developing fetus and breastfed infant rely on maternal supply of DHA, adequacy of DHA in the maternal diet during pregnancy and lactation is critical.

Dietary intake of omega-3 PUFAs in many parts of the world, including the U.S., is low, while intake of omega-6 PUFAs has increased (10). Intake of n-3 PUFAs and DHA+ eicosapentaenoic acid (EPA, 20:5*n*-3) in the U.S. are an estimated 1.6 g/d and 0.1-0.2 g/d, respectively, and the ratio of dietary n-6:n-3 PUFAs is approximately 9.8:1 (11). Populations living in coastal countries such as Japan and Norway, where fish is widely consumed, have higher dietary intake of n-3 PUFAs and correspondingly high concentrations of DHA in their breast milk (10, 12). Although there is no official dietary recommendation for EPA and DHA intake in the United States, several expert groups suggest DHA intakes of at least 200-220 mg/d (up to 1 g/d) for pregnant and lactating

women, and 2.7 g/d of n-3 PUFAs, and the suggested n-6:n-3 ratio is approximately 2-5:1 (11, 13, 14). Since breast milk DHA concentrations are dependent on maternal diet and stores, low DHA intake, as seen in many populations, results in low concentration of DHA in breast milk. Consequently, low DHA concentration in breast milk can negatively influence growth and development of the breastfed infant.

This study investigated the influence of DHA supplementation from gestation weeks 18-22 through parturition on breast milk fatty acid composition 1 month post-partum. To our knowledge, this is the first study to assess the impact of algae-derived DHA supplements during the latter half of pregnancy on breast milk composition.

METHODS

Study population and setting

Study participants were recruited at the Mexican Institute of Social Security (Instituto Mexicano del Seguro Social or IMSS) General Hospital I, a large hospital located in Cuernavaca, Mexico, and 3 small health clinics within the IMSS system in Cuernavaca during routine prenatal care visits between February 2005 and February 2007. The IMSS health care system provides employed persons access to medical care. Women were considered for inclusion in the study if they were in gestation weeks 18-22, planned to predominantly breastfeed for at least 3 months, were 18-35 years old, planned to deliver at the IMSS General Hospital in Cuernavaca, and planned to live in the area for 2 years after delivery. Women were excluded if any of the following criteria were present: 1) high risk pregnancy, 2) lipid metabolism or absorption disorders, 3) regular intake of fish oil or DHA supplements, or 4) chronic use of certain medications, such as

medications for epilepsy. Written informed consent was obtained from each participant after a thorough explanation of the study details, and participants were free to withdraw from the study at any time.

Ethics: The study protocol and all informed consent documents were approved by Emory University's Human Investigations Board and by the Instituto Nacional de Salud Pública (INSP) and the IMSS General Hospital I's Human Subjects Boards. The welfare of the subjects was monitored by an external Data Safety Monitoring Committee. This study is an ongoing collaborative project between the INSP in Cuernavaca, Mexico and Emory University, and is a registered clinical trial (registered at INSP in Mexico: #CI-011, and at clinicaltrials.gov: NCT00646360).

Study Design

Women were assigned to receive either 400 mg DHA or placebo daily, from gestation weeks 18-22 through parturition. The DHA capsules contained 200 mg DHA each derived from an algal source (Martek Biosciences Corporation, Columbia, MD). The placebo capsules contained olive oil, and were similar in appearance and taste to the DHA capsules. Fieldworkers visited the women's homes weekly to deliver a new bottle of 14 capsules, and compliance was monitored by counting any remaining pills and through interviews with participants.

All participants and members of the study team were blinded to the treatment scheme throughout the intervention period of the study. The blinding code was placed in sealed envelopes at the beginning of the study, and these envelopes were held by investigators at Emory who were not involved in the study. Data were un-blinded for the

analytical study team after the last baby in the study was born and had completed 6 months of age, at which time participants were no longer taking supplements. As the study is ongoing for follow-up of child development, participants and field workers in Mexico remain blinded to the treatment allocation.

All eligible women were randomized to either the treatment or the control group using a computer-generated list created by the study biostatistician at Emory University. Women were randomized to either the treatment or the control group using the block randomization method to randomly create balanced replication of 4 treatments (2 colors for DHA and 2 for control) using a block size of 8. Success of randomization was assessed by comparison of a variety of baseline maternal characteristics in the two treatment groups.

Ascertainment of prenatal dietary intake

Using a validated food frequency questionnaire (FFQ), we assessed maternal dietary intake at 18-22 weeks' gestation. The 110 item FFQ included specific questions about consumption of sources of DHA such as fresh water fish, seafood, canned tuna and sardines, salmon, trout, cod liver oil, and dried fish called "charales" in the previous 3 months. Estimates of n-3 PUFA intake ascertained using this FFQ correlate reasonably well with erythrocyte membrane n-3 PUFA phospholipid concentration (15).

Blood and milk samples

Breast milk samples were collected from all women who agreed to provide samples at the IMSS hospital during routine study visits at 1 month post-partum. The milk samples were taken during a morning feed between 8AM and 12PM, and mothers were asked to feed their infant before coming to the hospital so that the milk sample was not from the first feed of the day. Initially, 5 ml of foremilk was hand-expressed before infants were allowed to suckle the nipple for 10 minutes; afterwards an expression of 5 ml of hindmilk was collected into the same 14 ml plastic test tube and was thoroughly mixed with the initial 5 ml foremilk sample. The samples were then aliquoted into multiple cryovials and frozen in a nitrogen environment -70° C.

Seven ml of maternal blood was obtained by venipuncture by trained technicians at the IMSS hospital at 18-22 weeks' gestation and at delivery. All samples were collected into tubes containing EDTA. Plasma and erythrocytes were separated by centrifugation at 800 G for 10 minutes at room temperature. Plasma and erythrocytes were then aliquoted into multiple cryovials, and were immediately frozen in a nitrogen environment and stored at -70° C.

The total fat from milk and plasma was extracted with a chloroform-methanol mixture (2:1). The yield was determined by gravimetry following a modification of the method described by Folch and was expressed as g/100 g of serum or milk (16). Fatty acids were derivatized using borum trifluride and were then extracted with pure hexane. The extracts were injected into a gas chromatograph Hewlett Packard (USA) Mod. 5890 Series II, using a 100 m length x 0.25 mm internal diameter Supelco SP 2560 (USA) column. The chromatographic peaks were identified using true reference standards for

37 fatty acids, (Supelco, St. Louis, MO, USA). The fatty acid content is expressed as percentage of total fat.

Statistical analysis

We conducted the analysis according to the intention-to-treat principle, and analyzed a random sub-sample of 174 human milk samples collected at 1 month post-partum. Continuous baseline characteristics and characteristics at birth were tested for normality, and group differences were calculated using T-Tests or Wilcoxon rank sum tests, as appropriate. Categorical characteristics were tested using Chi square tests. Extreme outliers in the main outcomes variables (breast milk PUFA concentrations) were deleted using the criteria: quartile 3 + (3*inter-quartile range). Between-group differences in between breast milk fatty acid concentrations were evaluated using either Wilcoxon rank sum tests or T-Tests, as appropriate. We computed Spearman correlation coefficients to describe the relationship between breast milk fatty acids and dietary intake and plasma DHA, EPA and AA levels. We conducted statistical analyses using TTEST, NPAR1WAY and CORR procedures in the Statistical System Software version 9.2, SAS Institute, Cary, North Carolina. Statistical significance was defined as $p \leq 0.05$.

Role of the funding source: The National Institutes of Health and The March of Dimes Foundation provided funding for the study. Martek Biosciences Corporation provided the DHA supplement. None of the sponsors had any role in the design or conduct of the study, or in the analysis and interpretation of the data.

RESULTS

CONSORT: Among the 1836 women screened, 1762 were eligible to participate in the study and 1094 were randomized to treatment (Figure 1). Of women randomized, 523 in the DHA group and 517 in the placebo group received at least one dose of treatment. Four hundred and eighty five women in the DHA group and 488 women in the placebo group completed treatment by remaining in the study through parturition. Among women who received treatment, 6.4% were lost to follow up before delivery. Select reasons for the 67 losses to follow up between receiving treatment and parturition included moving out of the area, refusal to participate, the husband did not want the woman to continue with the study and the supplement made the woman feel bad. We analyzed a random sub-sample of 174 breast milk samples obtained at 1 month.

Baseline characteristics: Baseline maternal characteristics for the sub-sample of 174 mothers who provided breast milk samples were similar between treatment groups (Table 1). Women were on average 26 years old and 20.6 weeks gestation, and 34.5% were primiparous at baseline. The women had completed an average of 11.6 years of school, and SES levels were similar across groups. Characteristics at delivery of the 174 infants whose mothers provided breast milk samples were also similar between groups. Daily dietary intake of the 174 women at 18-22 weeks gestation did not differ between treatment groups (Table 2).

Fatty acid composition of breast milk: Mean concentrations of both DHA and ALA in breast milk, expressed as % of total fatty acids, were higher in the DHA group, compared to the placebo group at 1 month post-partum (Table 3). Breast milk concentrations of other PUFAs did not differ between treatment groups. Correlations

between DHA, eicosapentaenoic acid (EPA), and arachidonic acid (AA) in breast milk and PUFA concentrations at baseline and delivery and dietary PUFA intake, by group, are shown in Table 4. Maternal plasma phospholipid DHA concentrations correlated positively with breast milk DHA at 1 month post-partum in both the placebo and DHA groups ($r=0.4$, $p<0.01$ for both treatment groups).

DISCUSSION

We showed that supplementing women with 400 mg/d of algae-derived DHA from 18-22 weeks' gestation through delivery resulted in significantly higher concentrations of both DHA and ALA in breast milk at 1 month post-partum. Breast milk concentrations of other PUFAs such as EPA and AA acid did not differ between groups at 1 month post-partum. We also found a positive correlation between DHA concentrations in maternal plasma and breast milk at 1 month post-partum. To our knowledge, this was the first study to examine the influence of algae-derived DHA supplementation in pregnancy alone on breast milk PUFA concentrations. Previous studies have examined the influence of maternal supplementation with n-3 PUFAs on PUFA breast milk content, but have focused on supplementation during lactation, supplementation during lactation and pregnancy, and/or supplementation with fish oil (DHA+EPA) rather than DHA alone.

Demonstrating the efficacy of algae-derived DHA in improving breast milk DHA concentrations has important implications; first, purified algae-derived DHA does not contain toxicants such as methylmercury, polychlorinated biphenyls (PCBs) and dioxins, and second, this source of DHA provides an option other than fish oil and fish for persons

who do not consume seafood. Also, the unique design of the current study allows us to examine the influence of prenatal DHA on breast milk DHA concentrations without having to consider EPA, the other n-3 PUFA contained in fish oil. This provides insight into the influence of an individual long-chain PUFAs, DHA, on outcomes of interest.

Although our findings show that DHA in pregnancy improved breast milk DHA concentrations, we do not know if this difference is biologically significant for the breastfed infant. However, since maternal DHA status declines in later pregnancy and early breastfeeding, any significant increase in breast milk DHA concentration should be deemed desirable. The concentration of breast milk DHA in the DHA group was 15% higher than in the placebo group and the effect size of the difference is approximately 0.5, indicating a medium effect size. Higher concentration of DHA in breast milk indicates better maternal DHA status, which could benefit both the infant and the mother. The infant could benefit because DHA in breast milk correlates positively with DHA nutriture of the infant, which has been shown to influence cognitive development (1). Additionally, improved DHA nutriture in the mother may provide benefits such as a lower risk of pre-eclampsia and post-partum depression (17-20).

We found higher concentrations of breast milk ALA in the DHA-supplemented group. This finding is inconsistent with other trials of fish oil supplementation in pregnancy and/or lactation, which overall have shown no influence of fish oil supplementation on breast milk ALA concentrations. Although saturation is one possible explanation of higher concentrations of ALA in the DHA-supplemented group, DHA conversion from ALA is inefficient and should not influence ALA concentrations. Maternal diet can also influence ALA and DHA concentrations in breast milk and

although diet did not differ between groups at baseline, we do not know at this time if diet differed at 3 months post-partum.

The present study was conducted in a developing country, in a population in which dietary DHA intake is lower than recommended (21). Our study setting and population were unique, as most of the n-3 PUFA supplementation trials have been conducted in Europe, Australia and the United States. The conduct of such trials in developing countries is especially important because DHA intake in many developing countries is particularly low due to the high cost of marine foods.

A recent study conducted in Australia by Dunstan *et al.* (2007) showed that supplementation of pregnant atopic women with 2.2g DHA and 1.1g EPA from 20 weeks' gestation through parturition resulted in higher breast milk DHA and EPA concentrations at 3 days and 6 weeks post-partum (5). By 6 months post-partum, there was no difference in breast milk PUFA composition between intervention and control groups. Similar to our study, women in this study were supplemented only during pregnancy; however, this study focused on atopic women, and the women were supplemented with fish oil. In another recent study conducted by Bergmann *et al.* German women were supplemented with either a multi-vitamin, a multi-vitamin plus fructo-oligosaccharides (prebiotics), or a multi-vitamin plus fructo-oligosaccharides plus 200 mg DHA from 21 through 36 weeks' gestation, and then from the second week after delivery through 3 months post-partum (22). This study showed that concentration of DHA in breast milk were twice as high in the DHA-supplemented women at 3 months post-partum, compared to the other two treatment groups. Dunstan *et al.* showed that supplementing pregnant women daily from 20 weeks gestation through delivery with fish

oil resulted in significantly higher breast milk DHA and EPA concentrations in the fish oil group at 3 days post-partum, compared to the control group (23). Helland *et al.* supplemented pregnant women with cod liver oil or corn oil from 17-19 weeks' gestation through 3 months post-partum and found higher concentrations of DHA in breast milk in the fish oil group, compared to the control group (24). Several studies that have focused on supplementation during lactation have shown higher breast milk DHA concentrations in the supplemented groups.

Jensen and colleagues supplemented 3 groups of lactating women from post-partum weeks 2 through 8 with either DHA derived from algae, high-DHA eggs, or marine oil (25). The three groups of supplemented women had higher breast milk DHA concentrations than the control group, and a dose-response was observed. The investigators noted a strong correlation between maternal plasma erythrocyte DHA concentration and breast milk DHA concentration. Lactating women were supplemented with two different doses of tuna oil or placebo in a study conducted by Hawkes *et al.*, and results showed that lactating women in the tuna oil groups had significantly higher DHA concentrations in their breast milk, compared to controls (26, 27). Similarly, Helland *et al.* showed that short-term supplementation with cod liver oil during lactation resulted in significantly higher DHA concentrations in breast milk, compared to placebo (28). Boris and colleagues supplemented Danish pregnant women with fish oil from the 30th week of pregnancy through 30 days post-partum, or through delivery (29). Women who consumed fish oil through 30 days post-partum had significantly higher breast milk DHA concentrations at 4, 16 and 30 days post-partum, compared to controls and to women who ceased supplementation at delivery. Contrary to our findings, women in this study who

received fish oil supplementation only during pregnancy did not have significantly higher concentrations of breast milk DHA at 1 month post-partum. Lauritzen and colleagues found that supplementing Danish women with fish oil within one week of delivery through 4 months post-partum resulted in higher breast milk DHA concentration, compared to placebo (30, 31). Additionally, infants of fish oil-supplemented mothers had higher DHA erythrocyte concentrations.

The aforementioned studies demonstrate that maternal intake of DHA, most often in the form of fish oil, in pregnancy and/or lactation results in improved maternal, breast milk and infant n-3 PUFA status. Overall, fish oil supplementation from mid-pregnancy through early lactation results in larger increases in DHA concentrations in breast milk than fish oil supplementation in pregnancy alone and higher doses of fish oil results in higher breast milk DHA concentrations. Boris *et al.* specifically demonstrated that women who were supplemented with fish oil in pregnancy and lactation had higher concentrations of breast milk DHA at 30 days postpartum (1.4 % total fatty acids) compared to women supplemented during pregnancy alone (0.6 % total fatty acids). Also, women who were supplemented with the high dose of 2.1 g DHA from 20 weeks' gestation through delivery had a 40% higher concentration of breast milk DHA at 6 weeks postpartum than controls, with an effect size of 1.1, showing that higher concentrations of dietary DHA result in a large effect size (5).

CONCLUSIONS

Our current study showed that supplementing women from 18-22 weeks' gestation through parturition with 400 mg DHA/day resulted in higher concentrations of breast

milk DHA and ALA, compared to placebo. Adequate DHA concentration in breast milk is essential for the exclusively breastfed infant because breast milk provides the sole source of nutrition for the infant. Higher concentration of DHA in breast milk indicates better maternal DHA status, which could benefit both the infant and the mother. The infant could benefit because DHA in breast milk correlates positively with DHA nutriture of the infant, which has been shown to influence cognitive development (1). Additionally, improved DHA nutriture in the mother may provide benefits such as a lower risk of pre-eclampsia and post-partum depression (17-20). Our study demonstrated that improvements in breast milk DHA status can be improved with a reasonable dose, equivalent to about 2 fish meals per week, of algae-derived DHA. Intake of DHA is inadequate in many populations, including pregnant women in the United States, and dietary guidance and/or supplementation might be indicated to ensure an adequate supply of DHA for both the mother and her infant.

Literature cited

1. Innis SM. Dietary omega 3 fatty acids and the developing brain. Brain Res. 2008;Spetember 9 [ahead of print].
2. Innis SM. Polyunsaturated fatty acids in human milk: an essential role in infant development. Adv Exp Med Biol 2004;554:27-43.
3. Innis SM. Dietary (n-3) fatty acids and brain development. J Nutr 2007;137(4):855-9.
4. Hanebutt FL, Demmelmair H, Schiessl B, Larque E, Koletzko B. Long-chain polyunsaturated fatty acid (LC-PUFA) transfer across the placenta. Clin Nutr 2008;27(5):685-93.
5. Dunstan JA, Mitoulas LR, Dixon G, Doherty DA, Hartmann PE, Simmer K, *et al.* The effects of fish oil supplementation in pregnancy on breast milk fatty acid composition over the course of lactation: a randomized controlled trial. Pediatr Res 2007;62(6):689-94.
6. Innis SM. Human milk: maternal dietary lipids and infant development. Proc Nutr Soc 2007;66(3):397-404.
7. Makrides M, Simmer K, Neumann M, Gibson R. Changes in the polyunsaturated fatty acids of breast milk from mothers of full-term infants over 30 wk of lactation. Am J Clin Nutr 1995;61(6):1231-3.
8. Bonham MP, Duffy EM, Wallace JM, Robson PJ, Myers GJ, Davidson PW, *et al.* Habitual fish consumption does not prevent a decrease in LCPUFA status in pregnant women (the Seychelles Child Development Nutrition Study). Prostaglandins Leukotrienes & Essential Fatty Acids 2008;78(6):343-50.
9. Makrides M, Gibson RA. Long-chain polyunsaturated fatty acid requirements during pregnancy and lactation. Am J Clin Nutr 2000;71(1 Suppl):307S-11S.
10. Hibbeln JR, Nieminen LRG, Blasbalg TL, Riggs JA, Lands WEM. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. American Journal of Clinical Nutrition 2006;83(6 Suppl):1483S-1493S.
11. Kris-Etherton PM, Taylor DS, Yu-Poth S, Huth P, Moriarty K, Fishell V, *et al.* Polyunsaturated fatty acids in the food chain in the United States. Am J Clin Nutr 2000;71(1 Suppl):179S-88S.
12. Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. American Journal of Clinical Nutrition 2007;85(6):1457-64.
13. Koletzko B, Cetin I, Brenna JT. Dietary fat intakes for pregnant and lactating women. Br J Nutr 2007;98(5):873-7.
14. Koletzko B, Lien E, Agostoni C, Bohles H, Campoy C, Cetin I, *et al.* The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. J Perinat Med 2008;36(1):5-14.
15. Parra MS, Schnaas L, Meydani M, Perroni E, Martinez S, Romieu I. Erythrocyte cell membrane phospholipid levels compared against reported dietary intakes of polyunsaturated fatty acids in pregnant Mexican women. Public Health Nutr 2002;5(6A):931-7.

16. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226(1):497-509.
17. Hibbeln JR. Seafood consumption, the DHA content of mothers' milk and prevalence rates of postpartum depression: a cross-national, ecological analysis. J Affect Disord 2002;69(1-3):15-29.
18. Su KP, Huang SY, Chiu TH, Huang KC, Huang CL, Chang HC, *et al.* Omega-3 fatty acids for major depressive disorder during pregnancy: results from a randomized, double-blind, placebo-controlled trial. J Clin Psychiatry 2008;69(4):644-51.
19. Freeman MP. Omega-3 fatty acids and perinatal depression: a review of theliterature and recommendations for future research. Prostaglandins Leukot Essent Fatty Acids 2006;75(4-5):291-7.
20. Olsen SF, Secher NJ. A possible preventive effect of low-dose fish oil on early delivery and pre-eclampsia: indications from a 50-year-old controlled trial. Br J Nutr 1990;64(3):599-609.
21. Parra-Cabrera S, Flores R, Stein A, Martorell R, Ramakrishnan U. Dietary Intakes of Omega-3 Fatty acids in Mexican Pregnant Women In: 10th European Nutrition Conference; 2006; Paris; 2006.
22. Bergmann RL, Haschke-Becher E, Klassen-Wigger P, Bergmann KE, Richter R, Dudenhausen JW, *et al.* Supplementation with 200 mg/day docosahexaenoic acid from mid-pregnancy through lactation improves the docosahexaenoic acid status of mothers with a habitually low fish intake and of their infants. Ann Nutr Metab 2008;52(2):157-66.
23. Dunstan JA, Roper J, Mitoulas L, Hartmann PE, Simmer K, Prescott SL. The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition. Clin Exp Allergy 2004;34(8):1237-42.
24. Helland IB, Saugstad OD, Saarem K, Van Houwelingen AC, Nylander G, Drevon CA. Supplementation of n-3 fatty acids during pregnancy and lactation reduces maternal plasma lipid levels and provides DHA to the infants. J Matern Fetal Neonatal Med 2006;19(7):397-406.
25. Jensen CL, Maude M, Anderson RE, Heird WC. Effect of docosahexaenoic acid supplementation of lactating women on the fatty acid composition of breast milk lipids and maternal and infant plasma phospholipids. Am J Clin Nutr 2000;71(1 Suppl):292S-9S.
26. Hawkes JS, Bryan DL, Makrides M, Neumann MA, Gibson RA. A randomized trial of supplementation with docosahexaenoic acid-rich tuna oil and its effects on the human milk cytokines interleukin 1 beta, interleukin 6, and tumor necrosis factor alpha. Am J Clin Nutr 2002;75(4):754-60.
27. Hawkes JS, Bryan DL, Neumann MA, Makrides M, Gibson RA. Transforming growth factor beta in human milk does not change in response to modest intakes of docosahexaenoic acid. Lipids 2001;36(10):1179-81.
28. Helland IB, Saarem K, Saugstad OD, Drevon CA. Fatty acid composition in maternal milk and plasma during supplementation with cod liver oil. Eur J Clin Nutr 1998;52(11):839-45.
29. Boris J, Jensen B, Salvig JD, Secher NJ, Olsen SF. A randomized controlled trial of the effect of fish oil supplementation in late pregnancy and early lactation on the n-3 fatty acid content in human breast milk. Lipids 2004;39(12):1191-6.

