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Docosahexaenoic Acid Supplementation to Mothers to Improve Preterm Infant Nutrition and Immune Homeostasis
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Abstract

Background:
Preterm infants miss the last trimester of nutrient accretion. Inflammatory morbidities are common in this population. The American Academy of Pediatrics recommends intrauterine accretion for nutrient goals. Docosahexaenoic (DHA) is an anti-inflammatory dietary fatty acid and is accreted over the last trimester at 65mg/day.
Preliminary data: We measured DHA levels in breast milk from 38 mothers and found lower concentrations than those recommended (0.1 mol wt %). Dietary intake of DHA was lower than that recommended for nursing mothers (23 vs 200 mg/day, $p<0.001$). When we supplemented with a placebo (n=5) vs. 1 gram of DHA (n=6) mother's tolerated it well. The daily intake by a preterm infant was improved to a concentration that mimicked intrauterine intake when the mother took 1 gram of DHA per day.
Hypothesis: We designed a prospective, randomized trial to test the hypotheses that DHA supplementation to human milk providers will increase DHA concentration in milk and infant blood levels; and that infants receiving milk from DHA-supplemented providers will be receiving a more appropriate enteral intake to better mimic intrauterine accretion. In addition, we hypothesize that pro-inflammatory cytokines will be reduced in the mothers and infants receiving the higher DHA diet.
Study Design: Breast milk providers for enterally-fed preterm infants <29 weeks gestational age will be randomized to receive daily oral supplementation with DHA at 200mg or 1000mg. Based on our previous study with a placebo, it was apparent that even motivated mothers had a paucity of DHA in the diet and in their prenatal supplement therefore we felt it important to randomize to at least the baseline 200mg per day and then added the other dose as the target optimal dose for milk concentrations to achieve 0.8 mol weight percent. This concentration of DHA would provide intrauterine accretion when fed at 150ml/kg per day in the NICU. DHA concentration will be analyzed by gas chromatography in milk and infant cell membranes. Milk and infant plasma cytokine levels and cytokine production capacity will be measured. All samples will be obtained at baseline (within first week of life), weeks 2, 4, and 8. Inflammatory complications (BPD, NEC, and ROP) will be tracked. Cytokine gene expression from RNA extracted from the Week 4 milk samples will also be examined. Stool samples will not only have fatty acids but also bacteria and yeast analyzed.

Significance: Our unique investigation of maternal supplementation to improve DHA delivery to preterm infants represents the first study of its kind.
Purpose
The objective of this project is to improve preterm infant intake of DHA that approximate the levels obtained by fetal accretion during the third trimester of pregnancy and determine if there are functional relationships to inflammation, growth, and development.

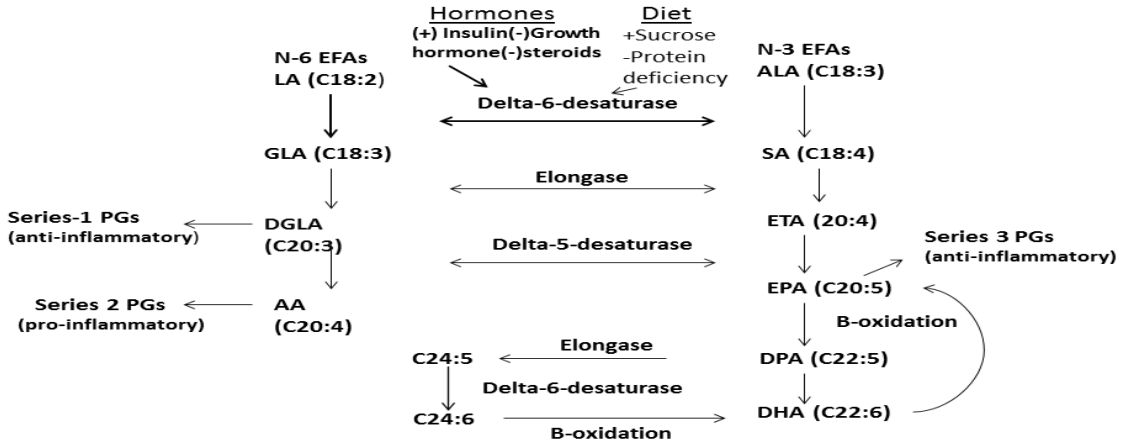
The following specific aims will test the hypotheses that:

Aim 1: Maternal DHA supplementation will improve plasma and milk DHA concentration in the mother and preterm infant
Aim 2: Higher intakes of dietary DHA will decrease expression of pro-inflammatory cytokines and increase expression of anti-inflammatory cytokines in the plasma and milk samples of

breast feeding mother - infant dyads through the alteration of sRAGE signaling. This will result in an improved microbiome as evidence of bacteria and yeast in stool samples.

Background:
Preterm infants miss the last trimester of nutrient accretion and may be deficient in essential molecules. These deficiencies may contribute to increased rates of infection and make these infants more vulnerable to disease [1]. Long chain omega-3 fatty acids, in particular docosahexaenoic acid (DHA), are preferentially transported across the placenta and provide components of membrane phospholipids that have been associated with improved markers of brain and retinal development [2-4]. Because the bulk of this transport occurs in the third trimester of pregnancy, premature infants do not benefit from this increase in DHA delivery. In adults, DHA can be synthesized from its precursor, linolenic acid - an essential fatty acid - through a series of elongase and desaturase enzyme activities that may be limited in the preterm infant [5]. The preterm infant may be particularly vulnerable to biosynthesis of DHA because of dietary protein limitations and steroid use thereby making a preformed dietary DHA source imperative – figure 1 (Valentine, Adv. Nutr, 2012).

Figure 1. Re-examination of the role diet and hormones may have in preterm infant DHA biosynthesis and FX consequences.



Likewise, these DHA synthesizing enzymes are competitively shared by the omega-6 fatty acid series. Both the omega 6 and the omega 3 fatty acids, provide substrates for a host of pro- and anti-inflammatory lipid mediators. Omega 6 fatty acids are closely associated with the production of pro