30. Lauritzen L, Jorgensen MH, Mikkelsen TB, Skovgaard M, Straarup EM, Olsen SF, *et al.* Maternal fish oil supplementation in lactation: effect on visual acuity and n-3 fatty acid content of infant erythrocytes. Lipids 2004;39(3):195-206.
31. Lauritzen L, Jorgensen MH, Olsen SF, Straarup EM, Michaelsen KF. Maternal fish oil supplementation in lactation: effect on developmental outcome in breast-fed infants. Reprod Nutr Dev 2005;45(5):535-47.

TABLE 1 **Selected characteristics at 18-22 weeks' gestation and at delivery of 174 women who provided breast milk samples at 1 month post-partum**

	DHA Group (*n*=87)	**Placebo Group (*n*=87)**	***P*-value**
At Baseline			
Age, mean (SD), y	26.4 (5.1)	25.8 (4.8)	0.42
Gestational age, mean (SD), wk	20.7 (2.0)	20.6 (1.9)	0.74
Primipara, %	32.2	36.8	0.52
Parity, mean (SD)	2.1 (1.0)	2.0 (1.1)	0.57
Weight, mean (SD), kg	61.1 (9.9)	62.0 (10.0)	0.56
Height, mean (SD), cm	154.4 (5.9)	155.6 (5.4)	0.16
BMI, mean (SD), kg/m^2	25.6 (3.6)	25.6 (3.7)	0.95
Schooling, mean (SD), y	11.4 (3.6)	11.7 (3.4)	0.66
SES, mean (SD), PCA	-0.13 (1.0)	-0.08 (1.0)	0.76
At Delivery			
Gestational age, mean (SD), wk	39.1 (2.0)	39.4 (1.8)	0.34
Premature, %, <37 wk	8.1	7.0	0.79
Birth weight, mean (SD), g	3246 (445.5)	3254 (455.4)	0.90

PCA indicates principal components analysis. Chi square tests, T-tests or Wilcoxon rank sum tests were used to assess significant differences between groups

TABLE 2 **Dietary intakes in women 18-22 weeks' gestation evaluated by a food frequency questionnaire**

	DHA Group (*n*=87)	Placebo Group (*n*=87)	*P*-value
Energy, median (Q1, Q3), Kcal/d	3197 (2722, 4014)	3444 (2901, 4170)	0.41
Total fat, median (Q1, Q3), g/d	89.3 (71.2, 121.8)	97.4 (79.3, 121.8)	0.51
Saturated fat, median (Q1, Q3), g/d	28.1 (21.2, 37.9)	29.3 (24.4, 37.7)	0.36
Monounsaturated fatty acids, median (Q1, Q3), g/d	32.3 (26.0, 45.9)	34.8 (26.9, 45.7)	0.77
Polyunsaturated fatty acids, median (Q1, Q3), g/d	19.2 (14.4, 26.1)	20.6 (15.9, 26.4)	0.48
∑n-6 fatty acids, median (Q1, Q3), g/d	18.3 (13.0, 23.4)	18.7 (14.5, 23.4)	0.57
18:2n-6 (LA) , median (Q1, Q3), g/d	18.2 (12.9, 23.0)	18.5 (14.4, 23.3)	0.44
20:4n-6 (AA) , median (Q1, Q3), g/d	0.14 (0.09, 0.19)	0.13 (0.10, 0.18)	0.79
∑n-3 fatty acids, median (Q1, Q3), g/d	1.51 (1.07, 2.03)	1.60 (1.10, 2.34)	0.12
18:3n-3 (ALA) , median (Q1, Q3), g/d	1.35 (1.02, 1.85)	1.54 (0.99, 2.17)	0.30
20:5n-3 (EPA) , median (Q1, Q3), g/d	0.02 (0.01, 0.03)	0.02 (0.01, 0.04)	0.21
22:6n-3 (DHA) , median (Q1, Q3), g/d	0.06 (0.03, 0.08)	0.05 (0.04, 0.10)	0.43
n-6:n-3, median (Q1, Q3), ratio of n-6 to n-3 fatty acids	12.6 (8.7, 16.7)	11.9 (9.6, 14.9)	0.40

Q1, Q3 indicates quartile 1 and quartile 3. T-tests or Wilcoxon rank sum tests, as appropriate, were used to assess differences between groups

TABLE 3 **Mean fatty acid concentrations in breast milk (% of total fatty acids) measured 1 month post-partum**

	% of total fatty acids in breast milk at 1 month post-partum		
Fatty acid	**DHA Group (*n*=87)**	**Placebo Group (*n*=87)**	***P*-value**
Saturated, mean (SD)	40.41 (4.61)	41.46 (5.05)	0.15
Unsaturated, mean (SD)	39.62 (3.05)	39.51 (3.14)	0.82
∑n-6, mean (SD)	18.06 (3.15)	17.24 (3.52)	0.11
18:2n-6 (LA), mean (SD)	16.53 (2.98)	15.75 (3.41)	0.11
20:2n-6, mean (SD)	0.45 (0.10)	0.47 (0.12)	0.88
20:3n-6, mean (SD)	0.43 (0.09)	0.45 (0.11)	0.14
20:4n-6 (AA), mean (SD)	0.41 (0.12)	0.43 (0.12)	0.23
∑n-3, mean (SD)	1.92 (0.57)	1.79 (0.58)	0.16
18:3n-3 (ALA), mean (SD)	1.38 (0.47)	1.24 (0.46)	0.02
20:5n-3, (EPA) mean (SD)	0.14 (0.13)	0.16 (0.15)	0.25
22:5n-3, mean (SD)	0.10 (0.04)	0.12 (0.05)	0.06
22:6n-3, (DHA) mean (SD)	0.20 (0.06)	0.17 (0.07)	<0.01
n-6:n-3, mean (SD), ratio of n-6 to n-3 fatty acids	9.88 (2.00)	10.12 (2.12)	0.43

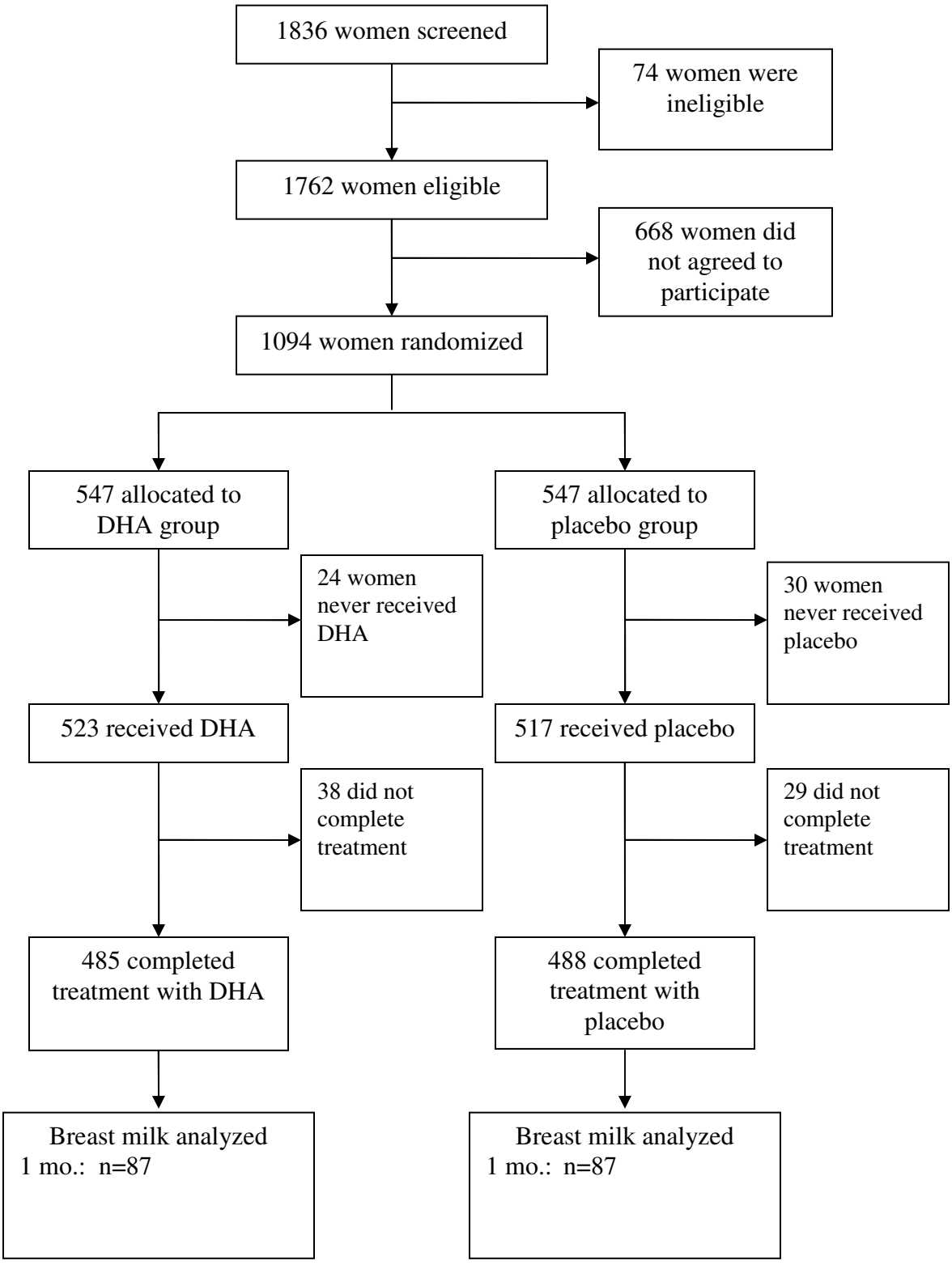
T-tests or Wilcoxon rank sum tests, as appropriate, were used to assess differences between groups.

TABLE 4 **Spearman rank-order correlation between breast milk DHA, EPA and AA concentration at 1 month post-partum and maternal intake of fatty acids in pregnancy, plasma phospholipid fatty acid concentrations at baseline, delivery and 1 month post-partum, by treatment group**

Breast milk PUFAs at 1 month PP (% of total fatty acids)	**Maternal DHA plasma baseline**	**Maternal DHA plasma delivery**	**Maternal DHA plasma 1 month PP**	**Dietary DHA intake base-line**	**Maternal AA plasma baseline**	**Maternal AA plasma delivery**	**Maternal AA plasma 1 month PP**	**Dietary AA intake base-line**	**Maternal EPA plasma baseline**	**Maternal EPA plasma delivery**	**Maternal EPA plasma 1 month PP**	**Dietary EPA intake base-line**
Placebo group (n=87)												
DHA	0.06	0.06	0.41[1]	0.22[2]	0.31[2]	0.22	0.22[2]	0.24	0.08	0.15	-0.06	0.22[2]
EPA	-0.13	0.00	-0.05	-0.12	0.12	0.04	0.08	-0.19	0.04	0.15	-0.03	-0.12
AA	-0.03	0.04	0.40[1]	0.18	0.34[1]	0.42[1]	0.54[1]	0.24[2]	0.23	0.18	-0.06	0.12
DHA group (n=86)												
DHA	-0.08	-0.00	0.41[1]	-0.05	-0.12	0.02	0.27[2]	-0.10	0.11	0.07	0.10	-0.07
EPA	-0.34	-0.19	0.11	-0.10	-0.20	0.11	0.08	0.00	0.32[2]	0.04	-0.04	-0.12
AA	0.01	-0.10	0.30[1]	-0.09	0.10	0.26[2]	0.47[1]	0.00	0.32[2]	0.04	0.05	-0.12

[1]*p*< 0.01; [2]*p*<0.05

Figure 1. Trial profile



CHAPTER 6

PRENATAL DOCOSAHEXAENOIC ACID SUPPLEMENTATION AND INFANT MORBIDITY: A DOUBLE-BLIND RANDOMIZED CONTROLLED TRIAL IN MEXICO

Prenatal docosahexaenoic acid supplementation and infant morbidity: a double-blind randomized controlled trial in Mexico[1]

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Running header: DHA PUFA prenatal supplementation infant illness

Abstract

Background: Long chain PUFAs such as docosahexaenoic acid (DHA) influence immune function and inflammation; however, the influence of maternal DHA intake on infant morbidity is unknown. **Objective**: We investigated the effect of prenatal DHA intake on infant morbidity at 1, 3 and 6 months. **Design:** In a double-blind RCT conducted in Mexico, pregnant women were supplemented daily with 400mg DHA or placebo from 18-22 weeks' gestation through parturition. At 1, 3 and 6 months caregivers reported occurrence and duration of illness symptoms in the preceding 15d. **Results:** Among 1040 women randomized to treatment, morbidity data were available at 1, 3 and 6 months for 849, 834 and 834 infants, respectively. The occurrence of specific illness symptoms did not differ between the groups at 1, 3 and 6 months; however, the occurrence of a combined measure of cold symptoms was lower in the DHA group (OR=0.76;95% CI=0.58,1.00) at 1 month. Duration of illness varied between groups at 1, 3 and 6 months. At 1 month, the DHA group experienced 26, 15 and 30% shorter duration of cough (P<0.01), phlegm (P=0.01) and wheezing (P<0.01), respectively, but had 22% longer duration of rash (P<0.01). At 6 months, infants exposed to DHA experienced 20, 13, 54, 22, and 25% shorter duration of fever (P=0.03), nasal secretion (P=0.02), difficulty breathing (P=0.02), rash (P<0.01) and "other illness" (P=0.01), respectively, but longer duration of vomiting (P<0.01). **Conclusion:** DHA intake during pregnancy decreased the occurrence of colds in 1-month-olds, and generally shortened illness symptom duration in 1- and 6-month-olds.

Introduction

Long chain polyunsaturated fatty acids (LCPUFAs), particularly docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), are integral for fetal neural and retinal development and accrete extensively in these tissues in the last trimester of pregnancy (1-3). LCPUFAs also serve as structural constituents of cell membranes and act as signaling molecules. LCPUFAs modulate immune function and inflammation through modification of the LCPUFA content of cell membranes and subsequent alteration in eicosanoid synthesis and lipid raft structure, alteration of cell membrane fluidity, regulation of nuclear transcription factors, modification of cytokine production and modification of cell adhesion molecule expression (4-12). Trials in adults and children focusing on the effect of supplementation with fish oil or LCPUFAs on immune function and inflammation have shown inconsistent results; some trials have shown no effect of LCPUFA supplementation on illness, while others have shown a decreased incidence or severity of illness (13-18). The specific clinical effects of n-3 PUFA supplementation in pregnancy on child morbidity are yet unknown; however, this question is important because the infant immune system begins to develop *in utero* and DHA intake is often inadequate in many populations of pregnant women.

Dietary intake of n-6 PUFAs such as AA in numerous populations worldwide is adequate while dietary intake of n-3 PUFAs such as DHA is often insufficient, especially in pregnant women whose stores are mobilized for fetal growth (19, 20). Maternal DHA status in pregnancy and breast milk PUFA levels correspond positively with infant status (21, 22). Further, n-3 PUFA supplementation improves maternal status in pregnancy, breast milk LCPUFA concentrations and infant LCPUFA status (23-27).

Infants are a vulnerable population born with immature immune systems and are unable to mount cell-mediated immune responses comparable to those mounted by adults, rendering them especially susceptible to infection (28, 29). Worldwide, >50% of mortality in children <5 is caused by infection; hence, healthy infant immune function is essential for child health and survival (30, 31). Numerous studies that have examined the potential influence of *in utero* and early postnatal nutritional insults on later immune function have shown varied results; however, compelling evidence that prenatal nutrition influences later immune function exists (32, 33). For example, a longitudinal study in the Philippines showed that, when challenged with vaccination, adolescents who had been prenatally undernourished had a lower probability of mounting an adequate immune response compared with adolescents who were adequately nourished during fetal development (33). This and other studies show that *in utero* insults such as maternal nutritional deficiencies potentially influence the development of the fetal and subsequent infant immune system (34, 35).

The influence of perinatal maternal LCPUFA nutriture on infant immune function is unclear; hence, we investigated the effect of DHA intake in the second half of pregnancy on morbidity in Mexican infants. Specifically, we investigated the influence of daily DHA supplementation (400 mg/day) from gestation weeks 18-22 through parturition on care giver-reported infant illness symptoms.

Methods

Participants and setting: Participants were recruited at the Mexican Institute of Social Security (Instituto Mexicano del Seguro Social or IMSS) General Hospital I, a

large hospital located in Cuernavaca, Mexico, and 3 small health clinics within the IMSS system in Cuernavaca during routine prenatal care visits between February 2005 and February 2007. The IMSS health care system provides employed persons access to medical care. Women were considered for inclusion in the study if they were in gestation weeks 18-22, planned to predominantly breastfeed for at least 3 months, were 18-35 years old, planned to deliver at the IMSS General Hospital I in Cuernavaca and planned to live in the area for 2 years after delivery. Women were excluded if any of the following criteria were present: 1) high risk pregnancy, 2) lipid metabolism or absorption disorders, 3) regular intake of fish oil or DHA supplements, or 4) chronic use of certain medications, such as medications for epilepsy. Written informed consent was obtained from each participant after a thorough explanation of the study details, and participants were free to withdraw from the study at any time.

Intervention: Women were assigned to receive either 400 mg DHA or placebo daily, from gestation weeks 18-22 through parturition. The DHA capsules contained 200 mg DHA each derived from an algal source (Martek Biosciences Corporation, Columbia, MD). The placebo capsules contained olive oil, and were similar in appearance and taste to the DHA capsules. Fieldworkers visited the women's homes weekly to deliver a new bottle of 14 capsules, and compliance was monitored by counting any remaining pills and through interviews with participants.

Blinding: All participants and members of the study team were blinded to the treatment scheme throughout the intervention period of the study. The blinding code was placed in sealed envelopes at the beginning of the study, and these envelopes were held by investigators at Emory who were not involved in the study. Data were un-blinded for

the analytical study team after the last baby in the study was born and had completed 6 months of age, at which time participants were no longer taking supplements. As the study is ongoing for follow-up of child development, participants and field workers in Mexico remain blinded to the treatment allocation.

Randomization: All eligible women were randomized to either the treatment or the control group using a computer-generated list created by the study biostatistician at Emory University. Women were randomized to either the treatment or the control group using the block randomization method to randomly create balanced replication of 4 treatments (2 colors for DHA and 2 for control) using a block size of 8. Success of randomization was assessed by comparison of a variety of baseline maternal characteristics in the two treatment groups.

Outcomes: Child morbidity information was captured using a 15-day recall questionnaire when mothers brought their infants to the hospital for study visits at 1, 3 and 6 months. Mothers were asked questions about the occurrence of illness symptoms, duration of symptoms and whether they sought medical care for their infant's symptoms. For example, mothers were asked "In the past 15 days, has your child experienced fever"; mothers who responded affirmatively were then asked "on what date did the symptoms begin, and on what date did they end" and "did you seek medical care for this illness?"

We created a variable of aggregate symptoms including phlegm, nasal congestion, nasal secretion, or cough, which we classified as an upper respiratory tract infection (henceforth denoted as "cold") (36). Mothers were asked at the 1 and 3 month study visits if they breast fed their child (yes/no); hence, breast feeding information is available through 3 months post-partum.

Ethics: The study protocol and all informed consent documents were approved by Emory University's Human Investigations Board and by the Instituto Nacional de Salud Pública (INSP) and the IMSS General Hospital I's Human Subjects Boards. The welfare of the subjects was monitored by an external Data Safety Monitoring Committee. This study is an ongoing collaborative project between the INSP in Cuernavaca, Mexico and Emory University in Atlanta, Georgia, and is a registered clinical trial (registered at INSP in Mexico: #CI-011, and at clinicaltrials.gov: NCT00646360).

Role of the funding source: The National Institutes of Health and The March of Dimes Foundation provided funding for the study. Martek Biosciences Corporation provided the DHA supplement and placebo. None of the sponsors had any role in the design or conduct of the study, or in the analysis and interpretation of the data.

Statistical analysis: We conducted the analysis according to the intention-to-treat principle, and analyzed the entire study population with available data at 1, 3 and 6 months post-partum. Continuous baseline characteristics and characteristics at birth were tested for normality, and group differences were calculated using T-Tests or Wilcoxon rank sum tests. Differences between categorical characteristics were tested using Chi square tests. Differences between reported illness symptoms in the previous 15 days, care seeking and severity of diarrhea were tested using Chi square and Fisher's exact tests, and unadjusted logistic regression models, which generated odds ratios (OR) and 95% CIs. Differences in duration of child illness were assessed using unadjusted Poisson regression models that included only children who experienced the specific illness symptom being tested and provided risk ratios (RR) and 95% CIs. If duration of illness for a reported symptom was missing, we did not include that child in that particular

analysis. We did not control for breast feeding status, or any demographic or outcome measures such as SES and birth weight because these characteristics were similar in the two groups. Using logistic and Poisson regression, we also conducted a secondary analysis testing whether breast feeding alone influenced occurrence and duration of illness. Statistical significance was defined as $P \leq 0.05$. We conducted statistical analyses using LOGISTIC and GENMOD procedures in the Statistical System Software version 9.2, SAS Institute, Cary, North Carolina.

Results

CONSORT: Figure 1 shows the trial profile. Among the 1836 women screened, 1762 were eligible to participate in the study and 1094 were randomized to treatment (547 to the DHA group and 547 to the placebo group). Of women randomized, 523 in the DHA group and 517 in the placebo group received at least one dose of treatment (54 women never received a supplement). Four hundred and eighty five women in the DHA group and 488 women in the placebo group completed treatment by remaining in the study through parturition. Among women who received treatment, 6.4% were lost to follow up before delivery. Select reasons for the 67 losses to follow up between receiving treatment and parturition included moving out of the area, refusal to participate, the husband did not want the woman to continue with the study and the supplement made the woman feel bad. Further losses to follow up occurred at 1, 3 and 6 months post-partum; 851, 835 and 834 women attended their 1, 3 and 6 month post-partum visits, respectively. Overall, loss to follow up of women who initially received treatment through 6 months post-partum was 19.8%, and was similar between the treatment groups

(P=0.7). The compliance rate, measured as the proportion of capsules consumed of the capsules distributed, was high (>94%) and was similar between the two groups (P=0.6).

Baseline demographics: Baseline maternal characteristics for the 851 mothers who attended the 1 month post-partum visit were similar between treatment groups (**Table 1**). At randomization women were on average 26 years old and 20.5 weeks' gestation. Approximately 38% were primiparous. The women completed an average of 12 years of school, and SES levels were similar across groups. Characteristics at delivery of the 851 infants whose mothers attended the 1 month post-partum visit were also similar across groups (**Table 2**). Fifty three percent of the children were male; birth weight and length were, on average, 3224.2 g and 50.4 cm, respectively. Average gestational age at birth was 39 weeks, and 9% of babies were premature (<37 weeks' gestation).

Numbers analyzed: We assessed infant morbidity at 1, 3 and 6 months of age. Morbidity data were available for 849, 834 and 834 infants, respectively at 1, 3 and 6 months; however, duration of illness was not available for each case of reported illness.

Outcomes: History of specific illness symptoms such as cough and nasal congestion in the previous 15 days did not differ significantly between the treatment groups at 1, 3 or 6 months; however, the higher occurrence of nasal secretion in the placebo group at 1 month approached significance (**Table 3**). For our combined measure of upper respiratory tract infection ("cold") symptoms, the DHA group reported a lower occurrence of cold symptoms than the placebo group at 1 month (OR=0.76, 95% CI 0.58, 1.00). Percentage time ill from any illness in the previous 15 days was similar between groups at 1 and 6 months; however, at 3 months, children in the DHA group experienced

14% less time ill than children in the placebo group (RR=0.86, 95% CI: 0.80, 0.93). At 3 months, the DHA group continued to report fewer cold symptoms in the previous 15 days; however, this difference was not statistically significant (OR=0.77, 95% CI=0.59, 1.02). Many symptoms, such as cough, phlegm, nasal secretion and fever, occurred more commonly in the 6-month-olds than in the 1 and 3-month-olds, whereas rash occurred more commonly in the 1-month-olds.

There was no difference in severity of diarrhea, measured by presence of blood or mucus in the stool or liquid stool consistency, between groups at 1, 3 and 6 months (using Chi square and Fisher's exact tests, as appropriate). Forty two, 71 and 10% of infants who had diarrhea had mucus in their stool, liquid stool or bloody stool, respectively, at 1 month. At 3 months, 71, 79 and 5% of infants who had diarrhea had mucus in their stool, liquid stool or bloody stool, respectively. Finally, at 6 months, 52, 73 and 5% of infants who had diarrhea had mucus in their stool, liquid stool or bloody stool, respectively.

Care seeking for illness symptoms, used as a measure of perceived illness severity, did not differ significantly between groups and was, overall, high among families with ill infants. At 1 month, 83, 62, 55, 46, 53, 44, 50, 61, 30, and 85% of mothers sought care if their infants had fever, cough, phlegm, nasal congestion, nasal secretion, wheezing, vomiting, diarrhea, rash or "other illness", respectively. At 3 and 6 months, >60% of caregivers sought care if their infant had fever, cough, phlegm, nasal congestion, wheezing, vomiting, diarrhea, or "other illness". At 1, 3 and 6 months, examples of "other illness" include conjunctivitis, ear infection, sore throat and reflux. All caregivers (100%) sought care if their infant had difficulty breathing, and care seeking for wheezing was higher in 3- and 6- month-olds.

At 1 month, median duration of cough, phlegm, nasal congestion, nasal secretion, rash and "other illness" was 4, 5, 5, 3, 9 and 11 days, respectively (**Table 4**). Children experienced longer duration of rash and "other illness" at 1 month compared to 3 and 6 months, while 6- month-olds experienced longer duration of cough. Median duration of wheezing at 1, 3 and 6 months was 4.0 days. **Table 5** shows the relative risk of longer duration of illness symptoms at 1, 3 and 6 months, in children who experienced illness. At 1 month, the DHA group experienced 26, 15 and 30% shorter duration of cough, phlegm and wheezing, respectively, but had 22% longer duration of rash, as compared to placebo. At 3 months, the DHA group experienced 23% shorter duration of "other illness" and 15% longer duration of nasal congestion. Six-month-olds experienced 20, 13, 54, 22, and 25% shorter duration of fever, nasal secretion, difficulty breathing, rash and "other illness", respectively, and longer duration of vomiting.

Secondary analysis: We did not control for breast feeding in our main analysis because breast feeding status at 1 and 3 months did not differ between the two groups; however, breast feeding alone was protective against various symptoms. In most cases, we found no significant interactions between breast feeding and group; hence, models were run without interaction terms. At 1 month, breast fed children were less likely to experience difficulty breathing (OR=0.22; 95% CI=0.068, 0.682) and had a shorter duration of wheezing (risk ratio=0.71; 95% CI=0.50, 1.00) and difficulty breathing (risk ratio=0.26; 95% CI=0.17, 0.39). Breast fed 3-month-olds had a shorter duration of fever (risk ratio=0.61; 95% CI=0.44, 0.85), wheezing (risk ratio=0.68; 95% CI=0.53, 0.86) and vomiting (risk ratio=0.44; 95% CI=0.30, 0.64).

Discussion

Key results: We found that DHA intake (400 mg/day) from 18-22 weeks' gestation through parturition reduced the occurrence of colds in 1-month-old Mexican infants and influenced illness duration at 1, 3 and 6 months. At 1 month, infants in the DHA group experienced shorter duration of cough, phlegm and wheezing, but longer duration of rash. Three-month-old infants in the DHA group experienced shorter duration of "other illness" such as ear infections and sore throats, but longer duration of nasal congestion. At 6 months, infants in the DHA group experienced shorter duration of nasal secretion, difficulty breathing, fever, rash and "other illness", but longer duration of vomiting. At 3 months, children in the DHA group experienced 14% less time ill from any illness than children in the placebo group. In general, the infants in the DHA group experienced shorter duration of illness symptoms. Rash at 1 month and vomiting at 6 months were longer in duration in the DHA group compared to the placebo group. Vomiting can be caused by either a viral or a bacterial illness, or by gastrointestinal upset ("spitting up") due to sensitive stomach or acid reflux. Allergic children might develop rash defined as atopic dermatitis, whereas other children might develop simple diaper rash caused by sensitive skin, diet, or wearing a soiled diaper for too long. Longer duration of these symptoms at certain time points was unexpected and we have no explanation for their occurrence; however, overall, children in the DHA group were generally healthier. A high proportion of mothers sought care for their infant's illness symptoms, which we believe was due to the free access to health care within the IMSS hospital system. To our knowledge, this is the first study to examine the influence of DHA in pregnancy on infant illness occurrence and duration.

Limitations: Illness symptoms were reported by the mother and not confirmed by a health care professional; therefore, it was not possible to distinguish between, for example, rash due to common diaper irritation and rash classified as clinical atopic dermatitis. Self-reporting of morbidity data using recall questionnaires can introduce bias due to memory loss; for example, Ramakrishnan et al. showed a rate of underreporting of diarrhea as high as 45% on days 7-13 of a morbidity recall (37). This finding was consistent with other studies that demonstrated underreporting of illness using recall instruments (38, 39). We saw no indication that illness was under- or over-reported by a particular treatment group in the trial since treatment groups shared similar baseline characteristics and double-blinding was maintained throughout the study (40). Additionally, the use of health calendars/diaries and the high rate of literacy in this study likely greatly aided in maternal recall of child illness during interviews (41).

To our knowledge, only one other randomized, controlled trial examining the effect of n-3 PUFA supplementation in pregnancy on infant immune function has been conducted (18, 42, 43). That study, in which atopic pregnant women were supplemented with fish oil (3.7 g n-3 PUFAs per day) from 20 weeks' gestation through delivery, showed that fish oil supplementation lowered neonatal cord blood concentrations of the plasma cytokine IL-13, modified neonatal neutrophil production, did not influence neonatal IgE concentrations and lowered the risk of a positive reaction to a specific skin prick test at 1 year. Children of women in the fish oil group tended to show less severe atopic dermatitis. The investigators also found a positive correlation between maternal n-3 PUFA concentration and IgA concentration in breast milk measured at 3 days post-partum, indicating that n-3 PUFAs in pregnancy might also modulate infant immune

function by means of breast milk immunoglobulin protection (44). Unlike our study, that study included only atopic mothers, the primary outcome of interest was allergic immune response, and the supplement was fish oil, rather than DHA. Our findings complement these findings because we examined clinical outcomes in the infants and also found a potential influence of n-3 PUFAs in pregnancy on infant immune function. Although our study was not originally designed to test the influence of DHA specifically in atopic women, the prevalence of atopy in the mothers was similar between the two treatment groups (33% based on specific IgE measurement), and secondary analyses are underway to examine the effects of DHA supplementation by maternal atopy status.

Several studies have evaluated the influence of essential fatty acid (EFA) supplementation in infancy and childhood on illness occurrence and severity and biological immunity outcomes (14, 15, 34, 45, 46). One study in which 1342 infants were assigned to receive either regular formula or formula supplemented with DHA and AA showed that the infants consuming the EFA-supplemented formula had a decreased incidence of bronchiolitis/bronchitis at 5, 7 and 9 months of age (15). A randomized trial in which term infants were given either regular formula or formula supplemented with long chain PUFAs showed that the LCPUFAs influenced the presence and function of infant immune cells such as CD3+ and CD44++ (45). A double-blind trial in which 5-7 year-olds were given a dietary supplement containing AA and DHA or placebo for 7 months showed alterations in immune cell phenotypes in the LCPUFA group, compared to placebo (46). A systematic review of randomized controlled trials of PUFA supplementation of infant formula in preterm babies found no difference in rates of sepsis or necrotizing enterocolitis between the two groups (16). In a double-blind study, dietary

EFA supplementation in children aged 36-49 months at risk of recurrent respiratory infections reduced the number of infective episodes, days with fever and days of school missed (14). Dietary n-3 PUFA intake has been shown to influence innate immune function in adults, as evidenced by a study in which men supplemented daily with the long chain n-3 PUFA eicosapentaenoic acid for 12 weeks exhibited modified neutrophil respiratory burst and prostaglandin production by mononuclear cells (17). Additionally, a 10 year follow-up study of >38,000 male U.S. health professionals showed that community-acquired pneumonia risk was lower in men who consumed higher amounts of dietary n-3 PUFAs (13). Our findings that DHA influenced respiratory outcomes such as wheezing are consistent with several studies focusing on n-3 PUFAs and atopic disorders. Overall, these supplementation trials in children and adults have shown varied results; however, several trials present compelling evidence of an influence of n-3 PUFAs on morbidity and/or biological immune measures.

Generalizability: Results from our study could be generalized to other populations of pregnant women of middle-to-lower socioeconomic status who have similarly low dietary DHA intakes. A study of pregnant women in Mexico City showed that their mean daily intake of DHA was 0.14 grams per day, well below the recommended intake and similar to intake in many populations around the world (19, 44). Intake of omega-3 PUFAs in the United States is an estimated 1.6 grams per day, and intake of DHA is approximately 0.1-0.2 grams per day (48). A study conducted in Canada indicated that a sample of pregnant women consumed 0.08 grams of DHA per day (49). Worldwide estimates of omega-3 PUFA intake show that the majority of countries' populations have lower than recommended intake of DHA, with the exception

of countries such as Japan, Iceland, Norway, Finland and Malaysia where fish is consumed frequently (19). Our study population has lower infectious disease rates than many developing countries, in particular for diarrhea and fever. Therefore, our results can only be generalized to population with similar nutritional status and disease rates.

Our findings contribute to the mounting evidence that perinatal n-3 PUFA nutrition can influence the development of fetal and neonatal immune function. We demonstrated that DHA supplementation in pregnancy influenced illness symptom duration in Mexican infants. n-3 PUFAs could potentially influence fetal and infant immune function development *in utero*, via breast milk, or a combination of both the prenatal and perinatal factors (50). Further studies designed specifically to examine the influence of perinatal n-3 PUFA nutriture on infant immune function, including both biological and clinically relevant outcomes, are necessary to evaluate the potential value of dietary modification or n-3 PUFA supplementation during pregnancy and/or during lactation.

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The authors' contributions were as follows: BIK: prepared the draft manuscript and conducted all statistical analyses, involved in the design of the morbidity-specific aspect of the study, ADS: reviewed the accuracy of the statistical methods, involved in the conception and design of the parent study, RM: involved in the conception and design

of the parent study, SPC: involved in the conception and design of the parent study and study supervision in Mexico, IR: involved in design of the morbidity-specific aspect of the study, UR: responsible for the conception and design of the parent study, acts as principal investigator of the study, obtained funding for the study. All authors critically reviewed the drafts and approved the final version of the manuscript. None of the authors had a personal or financial conflict of interest.

Literature cited

1. McCann JC, Ames BN. Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals.[see comment]. American Journal of Clinical Nutrition 2005;82:281-95.

2. Uauy R, Mena P, Rojas C. Essential fatty acids in early life: structural and functional role. Proc Nutr Soc 2000;59:3-15.

3. Al MD, van Houwelingen AC, Hornstra G. Long-chain polyunsaturated fatty acids, pregnancy, and pregnancy outcome. Am J Clin Nutr 2000;71:285S-91S.

4. Calder PC. Dietary modification of inflammation with lipids. Proc Nutr Soc 2002;61:345-58.

5. Calder PC, Grimble RF. Polyunsaturated fatty acids, inflammation and immunity. Eur J Clin Nutr 2002;56 Suppl 3:S14-9.

6. Yaqoob P. Lipids and the immune response. Curr Opin Clin Nutr Metab Care 1998;1:153-61.

7. Yaqoob P. Fatty acids and the immune system: from basic science to clinical applications. Proc Nutr Soc 2004;63:89-104.

8. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. Lipids 2001;36:1007-24.

9. Calder PC. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. Lipids 2003;38:343-52.

10. Calder PC. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 2007;77:327-35.

11. Deckelbaum RJ, Worgall TS, Seo T. n-3 fatty acids and gene expression. Am J Clin Nutr 2006;83:1520S-1525S.

12. Gottrand F. Long-chain polyunsaturated Fatty acids influence the immune system of infants. J Nutr 2008;138:1807S-1812S.

13. Merchant AT, Curhan GC, Rimm EB, Willett WC, Fawzi WW. Intake of n-6 and n-3 fatty acids and fish and risk of community-acquired pneumonia in US men. Am J Clin Nutr 2005;82:668-74.

14. Venuta A, Spano C, Laudizi L, Bettelli F, Beverelli A, Turchetto E. Essential fatty acids: the effects of dietary supplementation among children with recurrent respiratory infections. J Int Med Res 1996;24:325-30.

15. Pastor N, Soler B, Mitmesser SH, Ferguson P, Lifschitz C. Infants fed docosahexaenoic acid- and arachidonic acid-supplemented formula have decreased incidence of bronchiolitis/bronchitis the first year of life. Clin Pediatr (Phila) 2006;45:850-5.

16. Smithers LG, Gibson RA, McPhee A, Makrides M. Effect of long-chain polyunsaturated fatty acid supplementation of preterm infants on disease risk and neurodevelopment: a systematic review of randomized controlled trials. Am J Clin Nutr 2008;87:912-20.

17. Rees D, Miles EA, Banerjee T, *et al.* Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men.[see comment]. American Journal of Clinical Nutrition 2006;83:331-42.

18. Dunstan JA, Mori TA, Barden A, *et al.* Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol 2003;112:1178-84.

19. Hibbeln JR, Nieminen LRG, Blasbalg TL, Riggs JA, Lands WEM. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. American Journal of Clinical Nutrition 2006;83:1483S-1493S.

20. Koletzko B, Cetin I, Brenna JT. Dietary fat intakes for pregnant and lactating women. Br J Nutr 2007;98:873-7.

21. Al MD, van Houwelingen AC, Kester AD, Hasaart TH, de Jong AE, Hornstra G. Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. Br J Nutr 1995;74:55-68.

22. Jensen CL, Maude M, Anderson RE, Heird WC. Effect of docosahexaenoic acid supplementation of lactating women on the fatty acid composition of breast milk lipids and maternal and infant plasma phospholipids. Am J Clin Nutr 2000;71:292S-9S.

23. Dunstan JA, Mitoulas LR, Dixon G, *et al.* The effects of fish oil supplementation in pregnancy on breast milk fatty acid composition over the course of lactation: a randomized controlled trial. Pediatr Res 2007;62:689-94.

24. Dunstan JA, Mori TA, Barden A, *et al.* Effects of n-3 polyunsaturated fatty acid supplementation in pregnancy on maternal and fetal erythrocyte fatty acid composition. Eur J Clin Nutr 2004;58:429-37.
25. Krauss-Etschmann S, Shadid R, Campoy C, *et al.* Effects of fish-oil and folate supplementation of pregnant women on maternal and fetal plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid: a European randomized multicenter trial. American Journal of Clinical Nutrition 2007;85:1392-400.

26. Bergmann RL, Haschke-Becher E, Klassen-Wigger P, *et al.* Supplementation with 200 mg/day docosahexaenoic acid from mid-pregnancy through lactation improves the docosahexaenoic acid status of mothers with a habitually low fish intake and of their infants. Ann Nutr Metab 2008;52:157-66.

27. Koletzko B, Lien E, Agostoni C, *et al.* The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. J Perinat Med 2008;36:5-14.

28. Field CJ, Clandinin MT, Van Aerde JE. Polyunsaturated fatty acids and T-cell function: implications for the neonate. Lipids 2001;36:1025-32.

29. Szepfalusi Z. The maturation of the fetal and neonatal immune system. J Nutr 2008;138:1773S-1781S.

30. www.who.int.

31. Christian P. Infant Mortality. In: Semba R, Bloem M, eds. Nutrition and Health in Developing Countries. Totowa: Humana Press, 2008.

32. Moore SE, Cole TJ, Collinson AC, Poskitt EM, McGregor IA, Prentice AM. Prenatal or early postnatal events predict infectious deaths in young adulthood in rural Africa. Int J Epidemiol 1999;28:1088-95.

33. McDade TW, Beck MA, Kuzawa C, Adair LS. Prenatal undernutrition, postnatal environments, and antibody response to vaccination in adolescence. Am J Clin Nutr 2001;74:543-8.

34. Prescott SL, Dunstan JA. Prenatal fatty acid status and immune development: the pathways and the evidence. Lipids 2007;42:801-10.

35. Calder PC, Krauss-Etschmann S, de Jong EC, *et al.* Early nutrition and immunity - progress and perspectives. Br J Nutr 2006;96:774-90.

36. Dewey KG, Romero-Abal ME, Quan de Serrano J, *et al.* A randomized intervention study of the effects of discontinuing coffee intake on growth and morbidity of iron-deficient Guatemalan toddlers. J Nutr 1997;127:306-13.

37. Ramakrishnan R, Venkatarao T, Koya PK, Kamaraj P. Influence of recall period on estimates of diarrhoea morbidity in infants in rural Tamilnadu. Indian J Public Health. 1999 Oct-Dec;43(4):136-9.

38. Alam N, Henry FJ, Rahaman MM.Reporting errors in one-week diarrhea recall surveys: experience from a prospective study in rural Bangladesh. Int J Epidemiol. 1989 Sep;18(3):697-700.

39. Martorell R, Habicht JP, Yarbrough C, Lechtig A, Klein RE. Underreporting in fortnightly recall morbidity surveys. J Trop Pediatr Environ Child Health. 1976 Jun;22(3):129-34.

40. Manesh AO, Sheldon TA, Pickett KE, Carr-Hill R. Accuracy of child morbidity data in demographic and health surveys. Int J Epidemiol 2008;37:194-200.

41. Goldman N, Vaughan B, Pebley AR. The use of calendars to measure child illness in health interview surveys. Int J Epidemiol 1998;27:505-12.

42. Dunstan JA, Mori TA, Barden A, *et al.* Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clin Exp Allergy 2003;33:442-8.

43. Prescott SL, Barden AE, Mori TA, Dunstan JA. Maternal fish oil supplementation in pregnancy modifies neonatal leukotriene production by cord-blood-derived neutrophils. Clin Sci (Lond) 2007;113:409-16.

44. Dunstan JA, Roper J, Mitoulas L, Hartmann PE, Simmer K, Prescott SL. The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition. Clin Exp Allergy 2004;34:1237-42.

45. Field CJ, Van Aerde JE, Robinson LE, Clandinin MT. Effect of providing a formula supplemented with long-chain polyunsaturated fatty acids on immunity in full-term neonates. Br J Nutr 2008;99:91-9.

46. Mazurak VC, Lien V, Field CJ, Goruk SD, Pramuk K, Clandinin MT. Long-chain polyunsaturated fat supplementation in children with low docosahexaenoic acid intakes alters immune phenotypes compared with placebo. J Pediatr Gastroenterol Nutr 2008;46:570-9.

47. Parra MS, Schnaas L, Meydani M, Perroni E, Martinez S, Romieu I. Erythrocyte cell membrane phospholipid levels compared against reported dietary intakes of polyunsaturated fatty acids in pregnant Mexican women. Public Health Nutr 2002;5:931-7.

48. Kris-Etherton PM, Taylor DS, Yu-Poth S, *et al.* Polyunsaturated fatty acids in the food chain in the United States. Am J Clin Nutr 2000;71:179S-88S.

49. Denomme J, Stark KD, Holub BJ. Directly quantitated dietary (n-3) fatty acid intakes of pregnant Canadian women are lower than current dietary recommendations. J Nutr 2005;135:206-11.

50. Laitinen K, Hoppu U, Hamalainen M, Linderborg K, Moilanen E, Isolauri E. Breast milk fatty acids may link innate and adaptive immune regulation: analysis of soluble CD14, prostaglandin E2, and fatty acids. Pediatric Research 2006;59:723-7.

Table 1. Selected characteristics at randomization of 851 women who attended the 1 month post-partum study visit

	DHA n=423	Placebo n=428	P-value
Age in years[1]	26.3 ± 4.9	26.4 ± 4.6	0.86
Gestational age in wks[1]	20.5 ± 1.9	20.6 ± 2.0	0.56
Primipara[2]	37.4	39.0	0.62
SES using PCA[1, 3]	0.06 ± 1.0	0.06 ± 1.0	0.90
Schooling in years[1]	12.0 ± 3.5	12.0 ± 3.6	0.73

[1]Mean ± SD

[2]Proportion

[3]Principal components analysis

Chi square tests, T-tests or Wilcoxon rank sum tests were used to assess significant differences between groups

Table 2. Selected characteristics of 851 infants of women who attended the 1 month post-partum study visit

	DHA n=423	Placebo n=428	P-value
Sex (male)[2]	53.7	53.0	0.85
Birth weight (g)[1]	3225 ± 425	3224 ± 451	0.98
Birth length (cm)[1]	50.4 ± 2.1	50.4 ± 2.6	0.79
Gestational age at birth (mean wks)[1]	39.1 ± 1.7	39.1 ± 1.7	0.81
Premature (<37 wks) [2]	9.5	8.2	0.52
Breast fed at 1 month (yes)[2]	93.1	93.8	0.65
Breast fed at 3 months (yes)[2]	83.0	82.2	0.75

[1]Mean ± SD

[2]Proportion

Chi square tests, T-tests or Wilcoxon rank sum tests were used to assess significant differences between groups

Table 3. History of illness symptoms among infants, as reported by the infant's mother in the previous 15 days at 1, 3 and 6 months

Symptoms	1 month			3 months			6 months		
	DHA n=422 (%)	Placebo n=427 (%)	OR (95% CI)	DHA n=415 (%)	Placebo n=419 (%)	OR (95% CI)	DHA n=420 (%)	Placebo n=414 (%)	OR (95% CI)
Cough	9.5	11.0	0.85 (0.54, 1.32)	19.3	23.9	0.76 (0.55, 1.06)	33.1	32.9	1.01 (0.76, 1.35)
Phlegm	16.8	19.2	0.85 (0.60, 1.21)	19.5	18.6	1.06 (0.75, 1.50)	23.9	24.2	0.98 (0.72, 1.35)
Nasal congestion	28.2	32.8	0.81 (0.60, 1.08)	25.1	28.4	0.83 (0.62, 1.15)	29.6	28.0	1.08 (0.80, 1.46)
Nasal secretion	7.1	10.8	0.64 (0.39, 1.03)	14.9	17.8	0.85 (0.58, 1.23)	28.2	29.5	0.94 (0.70, 1.27)
Cold*	37.6	44.6	0.76 (0.58, 1.00)	37.8	62.2	0.77 (0.59, 1.02)	46.2	46.6	0.98 (0.75, 1.29)
Wheezing	8.3	7.0	1.19 (0.72, 1.98)	7.0	8.1	0.85 (0.51, 1.43)	11.9	10.9	1.11 (0.72, 1.70)
Difficulty breathing	2.4	2.3	1.01 (0.42, 2.46)	2.9	2.4	1.22 (0.52, 2.85)	1.4	1.7	0.85 (0.28, 2.54)
Fever	3.6	3.3	1.03 (0.49, 2.19)	8.4	10.5	0.79 (0.49, 1.25)	18.3	18.6	0.98 (0.69, 1.39)
Vomiting	5.5	3.5	1.58 (0.81, 3.08)	4.1	2.9	1.45 (0.68, 3.07)	5.5	4.1	1.36 (0.71, 2.58)
Diarrhea	3.3	4.0	0.83 (0.40, 1.70)	4.6	5.5	0.83 (0.44, 1.54)	7.6	7.5	1.02 (0.61, 1.71)
Rash	29.0	26.1	1.16 (0.86, 1.57)	8.4	10.3	0.81 (0.50, 1.29)	10.7	9.4	1.16 (0.74, 1.82)
Other Illness	6.9	5.9	1.19(0.69, 2.07)	5.1	5.3	0.96 (0.52, 1.78)	6.7	5.8	1.16 (0.66, 2.04)

*Cold (any of the following: cough, phlegm, nasal congestion, nasal secretion)

Table 4. Duration of symptoms in infants who experienced illness

Symptoms	1 month Days ill: median (25th and 75th percentiles)		3 months Days ill: median (25th and 75th percentiles)		6 months Days ill: median (25th and 75th percentiles)	
	DHA	**Placebo**	**DHA**	**Placebo**	**DHA**	**Placebo**
Cough	4.0 (3, 5)	5.0 (3, 9)	5.0 (3, 7)	4.0 (3, 7)	5.0 (3, 9)	5.0 (3, 9)
Phlegm	4.0 (3, 10)	6.0 (3, 14)	5.5 (3, 10)	5.0 (3, 10)	5.0 (3, 10)	5.0 (3, 9)
Nasal congestion	4.0 (3, 9)	5.0 (3, 12)	5.0 (3, 9)	4.0 (3, 8)	4.0 (3, 7)	5.0 (3, 7)
Nasal secretion	3.0 (2, 4)	4.0 (1, 6)	4.0 (3, 7)	3.0 (2, 6)	3.0 (3, 5)	4.0 (3, 7)
Wheezing	3.0 (2, 7)	5.0 (3, 15)	4.5 (2, 8)	4.0 (2, 8)	4.0 (3, 10)	3.0 (2, 8)
Difficulty breathing	3.5 (2, 10)	3.0 (2, 9)	3.0 (2, 7)	2.0 (1, 4)	2.0 (2, 3)	3.5 (1, 10)
Fever	2.0 (1, 3)	2.0 (1, 3)	2.0 (1, 3)	2.0 (1, 3)	2.0 (1, 3)	2.0 (1, 4)
Vomiting	3.0 (2, 15)	2.0 (1, 4)	2.0 (2, 8)	4.0 (2, 12)	3.0 (1, 4)	2.0 (1, 4)
Diarrhea	3.5 (3, 5)	2.5 (2, 10)	3.0 (2, 3)	3.0 (2, 6)	3.0 (1, 4)	3.5 (2, 5)
Rash	11.0 (6, 15)	7.0 (4, 15)	5.0 (2.5, 10)	5.0 (3, 10)	3.5 (2, 7)	4.0 (3, 9)
Other illness	12.0 (5, 15)	10.5 (5, 15)	7.0 (4, 12)	11.0 (5, 15)	5.0 (3, 6)	8.0 (4, 14)

Table 5. Relative risk of longer duration of illness at 1, 3 and 6 months, in children who experienced illness[1]

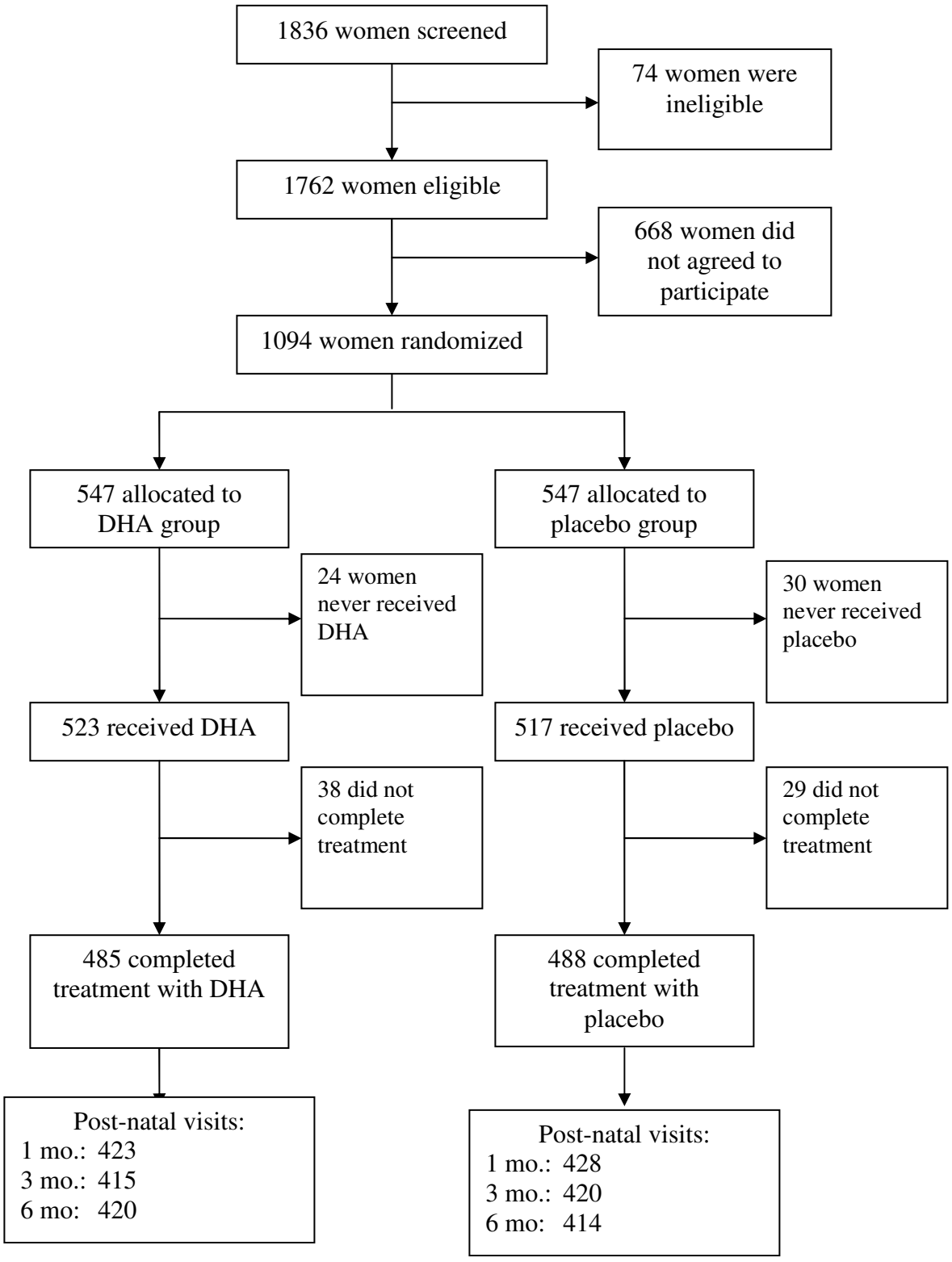
Symptoms	1 month			3 months			6 months		
	RR	**95% CI**	**P-value**	**RR**	**95% CI**	**P-value**	**RR**	**95% CI**	**P-value**
Cough	0.74	0.61, 0.90	<0.01	1.00	0.88, 1.14	0.95	1.00	0.91, 1.10	0.93
Phlegm	0.85	0.74, 0.96	0.01	1.06	0.93, 1.20	0.40	1.01	0.90, 1.14	0.83
Nasal congestion	0.98	0.89, 1.09	0.72	1.15	1.03, 1.28	0.02	0.94	0.84, 1.05	0.25
Nasal secretion	0.69	0.46, 1.03	0.07	1.07	0.92, 1.25	0.38	0.87	0.77, 0.98	0.02
Wheezing	0.70	0.57, 0.86	<0.01	0.99	0.79, 1.24	0.93	1.12	0.94, 1.33	0.21
Difficulty breathing	1.05	0.69, 1.58	0.83	1.59	0.96, 2.62	0.07	0.46	0.24, 0.87	0.02
Fever	0.99	0.57, 1.73	0.98	1.14	0.85, 1.52	0.40	0.80	0.66, 0.98	0.03
Vomiting	1.51	0.64, 3.59	0.35	0.73	0.51, 1.04	0.08	1.74	1.19, 2.54	<0.01
Diarrhea	0.74	0.52, 1.06	0.10	0.90	0.65, 1.24	0.51	0.86	0.65, 1.13	0.27
Rash	1.22	1.05, 1.41	<0.01	0.95	0.79, 1.14	0.56	0.78	0.64, 0.94	<0.01
Other illness	1.01	0.84, 1.22	0.89	0.77	0.62, 0.95	0.02	0.75	0.59, 0.94	0.01

All illnesses[2]	1.04	0.97, 1.1	0.26	0.86	0.80, 0.93	<0.0001	1.01	0.94, 1.1	0.88

[1]Relative risk was estimated by exponentiating β using Poisson regression modeling.

[2]Total days ill in previous 15 days, includes all children

Figure 1. Trial profile



CHAPTER 7

PRENATAL DOCOSAHEXAENOIC ACID SUPPLEMENTATION AND INFANT RESPONSE TO HEPATITIS B AND TETANUS VACCINATION IN A DOUBLE-BLIND RANDOMIZED, CONTROLLED TRIAL IN MEXICO

PRENATAL DOCOSAHEXAENOIC ACID SUPPLEMENTATION AND INFANT RESPONSE TO HEPATITIS B AND TETANUS VACCINATION IN A DOUBLE-BLIND RANDOMIZED, CONTROLLED TRIAL IN MEXICO

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Abstract

Objective: Omega-3 PUFAs, specifically long-chain PUFAs such as docosahexaenoic acid (DHA, 22:6*n*-3), modulate immune function and inflammation; however, their role in the development of infant immune function is yet unknown. Infants are born with immature immune systems and factors such as perinatal nutrition may influence infant immune development and response. We sought to determine the influence of DHA in pregnancy on infant response to hepatitis B (HepB) and tetanus vaccination. **Methods**: In a double-blind randomized, controlled trial in Mexico, pregnant women were supplemented daily with 400 mg algae-derived DHA or placebo from 18-22 weeks' gestation through parturition. Infants were routinely vaccinated with HepB at birth and at 2 months of age, and with tetanus at 2 months of age. Using flow cytometry (Luminex technology), we measured anti-HepB and anti-tetanus IgM and IgG antibody concentrations in infant plasma as functional markers of immunocompetence at 3 months of age. **Results**: Baseline maternal characteristics and infant outcomes such as birth weight and gestational age were similar between treatment groups (n=562). Concentration of anti-tetanus IgM and IgG and anti-HepB IgM antibodies did not differ between groups; however, the concentration of anti-HepB IgG antibody was 12% lower in the DHA group (GM=525.3, 95% CI=484.4, 569.7), compared to the DHA group (GM=464.9, 95% CI=430.0, 502.7). We found positive Spearman correlations in the placebo group between anti-HepB IgG and SES (r=.1). In the DHA group, we found positive correlations between anti-HepB IgG and maternal age (r=.2) and parity (r=.1) and anti-tetanus IgG and birth weight (r=.2). **Conclusions**: The current study demonstrates that DHA supplementation in

pregnancy influenced infant humoral response to HepB vaccination. The implications of these findings on overall infant immune function, however, are unknown and merit further investigation.

Introduction

Long-chain polyunsaturated fatty acids (LCPUFAs), particularly the omega-3 LCPUFA docosahexaenoic acid (DHA, 22:6*n*-3), are found in high concentrations in brain and retinal cell membranes and accumulate rapidly in neural tissue during later gestation and infancy (1, 2). DHA is also found in cells of the immune system and modulates immunity and inflammation through various mechanisms. These specific mechanisms include: incorporation of DHA into cell membrane phospholipids and modification of membrane structure and function, subsequent alteration in eicosanoid synthesis, cell adhesion molecule expression, cytokine production and lipid raft structure, and modulation of factors involved in gene expression such as nuclear factor kB (NFkB) (3-8). DHA has been shown to influence, among other components of immune response, phagocytosis, B- and T-cell proliferation, T-cell signaling, and antigen presentation (7, 9). Because of DHA's key role in neural development and immune function, adequate fetal and infant DHA nutriture is essential.

DHA is preferentially transferred across the placenta and is present in breast milk and availability of DHA to the developing fetus and its concentration in breast milk is dependent upon maternal diet and PUFA stores (10-13). Several expert groups recommend a daily dietary intake of at least 200 mg DHA/d for pregnant and lactating women; however, many populations do not consume this minimum amount (14, 15). Pre-formed DHA is found in high concentrations in oily fish such as salmon and herring and although DHA can be synthesized from the precursor α-linolenic acid (ALA; 18:3n-3), synthesis is limited in humans (16). Low intake of n-3 PUFAs may influence health

outcomes including cognitive development in children, post-partum depression, certain chronic inflammatory diseases and immune function.

Development of the immune system begins *in utero* and continues throughout early childhood. Although immune response in the fetus is down-regulated to avoid harmful immunological reactions between the mother and the fetus, fetuses indeed exhibit immune responses (17) . For example, fetuses have been shown to produce IgM *in utero* in response to maternal vaccination, although class switching to IgG does not occur (18). Although infants have relatively naïve immune systems at birth, they have the capacity to mount, albeit sometimes weak and fleeting, humoral and cell-mediated immune responses (19-21). Infants enjoy passive immunity conferred by their mother, specifically, maternal IgG is transferred across the placenta to the fetus, and breast milk contains sIgA, IgG and IgM antibodies (17). The immune system matures rapidly throughout the first few years of life and its development can be influenced by genetic factors and by environmental factors, such as nutrition.

Several studies in animals and in humans have shown that dietary n-3 PUFAs in pregnancy may influence *in utero* immune programming and subsequent maturation of the immune system; however, the influence of n-3 PUFAs on immune response to vaccination is unknown (8, 22-27). The main objective of the present study was to determine the influence of DHA exposure in pregnancy on infant immune response. Specifically, we sought to evaluate the influence of algae-derived DHA from gestation weeks 18-22 through parturition on response to hepatitis-B and tetanus vaccination at 3 months of age.

Methods

Study population and setting

Study participants were recruited at the Mexican Institute of Social Security (Instituto Mexicano del Seguro Social or IMSS) General Hospital I, a large hospital located in Cuernavaca, Mexico, and 3 small health clinics within the IMSS system in Cuernavaca during routine prenatal care visits between February 2005 and February 2007. The IMSS health care system provides employed persons access to medical care. Women were considered for inclusion in the study if they were in gestation weeks 18-22, planned to predominantly breastfeed for at least 3 months, were 18-35 years old, planned to deliver at the IMSS General Hospital in Cuernavaca, and planned to live in the area for 2 years after delivery. Women were excluded if any of the following criteria were present: 1) high risk pregnancy, 2) lipid metabolism or absorption disorders, 3) regular intake of fish oil or DHA supplements, or 4) chronic use of certain medications, such as medications for epilepsy. Written informed consent was obtained from each participant after a thorough explanation of the study details, and participants were free to withdraw from the study at any time.

Ethics: The study protocol and all informed consent documents were approved by Emory University's Human Investigations Board and by the Instituto Nacional de Salud Pública (INSP) and the IMSS General Hospital I's Human Subjects Boards. The welfare of the subjects was monitored by an external Data Safety Monitoring Committee. This study is an ongoing collaborative project between the INSP in Cuernavaca, Mexico and Emory University, and is a registered clinical trial (registered at INSP in Mexico: #CI-011, and at clinicaltrials.gov: NCT00646360).

Study design

Women were assigned to receive either 400 mg DHA or placebo daily, from gestation weeks 18-22 through parturition. The DHA capsules contained 200 mg DHA each derived from an algal source (Martek Biosciences Corporation, Columbia, MD). The placebo capsules contained olive oil, and were similar in appearance and taste to the DHA capsules. Fieldworkers visited the women's homes weekly to deliver a new bottle of 14 capsules, and compliance was monitored by counting any remaining pills and through interviews with participants.

All participants and members of the study team were blinded to the treatment scheme throughout the intervention period of the study. The blinding code was placed in sealed envelopes at the beginning of the study, and these envelopes were held by investigators at Emory who were not involved in the study. Data were un-blinded for the analytical study team after the last baby in the study was born and had completed 6 months of age, at which time participants were no longer taking supplements. The randomization process has been described elsewhere. Success of randomization was assessed by comparison of a variety of baseline maternal characteristics in the two treatment groups.

Infants were routinely given hepatitis B vaccination (Probivac-B made by Probiomed) at birth and at 2 months of age, and tetanus vaccination at 2 months of age. Mothers were routinely immunized against tetanus during their third trimester of pregnancy.

Blood collection and immunoglobulin analysis

Five ml of infant blood was obtained by venipuncture by trained technicians at the IMSS hospital at 3 months of age. All samples were collected into tubes containing EDTA. Plasma and erythrocytes were separated by centrifugation at 800 G for 10 minutes at room temperature. Plasma was then aliquoted into multiple cryovials, and was immediately frozen in a nitrogen environment and stored at -70° C.

Anti-hepatitis B and anti-tetanus IgM and IgG antibody concentrations were determined using flow cytometry (Luminex technology). We included 42 samples and their duplicates and 3 internal standards per plate. Samples underwent a 1:50 dilution. We used Wally's reagent (from Quantaplex, 1:5 of diluted solution) as the detection reagent for the IgM assay and Donkey Antihuman reagent (1:100 of stock solution) for the IgG assay. Intra-assay variation was assessed by including replicate bead populations coated with the same antigen. Replication of results was good, as evidenced by similarity of results in the duplicate bead populations, and therefore intra-assay variation was low.

Statistical analysis

We conducted the analysis according to the intention-to-treat principle, and analyzed all available infant plasma samples collected at 3 months. Continuous baseline characteristics and birth outcomes were tested for normality, and group differences were calculated using T-Tests or Wilcoxon rank-sum tests, as appropriate. Categorical baseline characteristics were compared using Chi-square analyses. Immunoglobulin concentrations were natural log-transformed to achieve normal distributions, and concentrations were expressed as geometric means (GM) with 95% confidence intervals (CI). The effect of

treatment on immunoglobulin concentration was assessed using unadjusted generalized linear models, and estimates were exponentiated because of the initial log-transformation of the values. We computed Spearman correlation coefficients to describe the relationship between the immunoglobulins and variables that might influence immunoglobulin concentration (parity, mother's age, SES). Exclusive breastfeeding was defined as no intake of supplemental foods or liquids, including water. We conducted statistical analyses using TTEST, NPAR1WAY, CORR and GLM procedures in the Statistical System Software version 9.2, SAS Institute, Cary, North Carolina. Statistical significance was defined as $P < 0.05$.

Results

Trial profile: Among the 1836 women screened 1094 were randomized to treatment and 523 in the DHA group and 517 in the placebo group received at least one dose of treatment (Figure 1). Four hundred and eighty five women in the DHA group and 488 women in the placebo group completed treatment by remaining in the study through parturition. Among women who received treatment, 6.4% were lost to follow up before delivery. Select reasons for the 67 losses to follow up between receiving treatment and parturition included moving out of the area, refusal to participate, the husband did not want the woman to continue with the study and the supplement made the woman feel bad. Of the 877 infants who attended the 3 month study visit, we were able to obtain and analyze 562 infant plasma samples.

Maternal and neonatal characteristics: Maternal baseline characteristics and infant characteristics at birth were similar between treatment groups (Table 1). Women

were, on average, 26 years old at randomization, had >11 years of schooling and entered into the study at approximately 20 weeks' gestation. There was no significant difference in maternal atopic status at baseline (not shown). The proportion of premature babies (<37 weeks' gestation) was <10% and 18% and 16% of babies were exclusively breastfed at 1 and 3 months, respectively.

Immunoglobulin concentrations in infant plasma: Concentration of anti-HepB IgG antibody was 12% higher in the placebo group (GM=525.3, 95% CI=484.4, 569.7), compared to the DHA group (GM=464.9, 95% CI=430.0, 502.7), while concentrations of anti-HepB IgM and anti-tetanus IgG and IgM were similar between groups (Table 2). Spearman rank-order correlations between immunoglobulins and various maternal and infant characteristics revealed weak positive correlations between both maternal age (r=0.2) and parity (r=0.1) and anti-HepB IgG antibody concentration in the DHA group. Anti-HepB IgG antibody concentration weakly correlated with SES in the placebo group (r=0.1). Overall, we did not observe strong correlations between immunoglobulin concentrations and characteristics such as sex, birth weight, gestational age, SES, maternal age and parity.

Discussion

Our study demonstrated that DHA supplementation from 18-22 weeks' gestation through parturition influenced infant humoral immune response in 3 month-old Mexican infants. Anti-HepB IgG antibody concentration was 12% lower in the DHA group compared to placebo, while concentrations of anti-tetanus IgG and IgM and anti-HepB IgM antibodies did not differ between groups. The current study provides preliminary evidence that

neonatal DHA nutriture may influence antibody response to hepatitis B vaccination; however, the exact mechanism of action is yet unclear.

The influence of n-3 PUFAs on inflammation and immune function and potential mechanisms have been reviewed extensively (3, 5, 7, 8). Numerous studies have demonstrated that n-3 PUFA supplementation in healthy adults inhibits the production of pro-inflammatory cytokines and eicosanoids, while several studies have shown no effect (5). Fish oil (Eicosapentaenoic acid (EPA, 20:5*n*-3) +DHA) supplementation has been shown to result in the displacement of membrane-phospholipid n-6 PUFAs such as arachidonic acid (AA, 20:4*n*-6), which serves as a substrate for pro-inflammatory eicosanoids such as leukotriene B_4 and prostaglandin E_2. DHA's ability to influence synthesis of these inflammatory mediators provides one potential link between DHA nutriture and immunity and inflammation.

Although few studies have evaluated the effect of n-3 PUFAs in pregnancy on immune function and inflammation, two studies showed that fish oil supplementation in pregnancy altered the production of inflammatory leukotienes and cytokines (26). Dunstan *et al.* demonstrated that fish oil supplementation of atopic pregnant women from 20 weeks' gestation through delivery resulted in significantly reduced concentration of the Th2-related cytokine interleukin-13 (IL-13) in cord plasma, suggesting that fish oil in pregnancy alone can influence the production of cytokines involved in inflammation and immune response (22). An additional analysis of the same trial reported lower levels of Th2 cytokines (IL-5, IL-13 and IL-10) in cord blood plasma from the fish oil group compared to placebo; however, only IL-10 was significantly lower in the fish oil group (24). Another analysis of the same study reported significantly lower production in the

fish oil group of leukotriene B_4 by neonatal neutrophils (25). Finally, a fourth analysis within the same study showed significantly higher percentage of cord blood $CD34^+$ progenitor cells in the fish oil group compared to placebo (28). Similar to the aforementioned trial, in a recent multi-center trial titled the Nutraceuticals for Healthier Life (NUHEAL), infants of women who were supplemented with fish oil from 22 weeks' gestation through delivery had lower concentrations of plasma IL-13and IL-4 and higher concentrations of TGF-β in cord blood than infants of the non-supplemented women (3, 29). These investigators also reported presence of fewer natural killer cells and CD8+ cells in infants of the mothers given fish oil compared to placebo. Overall, these trials demonstrate that fish oil in pregnancy alone can alter various factors involved in immune response, including Th-2-related cytokines and leukotriene synthesis.

Maternal nutritional status has been shown in studies to influence response to vaccination in offspring. McDade *et al.* showed that prenatal undernutrition, characterized by intra-uterine growth retardation (IUGR), influenced immune response in 14-15 year-olds (30). Children who experienced IUGR had a lower probability of mounting a positive antibody immune response to typhoid vaccination than children who were better nourished *in utero*. Micronutrient status in pregnancy can influence infant immune function as reported by Tielsch *et al.*, who showed that children born to vitamin A deficient women, as evidenced by night blindness during pregnancy, suffered increased risk of diarrhea, acute respiratory infection and dysentery compared to children born to women without night blindness (31). These studies demonstrate the potentially important role of maternal nutritional status in *in utero* programming of immune function.

Studies have also shown that the micronutrient status of the child may influence response to vaccination. For example, Diness et al. showed that high doses of vitamin A resulted in weaker immune responsiveness to BCG vaccination in 2 month-old boy infants; however, this effect was transient and long-term immunity was not compromised (32). Semba et al. also found that administration of high doses of vitamin A resulted in lower seroconversion rates in response to measles vaccination at 6 months of age (33). Alternatively, Bhaskaram et al. showed that high doses of vitamin A enhanced seroconversion to the measles vaccination (34). Finally, Newton et al. found that administration of high doses of vitamin A at 6, 10 and 14 weeks of age enhanced immune response to hepatitis B vaccination and had no effect on response to *Haemophilus influenza* type b (35). Overall, these studies illustrate the potential influence of micronutrient status on infant response to vaccination.

The infant immune system is immature, and both T-cell and B-cell response to vaccination is different in infants compared to adults. Infants preferentially produce IgG rather than IgM in response to vaccination, and can mount a primary IgG response by 2-3 months of age (36). Maternal antibodies can influence infant immune response to a primary vaccination through 12 months of age; however, the infant's ability to mount a secondary IgG antibody response to a booster vaccination is not influenced by the presence of maternal antibodies (20, 36). We examined response to a booster hepatitis B vaccination (infants were given one dose at birth and another at 2 months of age), and assume that maternal antibodies did not dampen the infant's ability to mount a response. In general, infants exhibit a lower and more short-lived antibody response than adults, and are very limited in their ability to mount T-cell responses to intracellular pathogens.

However, infants mount a higher protective antibody response than adults when challenged with the hepatitis B vaccine (37, 38).

The hepatitis B vaccine elicits a Th2-driven immune response in infants, compared to a Th1-driven response in naïve adults, which might provide insight into a possible mechanism of DHA's influence on hepatitis B-specific antibody response to vaccination (17, 37, 38). As described above, DHA supplementation in pregnancy has been shown to influence production of the cytokines IL-13, IL-4 and IL-10. These "pro-inflammatory" cytokines, generally synthesized by Th2 cells, can activate B cells which subsequently proliferate, differentiate into plasma cells and produce antibodies (23, 39). Although we did not quantify IL-13 concentrations in cord blood, this is a potential mechanism by which DHA influenced anti-HepB IgG antibody concentration in our study. However, infant humoral immune response is complex and could be influenced by numerous factors including antibodies transferred to the infant through in breast milk (40).

The present study has several strengths, including its design as a large, double-blind randomized, controlled trial. Because treatment groups were randomly assigned, baseline maternal characteristics were similar between groups. Additionally, outcomes that might influence immune response to vaccination such as birth weight, sex, gestational age at delivery and breast feeding status were similar between groups. As such, we attribute differences in immune response to vaccination to the intervention.

The current analysis was limited by the inability to distinguish *de novo* fetal IgG from maternal-derived IgG, which is transferred across the placenta to the developing fetus. However, we assume that the treatment groups originated from the same population and therefore entered the study with similar immune profiles. A study examining the

seroprevalence of hepatitis B in pregnant Mexican women revealed that 6.4% had been vaccinated against hepatitis B and that 1.65% had active infections (41). This study population was very similar to ours, as the women were pregnant and utilized the same health care (IMSS system) as women in our study. We measured anti-HepB antibodies after the second vaccination, so isotype switching of IgM to IgG was possible in the infants (18, 19). Hence, although a proportion of the IgG could have been maternal in origin, infants synthesized *de novo* IgG in response to vaccination.

We were unable to collect samples from each infant at 3 months due to factors such as the technician's inability to draw the blood and mother's refusal to allow a blood draw; however, our sample of infant plasma at 3 months (58% of the 973 children born) is similar to the main study population so we assume no selection bias.

In summary, we found that DHA in pregnancy altered immunological response to hepatitis B vaccination in Mexican infants. The current study provides preliminary evidence that DHA exposure *in utero* may influence immune programming in the fetus. Mechanisms of DHA's action on immune response could include alteration of cytokine or eicosanoid synthesis, alteration of gene expression and/or alteration of membrane constituents such as lipid rafts. DHA might also influence infant immune response via modification of breast milk immunoglobulin profiles. Our findings support previous studies that demonstrated the potential immunomodulatory influence of fish oil in pregnancy, while providing new insight into DHA's role in humoral immune response. The clinical and long-term significance of the current findings are yet unknown and may be partially revealed within the context of the currently ongoing randomized, controlled trial; however, the differences in anti-HepB IgG antibody concentrations are indeed small

and likely do not have clinical implications. Because anti-HepB IgG antibodies were detected in all infant plasma samples indicating seroconversion, we speculate that differences in mean concentrations will not impact the infants' ability to mount a response to a potential future hepatitis B infection.

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Literature cited

1. Koletzko B, Lien E, Agostoni C, Bohles H, Campoy C, Cetin I, Decsi T, Dudenhausen JW, Dupont C, *et al.* The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. Journal of perinatal medicine. 2008;36:5-14.

2. Innis SM. Dietary omega 3 fatty acids and the developing brain. Brain Res. 2008;Spetember 9 [ahead of print].

3. Calder PC, Krauss-Etschmann S, de Jong EC, Dupont C, Frick JS, Frokiaer H, Heinrich J, Garn H, Koletzko S, *et al.* Early nutrition and immunity - progress and perspectives. Br J Nutr. 2006 Oct;96:774-90.

4. Deckelbaum RJ, Worgall TS, Seo T. n-3 fatty acids and gene expression. Am J Clin Nutr. 2006 Jun;83:1520S-5S.

5. Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proc Nutr Soc. 2007 May;66:237-59.

6. Calder PC. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr. 2006 Jun;83:1505S-19S.

7. Calder PC. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids. 2007 Nov-Dec;77:327-35.

8. Gottrand F. Long-chain polyunsaturated Fatty acids influence the immune system of infants. J Nutr. 2008 Sep;138:1807S-12S.

9. Harbige LS. Fatty acids, the immune response, and autoimmunity: a question of n-6 essentiality and the balance between n-6 and n-3. Lipids. 2003 Apr Apr;38:323-41.

10. Hanebutt FL, Demmelmair H, Schiessl B, Larque E, Koletzko B. Long-chain polyunsaturated fatty acid (LC-PUFA) transfer across the placenta. Clinical nutrition (Edinburgh, Scotland). 2008 Oct;27:685-93.

11. Innis SM. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. J Pediatr. 2003 Oct;143:S1-8.

12. Koletzko B, Larque E, Demmelmair H. Placental transfer of long-chain polyunsaturated fatty acids (LC-PUFA). Journal of perinatal medicine. 2007;35 Suppl 1:S5-11.

13. Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. American Journal of Clinical Nutrition. 2007 Jun;85:1457-64.

14. Hibbeln JR, Nieminen LRG, Blasbalg TL, Riggs JA, Lands WEM. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. American Journal of Clinical Nutrition. 2006 Jun;83:1483S-93S.

15. Kris-Etherton PM, Taylor DS, Yu-Poth S, Huth P, Moriarty K, Fishell V, Hargrove RL, Zhao G, Etherton TD. Polyunsaturated fatty acids in the food chain in the United States. Am J Clin Nutr. 2000 Jan;71:179S-88S.

16. Burdge GC, Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reproduction, Nutrition, Development. 2005 Sep-Oct;45:581-97.

17. M'Rabet L, Vos AP, Boehm G, Garssen J. Breast-feeding and its role in early development of the immune system in infants: consequences for health later in life. J Nutr. 2008 Sep;138:1782S-90S.

18. Dastur FD, Shastry P, Iyer E, Awatramani V, Raut S, Mehta SD, Irani SF. The foetal immune response to maternal tetanus toxoid immunization. J Assoc Physicians India. 1993 Feb;41:94-6.

19. Szepfalusi Z. The maturation of the fetal and neonatal immune system. J Nutr. 2008 Sep;138:1773S-81S.

20. Siegrist CA. The challenges of vaccine responses in early life: selected examples. J Comp Pathol. 2007 Jul;137 Suppl 1:S4-9.

21. Offit PA, Quarles J, Gerber MA, Hackett CJ, Marcuse EK, Kollman TR, Gellin BG, Landry S. Addressing parents' concerns: do multiple vaccines overwhelm or weaken the infant's immune system? Pediatrics. 2002 Jan;109:124-9.

22. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, Prescott SL. Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clin Exp Allergy. 2003 Apr;33:442-8.

23. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. Lipids. 2001 Sep;36:1007-24.

24. Dunstan JA, Mori TA, Barden A, Beilin LJ, Taylor AL, Holt PG, Prescott SL. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol. 2003 Dec;112:1178-84.

25. Prescott SL, Barden AE, Mori TA, Dunstan JA. Maternal fish oil supplementation in pregnancy modifies neonatal leukotriene production by cord-blood-derived neutrophils. Clin Sci (Lond). 2007 Nov;113:409-16.

26. Prescott SL, Dunstan JA. Prenatal fatty acid status and immune development: the pathways and the evidence. Lipids. 2007 Sep;42:801-10.

27. Auvin S, Collet F, Gottrand F, Husson MO, Leroy X, Beermann C, Guery BP. Long-chain polyunsaturated fatty acids modulate lung inflammatory response induced by Pseudomonas aeruginosa in mice. Pediatr Res. 2005 Aug;58:211-5.

28. Denburg JA, Hatfield HM, Cyr MM, Hayes L, Holt PG, Sehmi R, Dunstan JA, Prescott SL. Fish oil supplementation in pregnancy modifies neonatal progenitors at birth in infants at risk of atopy. Pediatr Res. 2005 Feb;57:276-81.

29. Krauss-Etschmann S, Hartl D, Rzehak P, Heinrich J, Shadid R, Del Carmen Ramirez-Tortosa M, Campoy C, Pardillo S, Schendel DJ, *et al.* Decreased cord blood IL-4, IL-13, and CCR4 and increased TGF-beta levels after fish oil supplementation of pregnant women. J Allergy Clin Immunol. 2008 Feb;121:464-70 e6.

30. McDade TW, Beck MA, Kuzawa C, Adair LS. Prenatal undernutrition, postnatal environments, and antibody response to vaccination in adolescence. Am J Clin Nutr. 2001 Oct;74:543-8.

31. Tielsch JM, Rahmathullah L, Katz J, Thulasiraj RD, Coles C, Sheeladevi S, Prakash K. Maternal night blindness during pregnancy is associated with low birthweight, morbidity, and poor growth in South India. J Nutr. 2008 Apr;138:787-92.

32. Diness BR, Fisker AB, Roth A, Yazdanbakhsh M, Sartono E, Whittle H, Nante JE, Lisse IM, Ravn H, Rodrigues A, Aaby P, Benn CS. Effect of high-dose vitamin A supplementation on the immune response to Bacille Calmette-Guerin vaccine. Am J Clin Nutr. 2007 Oct;86(4):1152-9.

33. Semba RD, Munasir Z, Beeler J, Akib A, Muhilal, Audet S, Sommer A. Reduced seroconversion to measles in infants given vitamin A with measles vaccination. Lancet. 1995 May 27;345(8961):1330-2.

34. Bhaskaram P, Rao KV. Enhancement in seroconversion to measles vaccine with simultaneous administration of vitamin A in 9-months-old Indian infants. Indian J Pediatr. 1997 Jul-Aug;64(4):503-9.

35. Newton S, Owusu-Agyei S, Ampofo W, Zandoh C, Adjuik M, Adjei G, Tchum S, Filteau S, Kirkwood BR. Vitamin A supplementation enhances infants' immune responses to hepatitis B vaccine but does not affect responses to Haemophilus influenzae type b vaccine. J Nutr. 2007 May;137(5):1272-7.

36. Siegrist CA. Neonatal and early life vaccinology. Vaccine. 2001 May 14;19:3331-

37. Marchant A, Goldman M. T cell-mediated immune responses in human newborns: ready to learn? Clin Exp Immunol. 2005 Jul;141:10-8.

38. Ota MO, Vekemans J, Schlegel-Haueter SE, Fielding K, Whittle H, Lambert PH, McAdam KP, Siegrist CA, Marchant A. Hepatitis B immunisation induces higher antibody and memory Th2 responses in new-borns than in adults. Vaccine. 2004 Jan 2;22:511-9.

39. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev. 2003 Aug;8:223-46.

40. Dunstan JA, Roper J, Mitoulas L, Hartmann PE, Simmer K, Prescott SL. The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition. Clin Exp Allergy. 2004 Aug;34:1237-42.

41. Vazquez-Martinez JL, Coreno-Juarez MO, Montano-Estrada LF, Attlan M, Gomez-Dantes H. Seroprevalence of hepatitis B in pregnant women in Mexico. Salud Publica Mex. 2003 May-Jun;45:165-70.

TABLE 1 Selected maternal and infant characteristics

	DHA Group (*n*=292)	**Placebo Group (*n*=270)**	***P*-value**
At Baseline			
Age, mean (SD), y	26.5 (4.9)	25.8 (4.7)	0.16
Gestational age, mean (SD), wk	20.6 (2.0)	20.6 (1.8)	0.91
Primipara, %	34.9	38.8	0.35
Parity, mean (SD)	2.1 (1.0)	2.0 (1.1)	0.36
Weight, mean (SD), kg	62.9 (11.6)	63.0 (11.8)	0.89
Height, mean (SD), cm	155.2 (5.6)	155.5 (5.6)	0.48
BMI, mean (SD), kg/m^2	26.1 (4.4)	26.0 (4.3)	0.82
Schooling, mean (SD), y	11.7 (3.4)	11.9 (3.4)	0.32
SES, mean (SD), PCA	0.05 (0.91)	-0.06 (1.0)	0.17
Infant outcomes			
Gestational age, mean (SD), wk	39.1 (1.8)	39.2 (1.7)	0.62
Premature, %, <37 wk	9.3	8.9	0.89
Birth weight, mean (SD), g	3237.5 (427.5)	3184.8 (449.9)	0.13
% male	57.2	54.5	0.51
% breastfed,1 mo	94.8	94.7	0.96
% exclusively breastfed, 1 mo	17.8	18.9	0.74
% breastfed, 3 mo	81.9	84.1	0.50
% exclusively breastfed, 3 mo	15.8	16.0	0.94

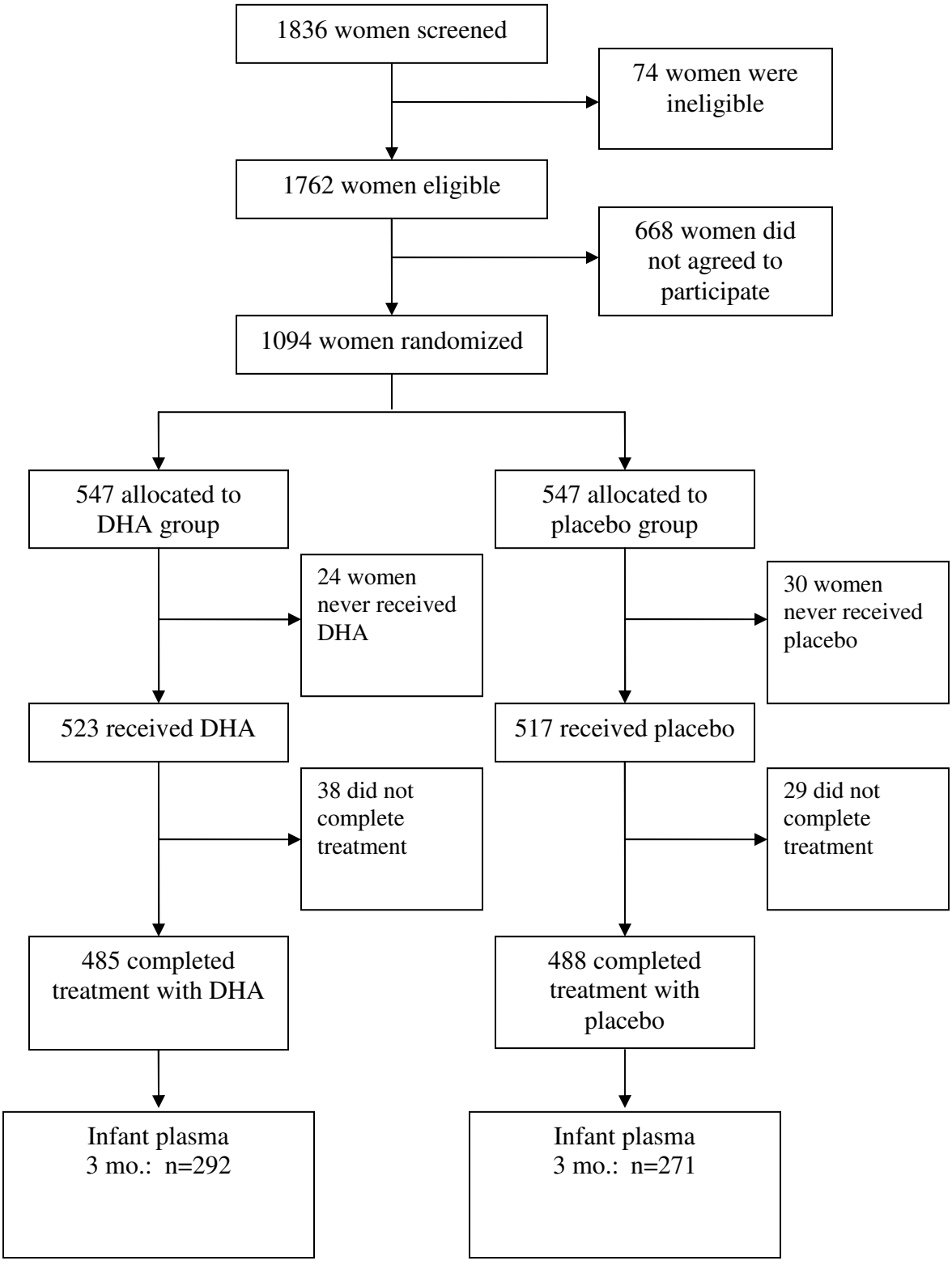
PCA indicates principal components analysis. Chi square tests, T-tests or Wilcoxon rank-sum tests were used to assess significant differences between groups.

TABLE 2 Anti-hepatitis B and anti-tetanus IgG and IgM antibody concentrations in infants at 3 months

	DHA Group (*n*=292) Median (Q1, Q3)	Placebo Group (*n*=270) Median (Q1, Q3)	Risk ratio (95% CI) *unadjusted*
Anti-hepatitis B IgG antibody (ug/ml)	428 (300, 638)	453 (309, 765)	0.88 (0.79, 0.99)[1]
Anti-tetanus IgG antibody (ug/ml)	2487 (1746, 3631)	2695 (1805, 4355)	0.92 (0.82, 1.02)[1]
Anti-hepatitis B IgM antibody (ug/ml)	518 (349, 836)	533 (345, 828)	0.97 (0.84, 1.11)[1]
Anti-tetanus IgM antibody (ug/ml)	443 (285, 720)	441 (297, 709)	0.95 (0.83, 1.10)[1]

[1]Group comparisons were made using natural log-transformed values; estimates were exponentiated.

Figure 1. Trial profile



CHAPTER 8: SUMMARY AND CONCLUSIONS

The current study was conducted within the context of an ongoing randomized, double-blind, placebo-controlled trial in Cuernavaca, Mexico, in which pregnant women were supplemented with 400 mg/day of algae-derived docosahexaenoic acid (DHA, 22:6n-3) from 18-22 weeks' gestation through parturition. We described the influence of prenatal DHA intake on 1) breast milk fatty acid concentrations at 1 month post-partum, 2) infant morbidity at 1, 3 and 6 months of age and 3) infant immune response to hepatitis B and tetanus vaccination at 3 months of age. To our knowledge, this was the first study of its kind to examine the influence of prenatal DHA supplementation on the aforementioned outcomes.

Key findings

DHA concentrations in breast milk

We showed that prenatal DHA supplementation resulted in significantly higher concentrations of both DHA and alpha-linolenic acid (ALA), expressed as % of total fatty acids, in breast milk at 1 month post-partum. Mean breast milk DHA concentrations in the DHA and placebo groups were 0.20 (SD=0.06) and 0.17 (SD=0.07), respectively (Δ=0.02, 95% CI=-0.04, -0.004), and mean ALA concentrations were 1.38 (SD=0.47) and 1.24 (SD=0.46), respectively (p=0.02). Maternal plasma DHA concentrations correlated positively with breast milk DHA at 1 month post-partum in both the placebo and DHA groups (r=0.4, p<0.01 for both treatment groups). Similarly, arachidonic acid (AA) concentrations in maternal plasma at 1 month post-partum correlated positively

with breast milk AA concentrations in both study arms ($r=0.5$, $p<0.01$ for both treatment groups).

The preceding findings agree with current literature on maternal DHA nutriture and its influence on breast milk DHA concentrations; however, our study contributes new information about the specific influence of DHA supplementation alone during the second half of pregnancy on breast milk PUFA concentrations. Our findings complement recent findings by Dunstan *et al.*, who showed that supplementation of pregnant atopic women with fish oil from 20 weeks' gestation through parturition resulted in higher breast milk DHA and EPA concentrations at 3 days and 6 weeks post-partum (46). Similar to our study, women in this study were supplemented only during pregnancy; however, this study focused on atopic women, and the women were supplemented with fish oil.

Our findings reveal that although DHA supplementation resulted in higher levels of DHA in breast milk, the concentration of DHA in breast milk in this population is low. Breast milk PUFA composition varies among different populations and DHA concentrations have been shown to range from 0.17 to 0.99 % of total fatty acids, with a mean concentration worldwide of 0.32% as estimated in a recent meta-analysis (43, 88). Mean breast milk DHA concentrations in our study population are similarly low as those seen in women in the United States and other countries where dietary DHA consumption is lower than recommended (42, 43).

Although our findings show that DHA in pregnancy improved breast milk DHA concentrations, we do not know if this difference is biologically significant for the breastfed infant. However, since maternal DHA status declines in later pregnancy and

early lactation, any significant increase in DHA concentration should be deemed desirable. Higher concentration of DHA in breast milk indicates better maternal DHA status, which could benefit both the infant and the mother. The infant could benefit because DHA in breast milk correlates positively with DHA nutriture of the infant, which has been shown to influence cognitive development (72, 74, 155). Additionally, improved DHA nutriture in the mother may provide benefits such as a lower risk of pre-eclampsia and post-partum depression (156-159). Since DHA was given to the mothers during pregnancy, any significant difference in outcomes in this study, such as infant morbidity, could be attributed to either DHA exposure *in utero*, DHA intake through breast milk, or a combination of *in utero* and early life exposure to DHA.

Infant morbidity

Analysis of infant morbidity revealed that the occurrence of specific illness symptoms did not differ between the groups at 1, 3 and 6 months; however, the occurrence of a combined measure of cold symptoms was lower in the DHA group (OR=0.76; 95% CI=0.58,1.00) at 1 month. Duration of illness varied between groups at 1, 3 and 6 months. At 1 month, the DHA group experienced 26, 15 and 30% shorter duration of cough (P<0.01), phlegm (P=0.01) and wheezing (P<0.01), respectively, but had 22% longer duration of rash (P<0.01). Although we did not find significant group differences in occurrence of symptoms at 3 months of age, the DHA group continued to experience, with borderline significance, lower occurrence of colds (OR=0.77; 95% CI=0.59, 1.02). Additionally, children in the DHA group experienced fewer total days ill from all symptoms than children in the placebo group (RR=0.86, 95% CI: 0.80, 0.93). At

6 months, infants exposed to DHA experienced 20, 13, 54, 22, and 25% shorter duration of fever (P=0.03), nasal secretion (P=0.02), difficulty breathing (P=0.02), rash (P<0.01) and "other illness" (P=0.01), respectively, but longer duration of vomiting (P<0.01).

In general, infants in the DHA group experienced shorter duration of illness symptoms, which is especially clinically relevant for symptoms such as difficulty breathing (the DHA group experienced 54% shorter duration). The lower occurrence of cold symptoms in the DHA group at 1 month is also clinically relevant because this is an immunologically naïve population that is more likely to experience complications from infections than older children and adults. Several studies have evaluated the influence of essential fatty acid (EFA) supplementation in infancy and childhood on illness and biological immunity outcomes and have found decreased risk of illnesses such as pneumonia, bronchitis, and respiratory infections with supplementation with LCPUFAs (34-36, 107, 135). However, our study was the first to examine the influence of DHA in pregnancy on infant morbidity. We also examined the influence of prenatal DHA on the infants' response to vaccination.

Infant response to hepatitis B and tetanus vaccination

Infants were vaccinated against hepatitis B at birth and at 2 months of age, and against tetanus at 2 months of age. Mothers were given a tetanus toxoid vaccination during pregnancy. We collected infant plasma at 3 months of age and determined concentrations of both anti-hepatitis B and anti-tetanus IgG and IgM antibodies. Concentration of anti-hepatitis B IgG antibody was 12% higher (RR: 0.88; 95% CI: 0.79, 0.99) in the placebo group (GM=525.3, 95% CI=484.4, 569.7), compared to the DHA

group (GM=464.9, 95% CI=430.0, 502.7), while concentrations of anti-hepatitis B IgM and anti-tetanus IgG and IgM were similar between groups. We found a weak positive Spearman correlation in the placebo group between anti-hepatitis B IgG and SES (r=.1). In the DHA group, we found weak positive correlations between anti-hepatitis B IgG and maternal age (r=.2) and parity (r=.1) and anti-tetanus IgG and birth weight (r=.2).

Since IgG is transferred to the fetus from the mother, we cannot definitively conclude that the anti-hepatitis B IgG in the infant plasma was generated by the infant. However, infants indeed begin to synthesize their own IgG in response to immunological challenges by 2-3 months of age, especially in the case of a booster vaccination (hepatitis B). Further, women were randomly assigned to the study groups, had similar baseline characteristics, and therefore presumably represent the study population and should have had similar baseline hepatitis-specific IgG concentrations.

Although our study was the first to examine the influence of DHA in pregnancy on infant response to vaccination, two previous studies examined the effect of fish oil in pregnancy on cytokine concentrations in cord blood. These two studies showed that fish oil supplementation during pregnancy resulted in lower cord blood IL-13 concentrations compared to controls, illustrating a potential pathway by which antibody production might be reduced by fish oil supplementation in pregnancy. IL-13, a Th-2-related cytokine, can stimulate antibody production; hence, reduced IL-13 could potentially result in dampening of antibody response (108, 160).

This dissertation study provides preliminary evidence that neonatal DHA nutriture may influence antibody response to hepatitis B vaccination; however, the exact mechanism of action is yet unclear.

Strengths and limitations

Our study has several strengths, including its design as a double-blind randomized, controlled trial (RCT). The design of an RCT allows researchers to draw direct conclusions about the effect of a treatment, such as prenatal DHA supplementation, on an outcome, such as infant morbidity. If the random allocation of treatment is indeed successful in an RCT, implying that the treatment groups originated from the same population, any group differences in outcomes should be attributable to the intervention without having to control for confounding covariates (161). Maternal baseline characteristics were similar between groups in our current study, implying that randomization of study participants was successful.

Double-blinding in an RCT ensures that, because the participants and investigators do not know the treatment scheme, both groups are treated equally throughout the study and the possibility of selection and other types of bias is therefore very low. True blinding of participants in fish oil trials has been questionable because fish oil capsules, for many people, have a very distinct after-taste. The DHA capsules used in our study were tasteless and indistinguishable from the control capsules, and unlike trials of fish oil capsules, the algae-derived DHA did not cause noticeable side effects such as the taste of fish that would identify the treatment group. To further eliminate bias, all analyses in the current study were conducted following intent-to-treat principles and no individuals were removed from the analyses because of factors such as low compliance.

Compliance, measured as the proportion of capsules consumed of the capsules distributed, was monitored weekly by trained field workers, and was high in our study. Approximately 94% of women in both treatment groups consumed the dose of capsules distributed to them (p=0.6). Overall, loss to follow up of women who initially received treatment through 6 months post-partum was 17%, and was similar between the treatment groups.

Another strength of our study is that it was conducted in a developing country, in a population in which dietary DHA intake is lower than recommended (162). Our study setting and population were unique, as most of the n-3 PUFA supplementation trials have been conducted in Europe, Australia and the United States. The conduct of such trials in developing countries is especially important because DHA intake in many developing countries is particularly low because of the high cost of marine foods and possible lack of knowledge about the potential benefits of intake of n-3 PUFAs during pregnancy.

Finally, all biological samples were handled carefully by the study team in Mexico and at Emory. Plasma was immediately frozen at the IMSS hospital in a nitrogen environment, was later transferred to freezers at the INSP laboratory, and was shipped from Mexico to the CDC on dry ice; all samples arrived at the CDC frozen. For the immunoglobulin analysis, samples were run in duplicate along with internal and external standards, and all samples were analyzed within a three week period.

The present study also had several limitations. Because our study was conducted within the context of an ongoing RCT whose main outcome was child growth and development, it was not initially designed to detect differences in infant morbidity and immune function. Although this is a potential limitation, we were able to obtain frozen

infant plasma from approximately 560 infants and accurate information on morbidity from >830 infants, which allowed us to conduct our analyses with sufficient power. Also, we created the morbidity questionnaires, which were administered from the onset of the parent study. Another potential weakness of our study was that illness symptoms were reported by the mother and not confirmed by a health care professional. Self-reporting of morbidity data can introduce bias, however we saw no indication that illness was under- or over-reported by a particular treatment group in the trial since treatment groups shared similar baseline characteristics and double-blinding was maintained throughout the study (163). Additionally, the use of health calendars/diaries in this study likely greatly aided in maternal recall of child illness during interviews (164).

Another limitation of the current study is that we do not yet have values of baseline maternal plasma DHA for all participants involved in our analyses. As of February 2009, the laboratory at INSP had determined maternal plasma DHA concentrations in a sub-sample of 217 women. If we had included this sub-sample of baseline maternal plasma DHA results in our breast milk analysis, we would have ignored at least 30% of our sample. Likewise, we would have ignored >75% of our morbidity sample and >65% of our sample for the IgG and IgM analysis. This was a limitation because we could not make group comparisons of baseline maternal DHA concentrations to ensure that the groups had similar DHA levels at the onset of the study. The two treatment groups had similar baseline characteristics for all variables tested (e.g. maternal age, weight, height, education, gestational age, SES and dietary intake of PUFAs) in each of our analyses, and it seems unlikely that all baseline characteristics except maternal DHA at baseline would be similar. However, a preliminary analysis of a

sub-group of maternal samples from the parent study suggests the possibility of a difference in baseline DHA concentrations between groups (P=0.046). The analyses are not final and the laboratory at INSP will likely analyze more of the available maternal plasma samples to determine if group differences truly exist. We currently do not know how the sub-sample of maternal plasma was generated, and whether the sub-sample was truly random. This issue will be addressed in the future by investigators in the parent study. Additionally, we would have ideally performed PUFA determinations on all available 1 month breast milk samples; however, limited study resources precluded this.

Implications of the study findings

Since the developing fetus and exclusively breastfed infant rely solely on maternal supply of DHA, adequacy of DHA in maternal diet during pregnancy and lactation is critical. We found that the dose of 400 mg/day influenced outcomes such as breast milk DHA concentrations, infant morbidity and infant response to vaccination. The dose of DHA needed to improve outcomes is important because of recent concerns about the safety of consumption of seafood by pregnant and lactating women and young children.

Seafood, especially large predatory fish such as swordfish, contains methylmercury, a ubiquitous environmental contaminant that becomes concentrated in the flesh of some marine animals. Additionally, the environmental contaminants polychlorinated biphenyls (PCBs) and dioxins, by-products of industrial activities, can accumulate in marine animal flesh. Excess exposure of a fetus or young child to any of these contaminants during gestation or via breast milk can cause harmful neurological

effects such as developmental delays (24). Accordingly, the U.S. Food and Drug Administration (FDA), jointly with the Environmental Protection Agency (EPA), recommends that women of childbearing age, pregnant and breastfeeding women, and young children consume no more than 12 ounces of fish, or approximately 2 servings, per week (165). Similarly, the European Food Safety Authority recommends that women of childbearing age consume 2 fish meals per week (24). The above recommendations state that fish intake should be limited to avoid excess exposure to contaminants; however, these guidelines correspond with recommended dietary intake of DHA. Sioen *et al.* (2008) recently determined using published nutrient and contaminant data that 2 fish meals per week, including fatty fish, is safe and does not exceed the tolerable weekly intake for methylmercury and dioxin-like compounds (166).

Although there is no official Dietary Reference Intake (DRI) for DHA set by the Institute of Medicine, several expert international groups and societies recommend that women of childbearing age and pregnant and lactating women consume at least 200 mg DHA/day (24, 41). Two servings of fish per week, which corresponds with the safe limit set by the FDA and EPA, would provide this amount of DHA. For example, a 6 ounce serving of cooked Alaskan salmon contains approximately 2.5 g of DHA (167). Two meals per week would provide 5 grams of DHA per week, or about 700 g of DHA/d. This estimation of daily intake would be lower for different types of fish, as salmon is one of the richest sources of DHA.

Our study will contribute to the formulation of future dietary recommendations by demonstrating that a dose of 400 mg/day, equivalent to approximately 2 fish meals per week, influenced breast milk DHA PUFA concentration. We also showed that 400

mg/day was well tolerated by pregnant women and caused no harm to the mothers or the children. The amount of DHA used in our study satisfies the recommended intake for women and children of at least 200 mg DHA/d, but is low enough to avoid the problem of potential intoxication by environmental contaminants if the DHA from seafood is consumed. Many of the other trials have used much higher doses of DHA. For example, in the study most similar to ours, by Dunstan *et al.* (2007) provided a dose of 2.2 g DHA/d (46). This is the equivalent of >2 fish meals per week, which would pose a potential risk for high exposure to contaminants if DHA is consumed in the form of seafood.

Additionally, our study showed that highly refined DHA derived from a single-celled organism (algae) influenced outcomes such as breast milk DHA concentration. Demonstrating the efficacy of algae-derived DHA has important implications; first, purified algae-derived DHA does not contain toxicants such as mercury, and second, this source of DHA provides an option other than fish oil and fish for persons who do not consume seafood. Arterburn *et al.* (2008) recently demonstrated that DHA from algal oil is bioequivalent to DHA from salmon by reporting similar erythrocyte and plasma concentrations in persons who had eaten salmon versus people who had consumed an algal oil DHA supplement (168). Also, the unique design of the current study allows us to examine the influence of prenatal DHA on outcomes without having to consider EPA, the other n-3 PUFA contained in fish oil. This provides insight into the influence of an individual long-chain PUFAs, DHA, on outcomes of interest.

These study results, along with results from other available studies, should help guide the construct of future dietary recommendations for DHA and recommendations on inclusion of n-3 PUFAs in prenatal vitamins.

Results from the immune function portion of this dissertation study further strengthen a proposed relationship between *in utero* exposure to n-3 PUFAs and development of immune function. These results do not have immediate policy implications because more research is necessary to further elucidate DHA's role in immune function. Our study did demonstrate, however, that DHA in pregnancy can potentially benefit the infant by shortening duration of illness. Our findings support previous studies that demonstrated the potential immunomodulatory influence of fish oil in pregnancy, while providing new insight into DHA's role in humoral immune response. We report lower anti-hepatitis B IgG concentrations in the DHA group, which is reasonably consistent with findings of DHA's role in potentially dampening Th2 polarization by decreasing production of Th2-related cytokines. The clinical and long-term significance of the finding that DHA modulated response to hepatitis B vaccination in our study population are yet unknown and may be partially revealed within the context of the currently ongoing randomized, controlled trial. Because anti-hepatitis B IgG antibodies were detected in all infant plasma samples indicating seroconversion, and because all infants will receive a third hepatitis B vaccination, the differences in mean anti-hepatitis B IgG concentrations will likely not impact the infants' ability to mount an adequate response to a potential future hepatitis B infection.

Generalizability

Results from our study could be generalized to other populations of pregnant women of middle-to-lower socioeconomic status who have similarly low dietary DHA intakes in both developed and developing countries. Our study population has lower infectious disease rates than many developing countries, in particular for diarrhea and fever. Therefore, our results on infant morbidity can only be generalized to population with similar nutritional status and disease rates.

Future analyses and studies

The current study was conducted within the context of a large ongoing RCT, and a wealth of data will continue to become available in the future. The following research questions, among many others, could be addressed within the context of the current RCT to complement the present dissertation analyses: 1) Do group differences in breast milk DHA concentrations remain at 3 months post-partum, 2) What is the relationship between breast milk PUFAs and infant PUFAs at 3 months of age, 3) Do concentrations of IgA in breast milk at 1 and 3 months differ between treatment groups 4) Do group differences in morbidity persist into later infancy and early childhood, and 5) Do group differences in anti-hepatitis B IgG antibody concentrations persist after the third vaccination at 4 months.

Because few studies have examined the influence of prenatal n-3 PUFAs on infant morbidity and immune function, more large RCTs designed specifically to detect differences in immune function are needed to further explore this relationship. Additionally, analysis of different markers of immune function, such as cytokine

concentrations and composition of cells involved in immunity, would provide additional information about the specific influence of prenatal DHA on immune function.

Further studies designed specifically to examine the influence of perinatal n-3 PUFA nutriture on infant immune function, including both biological and clinically relevant outcomes, are necessary to evaluate the potential value of dietary modification or n-3 PUFA supplementation during pregnancy and/or during lactation. We could gain important knowledge about optimal timing and influence of DHA supplementation in pregnancy and lactation through the conduct of the following 4-arm study: 1) placebo, 2) DHA supplementation in pregnancy only (20 weeks' gestation through parturition), 3) DHA supplementation in breastfeeding only (onset of breastfeeding through 4 months post-partum) and 4) DHA supplementation in pregnancy and lactation (20 weeks' gestation through 4 months post-partum). Results from this ideal study would provide additional information about the influence of DHA on *in utero* programming of immune function, early development of infant immune function, and a combination of both prenatal and postnatal factors.

Summary

In summary, this dissertation study provided novel information about the influence of DHA supplementation from 18-22 weeks' gestation through parturition on breast milk fatty acid concentration and morbidity and immune response in Mexican infants. We reported that DHA supplementation improved breast milk DHA concentration and overall, reduced the duration of illness symptoms in infants. We also found lower mean concentrations of anti-hepatitis B IgG in the DHA group at 3 months;

however, the clinical outcome of this difference is unknown. The finding of a higher concentration of breast milk DHA in the intervention group suggests that DHA's influence on infant immune function might begin *in utero*, where the immune system first begins to form, and continue later during breastfeeding, when the infant immune system continues to mature. These findings reinforce our conceptual framework proposing that infant immune function is influenced by both *in utero* exposure to DHA and exposure to DHA in breastfeeding. The DHA provided during pregnancy may have also altered maternal immune function both in pregnancy and lactation, which, in turn, could influence infant immune function. Our findings contribute to the mounting evidence that perinatal n-3 PUFA nutriture may influence the development and maturation of fetal and infant immune response.

LITERATURE CITED

(Chapters 1-4, 8)

1. www.who.int.
2. Jackson A, Calder P. Severe Undernutrition and Immunity In: Gershwin M, Nestel P, Keen C, eds. Handbook of Nutrition and Immunity: Humana Press Inc., 2004.
3. Keen C, Uriu-Adams J, Ensunsa J, Gershwin M. Trace Elements/Minerals and Immunity. In: Gershwin M, Nestel P, Keen C, eds. Handbook of Nutrition and Immunity: Humana Press Inc., 2004.
4. Ramakrishnan U, Webb A, Ologoudou K. Infection, Immunity, and Vitamins In: Gershwin M, Nestel P, Keen C, eds. Handbook of Nutrition and Immunity: Humana Press Inc., 2004.
5. Newton S, Owusu-Agyei S, Ampofo W, et al. Vitamin A supplementation enhances infants' immune responses to hepatitis B vaccine but does not affect responses to Haemophilus influenzae type b vaccine. J Nutr 2007;137:1272-7.
6. Bhandari N, Bahl R, Taneja S, et al. Effect of routine zinc supplementation on pneumonia in children aged 6 months to 3 years: randomised controlled trial in an urban slum. BMJ 2002;324:1358.
7. Bhandari N, Bahl R, Taneja S, et al. Substantial reduction in severe diarrheal morbidity by daily zinc supplementation in young north Indian children. Pediatrics 2002;109:e86.
8. Strand TA, Chandyo RK, Bahl R, et al. Effectiveness and efficacy of zinc for the treatment of acute diarrhea in young children. Pediatrics 2002;109:898-903.
9. Hussey GD, Klein M. A randomized, controlled trial of vitamin A in children with severe measles. N Engl J Med 1990;323:160-4.
10. Klein M, Hussey GD. Vitamin A reduces morbidity and mortality in measles. S Afr Med J 1990;78:56-8.
11. Tielsch JM, Rahmathullah L, Katz J, et al. Maternal night blindness during pregnancy is associated with low birthweight, morbidity, and poor growth in South India. J Nutr 2008;138:787-92.
12. Long KZ, Rosado JL, DuPont HL, Hertzmark E, Santos JI. Supplementation with vitamin A reduces watery diarrhoea and respiratory infections in Mexican children. Br J Nutr 2007;97:337-43.
13. Long KZ, Rosado JL, Fawzi W. The comparative impact of iron, the B-complex vitamins, vitamins C and E, and selenium on diarrheal pathogen outcomes relative to the impact produced by vitamin A and zinc. Nutr Rev 2007;65:218-32.
14. Villamor E, Fawzi WW. Effects of vitamin a supplementation on immune responses and correlation with clinical outcomes. Clin Microbiol Rev 2005;18:446-64.
15. Moore SE, Cole TJ, Collinson AC, Poskitt EM, McGregor IA, Prentice AM. Prenatal or early postnatal events predict infectious deaths in young adulthood in rural Africa. Int J Epidemiol 1999;28:1088-95.

16. McDade TW, Beck MA, Kuzawa C, Adair LS. Prenatal undernutrition, postnatal environments, and antibody response to vaccination in adolescence. Am J Clin Nutr 2001;74:543-8.
17. Christian P, West KP, Khatry SK, et al. Effects of maternal micronutrient supplementation on fetal loss and infant mortality: a cluster-randomized trial in Nepal. Am J Clin Nutr 2003;78:1194-202.
18. Field CJ, Clandinin MT, Van Aerde JE. Polyunsaturated fatty acids and T-cell function: implications for the neonate. Lipids 2001;36:1025-32.
19. Hanebutt FL, Demmelmair H, Schiessl B, Larque E, Koletzko B. Long-chain polyunsaturated fatty acid (LC-PUFA) transfer across the placenta. Clin Nutr 2008;27:685-93.
20. Crawford M. Placental delivery of arachidonic and docosahexaenoic acids: implications for the lipid nutrition of preterm infants. Am J Clin Nutr 2000;71:275S-84S.
21. McCann JC, Ames BN. Is docosahexaenoic acid, an n-3 long-chain polyunsaturated fatty acid, required for development of normal brain function? An overview of evidence from cognitive and behavioral tests in humans and animals.[see comment]. American Journal of Clinical Nutrition 2005;82:281-95.
22. Uauy R, Mena P, Rojas C. Essential fatty acids in early life: structural and functional role. Proc Nutr Soc 2000;59:3-15.
23. Al MD, van Houwelingen AC, Hornstra G. Long-chain polyunsaturated fatty acids, pregnancy, and pregnancy outcome. Am J Clin Nutr 2000;71:285S-91S.
24. Koletzko B, Lien E, Agostoni C, et al. The roles of long-chain polyunsaturated fatty acids in pregnancy, lactation and infancy: review of current knowledge and consensus recommendations. J Perinat Med 2008;36:5-14.
25. Calder PC. Dietary modification of inflammation with lipids. Proc Nutr Soc 2002;61:345-58.
26. Calder PC, Grimble RF. Polyunsaturated fatty acids, inflammation and immunity. Eur J Clin Nutr 2002;56 Suppl 3:S14-9.
27. Yaqoob P. Lipids and the immune response. Curr Opin Clin Nutr Metab Care 1998;1:153-61.
28. Yaqoob P. Fatty acids and the immune system: from basic science to clinical applications. Proc Nutr Soc 2004;63:89-104.
29. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. Lipids 2001;36:1007-24.
30. Calder PC. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. Lipids 2003;38:343-52.
31. Calder PC. Immunomodulation by omega-3 fatty acids. Prostaglandins Leukot Essent Fatty Acids 2007;77:327-35.
32. Deckelbaum RJ, Worgall TS, Seo T. n-3 fatty acids and gene expression. Am J Clin Nutr 2006;83:1520S-1525S.
33. Gottrand F. Long-chain polyunsaturated Fatty acids influence the immune system of infants. J Nutr 2008;138:1807S-1812S.
34. Merchant AT, Curhan GC, Rimm EB, Willett WC, Fawzi WW. Intake of n-6 and n-3 fatty acids and fish and risk of community-acquired pneumonia in US men. Am J Clin Nutr 2005;82:668-74.

35. Venuta A, Spano C, Laudizi L, Bettelli F, Beverelli A, Turchetto E. Essential fatty acids: the effects of dietary supplementation among children with recurrent respiratory infections. J Int Med Res 1996;24:325-30.
36. Pastor N, Soler B, Mitmesser SH, Ferguson P, Lifschitz C. Infants fed docosahexaenoic acid- and arachidonic acid-supplemented formula have decreased incidence of bronchiolitis/bronchitis the first year of life. Clin Pediatr (Phila) 2006;45:850-5.
37. Smithers LG, Gibson RA, McPhee A, Makrides M. Effect of long-chain polyunsaturated fatty acid supplementation of preterm infants on disease risk and neurodevelopment: a systematic review of randomized controlled trials. Am J Clin Nutr 2008;87:912-20.
38. Rees D, Miles EA, Banerjee T, et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older men.[see comment]. American Journal of Clinical Nutrition 2006;83:331-42.
39. Dunstan JA, Mori TA, Barden A, et al. Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. J Allergy Clin Immunol 2003;112:1178-84.
40. Hibbeln JR, Nieminen LRG, Blasbalg TL, Riggs JA, Lands WEM. Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. American Journal of Clinical Nutrition 2006;83:1483S-1493S.
41. Koletzko B, Cetin I, Brenna JT. Dietary fat intakes for pregnant and lactating women. Br J Nutr 2007;98:873-7.
42. Kris-Etherton PM, Taylor DS, Yu-Poth S, et al. Polyunsaturated fatty acids in the food chain in the United States. Am J Clin Nutr 2000;71:179S-88S.
43. Brenna JT, Varamini B, Jensen RG, Diersen-Schade DA, Boettcher JA, Arterburn LM. Docosahexaenoic and arachidonic acid concentrations in human breast milk worldwide. American Journal of Clinical Nutrition 2007;85:1457-64.
44. Al MD, van Houwelingen AC, Kester AD, Hasaart TH, de Jong AE, Hornstra G. Maternal essential fatty acid patterns during normal pregnancy and their relationship to the neonatal essential fatty acid status. Br J Nutr 1995;74:55-68.
45. Jensen CL, Maude M, Anderson RE, Heird WC. Effect of docosahexaenoic acid supplementation of lactating women on the fatty acid composition of breast milk lipids and maternal and infant plasma phospholipids. Am J Clin Nutr 2000;71:292S-9S.
46. Dunstan JA, Mitoulas LR, Dixon G, et al. The effects of fish oil supplementation in pregnancy on breast milk fatty acid composition over the course of lactation: a randomized controlled trial. Pediatr Res 2007;62:689-94.
47. Dunstan JA, Mori TA, Barden A, et al. Effects of n-3 polyunsaturated fatty acid supplementation in pregnancy on maternal and fetal erythrocyte fatty acid composition. Eur J Clin Nutr 2004;58:429-37.
48. Krauss-Etschmann S, Shadid R, Campoy C, et al. Effects of fish-oil and folate supplementation of pregnant women on maternal and fetal plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid: a European randomized multicenter trial. American Journal of Clinical Nutrition 2007;85:1392-400.

49. Bergmann RL, Haschke-Becher E, Klassen-Wigger P, et al. Supplementation with 200 mg/day docosahexaenoic acid from mid-pregnancy through lactation improves the docosahexaenoic acid status of mothers with a habitually low fish intake and of their infants. Ann Nutr Metab 2008;52:157-66.
50. Makrides M, Simmer K, Neumann M, Gibson R. Changes in the polyunsaturated fatty acids of breast milk from mothers of full-term infants over 30 wk of lactation. Am J Clin Nutr 1995;61:1231-3.
51. Bonham MP, Duffy EM, Wallace JM, et al. Habitual fish consumption does not prevent a decrease in LCPUFA status in pregnant women (the Seychelles Child Development Nutrition Study). Prostaglandins Leukotrienes & Essential Fatty Acids 2008;78:343-50.
52. Makrides M, Gibson RA. Long-chain polyunsaturated fatty acid requirements during pregnancy and lactation. Am J Clin Nutr 2000;71:307S-11S.
53. Picciano MF, McGuire MK. Use of dietary supplements by pregnant and lactating women in North America. Am J Clin Nutr 2009;89:663S-7S.
54. M'Rabet L, Vos AP, Boehm G, Garssen J. Breast-feeding and its role in early development of the immune system in infants: consequences for health later in life. J Nutr 2008;138:1782S-1790S.
55. Burdge GC, Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reproduction, Nutrition, Development 2005;45:581-97.
56. Arterburn LM, Hall EB, Oken H. Distribution, interconversion, and dose response of n-3 fatty acids in humans. American Journal of Clinical Nutrition 2006;83:1467S-1476S.
57. Brenna JT. Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. Curr Opin Clin Nutr Metab Care 2002;5:127-32.
58. Innis SM. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. J Pediatr 2003;143:S1-8.
59. Brenna JT, Salem N, Jr., Sinclair AJ, Cunnane SC. alpha-Linolenic acid supplementation and conversion to n-3 long-chain polyunsaturated fatty acids in humans. Prostaglandins Leukot Essent Fatty Acids 2009;80:85-91.
60. Goyens PLL, Spilker ME, Zock PL, Katan MB, Mensink RP. Conversion of alpha-linolenic acid in humans is influenced by the absolute amounts of alpha-linolenic acid and linoleic acid in the diet and not by their ratio. American Journal of Clinical Nutrition 2006;84:44-53.
61. Burdge GC, Wootton SA. Conversion of alpha-linolenic acid to eicosapentaenoic, docosapentaenoic and docosahexaenoic acids in young women. Br J Nutr 2002;88:411-20.
62. Ezaki O, Takahashi M, Shigematsu T, et al. Long-term effects of dietary alpha-linolenic acid from perilla oil on serum fatty acids composition and on the risk factors of coronary heart disease in Japanese elderly subjects. J Nutr Sci Vitaminol (Tokyo) 1999;45:759-72.
63. Ghafoorunissa, Vani A, Laxmi R, Sesikeran B. Effects of dietary alpha-linolenic acid from blended oils on biochemical indices of coronary heart disease in Indians. Lipids 2002;37:1077-86.

64. Jensen CL, Chen H, Fraley JK, Anderson RE, Heird WC. Biochemical effects of dietary linoleic/alpha-linolenic acid ratio in term infants. Lipids 1996;31:107-13.
65. Clark KJ, Makrides M, Neumann MA, Gibson RA. Determination of the optimal ratio of linoleic acid to alpha-linolenic acid in infant formulas. J Pediatr 1992;120:S151-8.
66. Al MD, Hornstra G, van der Schouw YT, Bulstra-Ramakers MT, Huisjes HJ. Biochemical EFA status of mothers and their neonates after normal pregnancy. Early Hum Dev 1990;24:239-48.
67. Portolesi R, Powell BC, Gibson RA. Competition between 24:5n-3 and ALA for Delta 6 desaturase may limit the accumulation of DHA in HepG2 cell membranes. J Lipid Res 2007;48:1592-8.
68. Innis SM, Elias SL. Intakes of essential n-6 and n-3 polyunsaturated fatty acids among pregnant Canadian women. American Journal of Clinical Nutrition 2003;77:473-8.
69. Gebauer SK, Psota TL, Harris WS, Kris-Etherton PM. n-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. Am J Clin Nutr 2006;83:1526S-1535S.
70. Sweeney B, Puri P, Reen DJ. Modulation of immune cell function by polyunsaturated fatty acids. Pediatr Surg Int 2005;21:335-40.
71. Szajewska H, Horvath A, Koletzko B. Effect of n-3 long-chain polyunsaturated fatty acid supplementation of women with low-risk pregnancies on pregnancy outcomes and growth measures at birth: a meta-analysis of randomized controlled trials. American Journal of Clinical Nutrition 2006;83:1337-44.
72. Innis SM. Dietary omega 3 fatty acids and the developing brain. Brain Res. 2008;Spetember 9 [ahead of print].
73. Carlson SE. Docosahexaenoic acid supplementation in pregnancy and lactation. Am J Clin Nutr 2009;89:678S-84S.
74. Ramakrishnan U, Imhoff-Kunsch B, Digirolamo A. Role of docosahexaenoic acid in maternal and child mental health. Am J Clin Nutr 2009.
75. Koletzko B, Larque E, Demmelmair H. Placental transfer of long-chain polyunsaturated fatty acids (LC-PUFA). J Perinat Med 2007;35 Suppl 1:S5-11.
76. Hornstra G. Essential fatty acids in mothers and their neonates. Am J Clin Nutr 2000;71:1262S-9S.
77. van Houwelingen AC, Sorensen JD, Hornstra G, et al. Essential fatty acid status in neonates after fish-oil supplementation during late pregnancy. Br J Nutr 1995;74:723-31.
78. Connor WE, Lowensohn R, Hatcher L. Increased docosahexaenoic acid levels in human newborn infants by administration of sardines and fish oil during pregnancy. Lipids 1996;31 Suppl:S183-7.
79. Velzing-Aarts FV, van der Klis FR, van der Dijs FP, et al. Effect of three low-dose fish oil supplements, administered during pregnancy, on neonatal long-chain polyunsaturated fatty acid status at birth. Prostaglandins Leukot Essent Fatty Acids 2001;65:51-7.
80. Montgomery C, Speake BK, Cameron A, Sattar N, Weaver LT. Maternal docosahexaenoic acid supplementation and fetal accretion. Br J Nutr 2003;90:135-45.

81. Dunstan JA, Mori TA, Barden A, et al. Maternal fish oil supplementation in pregnancy reduces interleukin-13 levels in cord blood of infants at high risk of atopy. Clin Exp Allergy 2003;33:442-8.
82. de Groot RH, Hornstra G, van Houwelingen AC, Roumen F. Effect of alpha-linolenic acid supplementation during pregnancy on maternal and neonatal polyunsaturated fatty acid status and pregnancy outcome. Am J Clin Nutr 2004;79:251-60.
83. Helland IB, Smith L, Saarem K, Saugstad OD, Drevon CA. Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. Pediatrics 2003;111:e39-44.
84. Smuts CM, Huang M, Mundy D, Plasse T, Major S, Carlson SE. A randomized trial of docosahexaenoic acid supplementation during the third trimester of pregnancy. Obstet Gynecol 2003;101:469-79.
85. Otto SJ, van Houwelingen AC, Hornstra G. The effect of supplementation with docosahexaenoic and arachidonic acid derived from single cell oils on plasma and erythrocyte fatty acids of pregnant women in the second trimester. Prostaglandins Leukot Essent Fatty Acids 2000;63:323-8.
86. Innis SM. Human milk: maternal dietary lipids and infant development. Proc Nutr Soc 2007;66:397-404.
87. Lauritzen L, Halkjaer LB, Mikkelsen TB, et al. Fatty acid composition of human milk in atopic Danish mothers. American Journal of Clinical Nutrition 2006;84:190-6.
88. Yuhas R, Pramuk K, Lien EL. Human milk fatty acid composition from nine countries varies most in DHA. Lipids 2006;41:851-8.
89. Dunstan JA, Roper J, Mitoulas L, Hartmann PE, Simmer K, Prescott SL. The effect of supplementation with fish oil during pregnancy on breast milk immunoglobulin A, soluble CD14, cytokine levels and fatty acid composition. Clin Exp Allergy 2004;34:1237-42.
90. Helland IB, Saugstad OD, Saarem K, Van Houwelingen AC, Nylander G, Drevon CA. Supplementation of n-3 fatty acids during pregnancy and lactation reduces maternal plasma lipid levels and provides DHA to the infants. J Matern Fetal Neonatal Med 2006;19:397-406.
91. Hawkes JS, Bryan DL, Makrides M, Neumann MA, Gibson RA. A randomized trial of supplementation with docosahexaenoic acid-rich tuna oil and its effects on the human milk cytokines interleukin 1 beta, interleukin 6, and tumor necrosis factor alpha. Am J Clin Nutr 2002;75:754-60.
92. Hawkes JS, Bryan DL, Neumann MA, Makrides M, Gibson RA. Transforming growth factor beta in human milk does not change in response to modest intakes of docosahexaenoic acid. Lipids 2001;36:1179-81.
93. Helland IB, Saarem K, Saugstad OD, Drevon CA. Fatty acid composition in maternal milk and plasma during supplementation with cod liver oil. Eur J Clin Nutr 1998;52:839-45.
94. Boris J, Jensen B, Salvig JD, Secher NJ, Olsen SF. A randomized controlled trial of the effect of fish oil supplementation in late pregnancy and early lactation on the n-3 fatty acid content in human breast milk. Lipids 2004;39:1191-6.

95. Lauritzen L, Jorgensen MH, Mikkelsen TB, et al. Maternal fish oil supplementation in lactation: effect on visual acuity and n-3 fatty acid content of infant erythrocytes. Lipids 2004;39:195-206.
96. Lauritzen L, Jorgensen MH, Olsen SF, Straarup EM, Michaelsen KF. Maternal fish oil supplementation in lactation: effect on developmental outcome in breast-fed infants. Reprod Nutr Dev 2005;45:535-47.
97. Offit PA, Quarles J, Gerber MA, et al. Addressing parents' concerns: do multiple vaccines overwhelm or weaken the infant's immune system? Pediatrics 2002;109:124-9.
98. Szepfalusi Z. The maturation of the fetal and neonatal immune system. J Nutr 2008;138:1773S-1781S.
99. Calder PC, Krauss-Etschmann S, de Jong EC, et al. Early nutrition and immunity - progress and perspectives. Br J Nutr 2006;96:774-90.
100. Siegrist CA. Neonatal and early life vaccinology. Vaccine 2001;19:3331-46.
101. Marchant A, Goldman M. T cell-mediated immune responses in human newborns: ready to learn? Clin Exp Immunol 2005;141:10-8.
102. Ota MO, Vekemans J, Schlegel-Haueter SE, et al. Hepatitis B immunisation induces higher antibody and memory Th2 responses in new-borns than in adults. Vaccine 2004;22:511-9.
103. Janeway C, Travers P, Walport M, Shlomchik M. Immuno Biology 5: Garland Publishing, 2001.
104. Yaqoob P. Lipids and the immune response: from molecular mechanisms to clinical applications. Curr Opin Clin Nutr Metab Care 2003;6:133-50.
105. Kelley DS. Modulation of human immune and inflammatory responses by dietary fatty acids. Nutrition 2001;17:669-73.
106. Das UN. Perinatal supplementation of long-chain polyunsaturated fatty acids, immune response and adult diseases. Med Sci Monit 2004;10:HY19-25.
107. Field CJ, Van Aerde JE, Robinson LE, Clandinin MT. Effect of providing a formula supplemented with long-chain polyunsaturated fatty acids on immunity in full-term neonates. Br J Nutr 2008;99:91-9.
108. Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003;8:223-46.
109. Calder PC, Yaqoob P, Thies F, Wallace FA, Miles EA. Fatty acids and lymphocyte functions. Br J Nutr 2002;87 Suppl 1:S31-48.
110. Calder PC, Yaqoob P. The level of protein and type of fat in the diet of pregnant rats both affect lymphocyte function in the offspring. Nutrition Reviews 2000;20:995-1005.
111. Berger A, German JB, Chiang BL, et al. Influence of feeding unsaturated fats on growth and immune status of mice. J Nutr 1993;123:225-33.
112. Rayon JI, Carver JD, Wyble LE, et al. The fatty acid composition of maternal diet affects lung prostaglandin E2 levels and survival from group B streptococcal sepsis in neonatal rat pups. J Nutr 1997;127:1989-92.
113. Lauridsen C, Stagsted J, Jensen SK. n-6 and n-3 fatty acids ratio and vitamin E in porcine maternal diet influence the antioxidant status and immune cell eicosanoid response in the progeny. Prostaglandins Other Lipid Mediat 2007;84:66-78.

114. Anderson M, Fritsche KL. (n-3) Fatty acids and infectious disease resistance. J Nutr 2002;132:3566-76.
115. Olive AP, Dudley M, Harari Y, Dudley A, Castro GA, Lifschitz CH. Fish oil supplementation does not impair the gut immune response to Trichinella spiralis infection in rats. J Pediatr Gastroenterol Nutr 1999;28:276-81.
116. Paul KP, Leichsenring M, Pfisterer M, et al. Influence of n-6 and n-3 polyunsaturated fatty acids on the resistance to experimental tuberculosis. Metabolism 1997;46:619-24.
117. Puertollano MA, Puertollano E, Ruiz-Bravo A, Jimenez-Valera M, De Pablo MA, De Cienfuegos GA. Changes in the immune functions and susceptibility to Listeria monocytogenes infection in mice fed dietary lipids. Immunol Cell Biol 2004;82:370-6.
118. Beli E, Li M, Cuff C, Pestka JJ. Docosahexaenoic acid-enriched fish oil consumption modulates immunoglobulin responses to and clearance of enteric reovirus infection in mice. J Nutr 2008;138:813-9.
119. Bassaganya-Riera J, Guri AJ, Noble AM, et al. Arachidonic acid-and docosahexaenoic acid-enriched formulas modulate antigen-specific T cell responses to influenza virus in neonatal piglets. Am J Clin Nutr 2007;85:824-36.
120. Byleveld PM, Pang GT, Clancy RL, Roberts DC. Fish oil feeding delays influenza virus clearance and impairs production of interferon-gamma and virus-specific immunoglobulin A in the lungs of mice. J Nutr 1999;129:328-35.
121. Pierre M, Husson MO, Le Berre R, et al. Omega-3 polyunsaturated fatty acids improve host response in chronic Pseudomonas aeruginosa lung infection in mice. Am J Physiol Lung Cell Mol Physiol 2007;292:L1422-31.
122. Auvin S, Collet F, Gottrand F, et al. Long-chain polyunsaturated fatty acids modulate lung inflammatory response induced by Pseudomonas aeruginosa in mice. Pediatr Res 2005;58:211-5.
123. McFarland CT, Fan YY, Chapkin RS, Weeks BR, McMurray DN. Dietary polyunsaturated fatty acids modulate resistance to Mycobacterium tuberculosis in guinea pigs. J Nutr 2008;138:2123-8.
124. Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. Br J Nutr 2003;89:679-89.
125. Kew S, Banerjee T, Minihane AM, et al. Lack of effect of foods enriched with plant- or marine-derived n-3 fatty acids on human immune function. Am J Clin Nutr 2003;77:1287-95.
126. Bechoua S, Dubois M, Vericel E, Chapuy P, Lagarde M, Prigent AF. Influence of very low dietary intake of marine oil on some functional aspects of immune cells in healthy elderly people. Br J Nutr 2003;89:523-31.
127. Miles EA, Banerjee T, Dooper MM, M'Rabet L, Graus YM, Calder PC. The influence of different combinations of gamma-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. Br J Nutr 2004;91:893-903.
128. Kew S, Mesa MD, Tricon S, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. Am J Clin Nutr 2004;79:674-81.

129. Kelley DS, Taylor PC, Nelson GJ, Mackey BE. Dietary docosahexaenoic acid and immunocompetence in young healthy men. Lipids 1998;33:559-66.
130. Kelley DS, Taylor PC, Nelson GJ, et al. Docosahexaenoic acid ingestion inhibits natural killer cell activity and production of inflammatory mediators in young healthy men. Lipids 1999;34:317-24.
131. Thies F, Miles EA, Nebe-von-Caron G, et al. Influence of dietary supplementation with long-chain n-3 or n-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. Lipids 2001;36:1183-93.
132. Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC. Dietary supplementation with gamma-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans. J Nutr 2001;131:1918-27.
133. Thies F, Nebe-von-Caron G, Powell JR, Yaqoob P, Newsholme EA, Calder PC. Dietary supplementation with eicosapentaenoic acid, but not with other long-chain n-3 or n-6 polyunsaturated fatty acids, decreases natural killer cell activity in healthy subjects aged >55 y. Am J Clin Nutr 2001;73:539-48.
134. Sijben JW, Calder PC. Differential immunomodulation with long-chain n-3 PUFA in health and chronic disease. Proceedings of the Nutrition Society 2007;66:237-59.
135. Mazurak VC, Lien V, Field CJ, Goruk SD, Pramuk K, Clandinin MT. Long-chain polyunsaturated fat supplementation in children with low docosahexaenoic acid intakes alters immune phenotypes compared with placebo. J Pediatr Gastroenterol Nutr 2008;46:570-9.
136. Nagakura T, Matsuda S, Shichijyo K, Sugimoto H, Hata K. Dietary supplementation with fish oil rich in omega-3 polyunsaturated fatty acids in children with bronchial asthma. Eur Respir J 2000;16:861-5.
137. Mihrshahi S, Peat JK, Webb K, Oddy W, Marks GB, Mellis CM. Effect of omega-3 fatty acid concentrations in plasma on symptoms of asthma at 18 months of age. Pediatr Allergy Immunol 2004;15:517-22.
138. Broughton KS, Johnson CS, Pace BK, Liebman M, Kleppinger KM. Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. Am J Clin Nutr 1997;65:1011-7.
139. Field CJ, Thomson CA, Van Aerde JE, et al. Lower proportion of CD45R0+ cells and deficient interleukin-10 production by formula-fed infants, compared with human-fed, is corrected with supplementation of long-chain polyunsaturated fatty acids. J Pediatr Gastroenterol Nutr 2000;31:291-9.
140. Auestad N, Scott DT, Janowsky JS, et al. Visual, cognitive, and language assessments at 39 months: a follow-up study of children fed formulas containing long-chain polyunsaturated fatty acids to 1 year of age. Pediatrics 2003;112:e177-83.
141. Damsgaard CT, Lauritzen L, Kjaer TM, et al. Fish oil supplementation modulates immune function in healthy infants. J Nutr 2007;137:1031-6.
142. Moore SE, Cole TJ, Poskitt EM, et al. Season of birth predicts mortality in rural Gambia. Nature 1997;388:434.

143. Moore SE, Collinson AC, Prentice AM. Immune function in rural Gambian children is not related to season of birth, birth size, or maternal supplementation status. Am J Clin Nutr 2001;74:840-7.
144. McDade TW, Beck MA, Kuzawa CW, Adair LS. Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. J Nutr 2001;131:1225-31.
145. McDade TW, Kuzawa CW, Adair LS, Beck MA. Prenatal and early postnatal environments are significant predictors of total immunoglobulin E concentration in Filipino adolescents. Clin Exp Allergy 2004;34:44-50.
146. Parra MS, Schnaas L, Meydani M, Perroni E, Martinez S, Romieu I. Erythrocyte cell membrane phospholipid levels compared against reported dietary intakes of polyunsaturated fatty acids in pregnant Mexican women. Public Health Nutr 2002;5:931-7.
147. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.
148. Dasso J, Lee J, Bach H, Mage RG. A comparison of ELISA and flow microsphere-based assays for quantification of immunoglobulins. J Immunol Methods 2002;263:23-33.
149. Earley MC, Vogt RF, Jr., Shapiro HM, et al. Report from a workshop on multianalyte microsphere assays. Cytometry 2002;50:239-42.
150. Vignali DA. Multiplexed particle-based flow cytometric assays. J Immunol Methods 2000;243:243-55.
151. Cohen J. Statistical Power Analysis for the Behavioral Sciences. Second Edition ed. Hillsdale, New Jersey: Lawrence Erlbaum Associates, Publishers, 1988.
152. van Gool CJ, Thijs C, Dagnelie PC, et al. Determinants of neonatal IgE level: parity, maternal age, birth season and perinatal essential fatty acid status in infants of atopic mothers. Allergy 2004;59:961-8.
153. Scirica CV, Gold DR, Ryan L, et al. Predictors of cord blood IgE levels in children at risk for asthma and atopy. J Allergy Clin Immunol 2007;119:81-8.
154. Dewey KG, Romero-Abal ME, Quan de Serrano J, et al. A randomized intervention study of the effects of discontinuing coffee intake on growth and morbidity of iron-deficient Guatemalan toddlers. J Nutr 1997;127:306-13.
155. Kidd PM. Omega-3 DHA and EPA for cognition, behavior, and mood: clinical findings and structural-functional synergies with cell membrane phospholipids. Altern Med Rev 2007;12:207-27.
156. Hibbeln JR. Seafood consumption, the DHA content of mothers' milk and prevalence rates of postpartum depression: a cross-national, ecological analysis. J Affect Disord 2002;69:15-29.
157. Su KP, Huang SY, Chiu TH, et al. Omega-3 fatty acids for major depressive disorder during pregnancy: results from a randomized, double-blind, placebo-controlled trial. J Clin Psychiatry 2008;69:644-51.
158. Freeman MP. Omega-3 fatty acids and perinatal depression: a review of the literature and recommendations for future research. Prostaglandins Leukot Essent Fatty Acids 2006;75:291-7.

159. Olsen SF, Secher NJ. A possible preventive effect of low-dose fish oil on early delivery and pre-eclampsia: indications from a 50-year-old controlled trial. Br J Nutr 1990;64:599-609.
160. Bost KL, Holton RH, Cain TK, Clements JD. In vivo treatment with anti-interleukin-13 antibodies significantly reduces the humoral immune response against an oral immunogen in mice. Immunology 1996;87:633-41.
161. Sibbald B, Roland M. Understanding controlled trials. Why are randomised controlled trials important? BMJ 1998;316:201.
162. Parra-Cabrera S, Flores R, Stein A, Martorell R, Ramakrishnan U. Dietary Intakes of Omega-3 Fatty acids in Mexican Pregnant Women 10th European Nutrition Conference. Paris, 2006.
163. Manesh AO, Sheldon TA, Pickett KE, Carr-Hill R. Accuracy of child morbidity data in demographic and health surveys. Int J Epidemiol 2008;37:194-200.
164. Goldman N, Vaughan B, Pebley AR. The use of calendars to measure child illness in health interview surveys. Int J Epidemiol 1998;27:505-12.
165. FDA U. What You Need to Know About Mercury in Fish and Shellfish. 2004.
166. Sioen I, De Henauw S, Verbeke W, Verdonck F, Willems JL, Van Camp J. Fish consumption is a safe solution to increase the intake of long-chain n-3 fatty acids. Public Health Nutr 2008;11:1107-16.
167. http://www.ars.usda.gov/ba/bhnrc/ndl.
168. Arterburn LM, Oken HA, Bailey Hall E, Hamersley J, Kuratko CN, Hoffman JP. Algal-oil capsules and cooked salmon: nutritionally equivalent sources of docosahexaenoic acid. J Am Diet Assoc 2008;108:1204-9.

APPENDICES

Appendix 1. Form 20: Infant feeding practices

EMORY UNIVERSITY SCHOOL OF PUBLIC HEALTH

EFECTO DE SUPLEMENTACION PRENATAL CON ÁCIDOS GRASOS OMEGA3 EN EL DESARROLLO NEUROCONDUCTUAL DEL INFANTE

PRACTICAS DE ALIMENTACION INFANTIL

1	**Tipo de Formulario:**	2 0
2	**Número de contacto**	
3	**Número del folio de la participante**	1
4	**Fecha de la entrevista**	d d m m a a
5	**Fecha de nacimiento del niño(a)**	d d m m a a
6	**Código del encuestador**	
7	Edad del niño en meses	
8	Sexo 1. Masculino 2. Femenino	
9	¿Qué número de hijo es? (*contando sólo los hijos nacidos vivos*)	
10	¿Le da pecho al niño? 1. Sí 2. No (*Pase a la pregunta 16*)	
11	¿Le da pecho cada vez que el niño lo pide? 1. Sí 2. No	
12	¿ Ha tenido problemas con los pechos? 1. Sí 2. No (*Pase a la pregunta 14*)	
13	¿Cuáles problemas ha tenido? a. Ausencia de pezón b. Pezón invertido c. Pezón corto d. Dolor e. Grietas f. Infección g. Bolas – Abscesos – Nódulos h. Otros _____ 1. Sí 2. No	a. b. c. d. e. f. g. h.

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EFECTO DE SUPLEMENTACION PRENATAL CON ÁCIDOS GRASOS OMEGA3 EN EL DESARROLLO NEUROCONDUCTUAL DEL INFANTE

PRACTICAS DE ALIMENTACION INFANTIL

14 ¿Cuántas veces le da pecho mientras usted está despierta? ☐☐

15 ¿Cuántas veces en la noche? (*pase a la pregunta 18*) ☐☐

SOLAMENTE SI NO LE ESTA DANDO PECHO

16 ¿Cuánto tiempo le dió pecho? (*días*) ☐☐

17 ¿Por qué le quitó el pecho al niño?

a. Leche insuficiente ☐
b. El niño lo rechazó ☐
c. Problemas con los pechos ☐
d. Recomendación del Doctor ☐
e. Recomendación del esposo ☐
f. Recomendación de la mamá/suegra ☐
g. Recomendación de otra persona ¿quién? _____ ☐
h. Trabajo fuera de casa ☐
i. Falta de tiempo ☐
j. Porque a la madre no le gustaba ☐
k. Porque el niño no subía de peso ☐
m. Enfermedad de la madre ☐
n. Enfermedad del niño ☐
o. Toma mamila y ya no quiere el pecho ☐
p. El niño come otras cosas ☐
q. Otra ¿Cuál? ☐

1. Sí
2. No

k

18 ¿ Le da otra leche diferente del pecho? ☐

1. Sí
2. No (*pase a la pregunta 24*)

19 ¿Cuál leche? (la mayoría de las veces)

a. Fórmula ¿Cuál marca? _____ ☐
b. Leche entera en polvo ☐
c. Leche diluida ☐
d. Leche de soya ☐
e. Otra _____ ☐

1. Sí
2. No

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PRACTICAS DE ALIMENTACION INFANTIL

20 ¿Con qué agua prepara la leche? (*la mayoría de las veces*)

a. Agua hervida
b. Agua de la llave sin hervir
c. Agua de garrafón
d. Agua de garrafón hervida
e. Agua de pozo o manantial
f. Ninguna porque es leche fluida y no le agrega agua
g. Otra _____

1. Sí
2. No

a. ☐ b. ☐ c. ☐ d. ☐ e. ☐ f. ☐ g. ☐

21 ¿Le agrega algún ingrediente más?

1. Sí
2. No (*pase a la pregunta 23*)

☐

22 ¿Qué ingrediente le agrega?

a. Azúcar o miel
b. Cereal ¿cuál marca?_____
c. Atole
d. Otro _____
e. Otro _____

1. Sí
2. No

a. ☐ b. ☐ c. ☐ d. ☐ e. ☐

23 ¿Con qué utensilio le da esta leche?
(*la mayoría de las veces*)

a. Cucharita
b. Biberón
c. Gotero
d. Taza
e. Jeringa
f. Otro

1. Sí
2. No

a. ☐ b. ☐ c. ☐ d. ☐ e. ☐ f. ☐

24 ¿Le da otros líquidos que no contengan leche?

1. Sí
2. No (*pase a la pregunta 27*)

☐

25 ¿Qué líquidos le da?

a. Agua simple
b. Agua con miel o azúcar
c. Té solo
d. Té con azúcar o miel
e. Atole con agua
f. Café solo
g. Café con miel o azúcar
h. Jugo
i. Otro: _____

1. Sí
2. No

a. ☐ b. ☐ c. ☐ d. ☐ e. ☐ f. ☐ g. ☐ h. ☐ i. ☐

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PRACTICAS DE ALIMENTACION INFANTIL

26 ¿Con qué agua prepara los líquidos la mayoría de las veces? (*ver la mismas opciones de la pregunta 20*) ☐

g. Otra _____

27 ¿Le da otros alimentos? ☐

1. Sí
2. No (*pase a la pregunta 29*)

28 ¿Cuáles?

a. Cereal ¿cuál marca?_____ ☐
b. Caldo de pollo ☐
c. Sopa de pasta ☐
d. Caldo de frijol ☐
e. Huevo ☐
f. Fruta cocida ☐
g. Verdura cocida ☐
h. Frijol ☐ (1. Sí / 2. No)
i. Tortilla ☐
j. Arroz ☐
k. Pollo ☐ (k)
m. Carne de res o cerdo ☐
n. Queso ☐
o. Verdura cruda ☐
p. Fruta cruda ☐
q. Otro _____ ☐
r. Otro _____ ☐

29 ¿Cuál es la talla (*aproximada*) del padre del niño? (*anote 999.9 si no aplica*) ☐☐☐.☐

30 ¿Cuál es el peso (aproximado) del padre del niño? (*anote 999.9 si no aplica*) ☐☐☐.☐

Observaciones_____

_____

VoBo_____

(Supervisora) dd/mm/aa

31 **Código de la supervisora** ☐☐

Appendix 2. Form 25: 15 day morbidity recall

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EFECTO DE SUPLEMENTACION PRENATAL CON ÁCIDOS GRASOS OMEGA3 EN EL DESARROLLO NEUROCONDUCTUAL DEL INFANTE

RECORDATORIO DE 15 DÍAS MORBILIDAD

Tipo de formulario 2 5

Número de contacto

Número del folio del niño 1

Fecha de nacimiento del niño

d d m m a a

Fecha de la entrevista

d d m m a a

Código del encuestador

En los últimos 15 días, el niño(a) ha padecido:

1) Fiebre
   1. Sí
   2. No *(pase a la pregunta 2)*
   9. No sé *(pase a la pregunta 2)*

   a. ¿Durante que días el niño(a) tuvo fiebre? __/__/__ dd/ mm /aa - __/__/__ dd/ mm /aa
   b. ¿Acudió al médico para que lo(a) atendieran?
      1. Sí
      2. No
      9. No sé

2) Tos
   1. Sí
   2. No *(pase a la pregunta 3)*
   9. No sé. *(pase a la pregunta 3)*

   a. ¿Durante que días el niño(a) tuvo tos? __/__/__ dd/ mm /aa - __/__/__ dd/ mm /aa
   b. ¿Acudió al médico para que lo(a) atendieran?
      1. Sí
      2. No
      9. No sé

3) Flema
   1. Sí
   2. No *(pase a la pregunta 4)*
   9. No sé *(pase a la pregunta 4)*

   a. ¿Durante que días el niño(a) tuvo flema? __/__/__ dd/ mm /aa - __/__/__ dd/ mm /aa
   b. ¿Acudió al médico para que lo(a) atendieran?
      1. Sí
      2. No
      9. No sé

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RECORDATORIO DE 15 DÍAS MORBILIDAD

4) Congestión nasal

1. Sí
2. No *(pase a la pregunta 5)*
9. No sé *(pase a la pregunta 5)*

a. ¿Durante que días el niño(a) tuvo congestión nasal? ___/___/___ (dd/ mm /aa) - ___/___/___ (dd/ mm /aa)

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

5) Secreción nasal

1. Sí
2. No *(pase a la pregunta 6)*
9. No sé *(pase a la pregunta 6)*

a. ¿Durante que días el niño(a) tuvo secreción nasal? ___/___/___ (dd/ mm /aa) - ___/___/___ (dd/ mm /aa)

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

6) Silbido de pecho

1. Sí
2. No (pase a la pregunta 7)
9. No sé (pase a la pregunta 7)

a. ¿Durante que días el niño(a) estuvo con silbido de pecho? ___/___/___ (dd/ mm /aa) - ___/___/___ (dd/ mm /aa)

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

7) Falta de aire

1. Sí
2. No *(pase a la pregunta 8)*
9. No sé *(pase a la pregunta 8)*

a. ¿Durante que días el niño(a) estuvo con síntomas de falta de aire? ___/___/___ (dd/ mm /aa) - ___/___/___ (dd/ mm /aa)

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

8) Vómito

1. Sí
2. No *(pase a la pregunta 9)*
9. No sé *(pase a la pregunta 9)*

a. ¿Durante que días el niño(a) tuvo vómito? ___/___/___ (dd/ mm /aa) - ___/___/___ (dd/ mm /aa)

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

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RECORDATORIO DE 15 DÍAS MORBILIDAD

9) Diarrea

1. Sí
2. No *(pase a la pregunta 10)*
9. No sé *(pase a la pregunta 10)*

☐

a. ¿Durante que días el niño(a) tuvo diarrea? ___/___/___ - ___/___/___
dd/ mm /aa dd/ mm /aa

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

☐

c. ¿La popo tiene moco?

1. Sí
2. No
9. No sé

☐

d. ¿La popo tiene sangre?

1. Sí
2. No
9. No sé

☐

e. ¿Fue de consistencia casi líquida?

1. Sí
2. No
9. No sé

☐

10) Salpullido, Erupción

1. Sí
2. No *(pase a la pregunta 11)*
9. No sé *(pase a la pregunta 11)*

☐

a. ¿Durante que días el niño(a) tuvo rash? ___/___/___ - ___/___/___
dd/ mm /aa dd/ mm /aa

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

☐

11) Otra enfermedad

1. Sí
2. No
9. No sé

☐

a. ¿Durante que días el niño(a) tuvo esta otra enfermedad? ___/___/___ - ___/___/___
dd/ mm /aa dd/ mm /aa

b. ¿Acudió al médico para que lo(a) atendieran?

1. Sí
2. No
9. No sé

☐

c. Describa como es la otra enfermedad :

_____

_____

12. **Código del supervisor** ☐☐ **Vo.Bo. Supervisor:** _____
dd/mm/aa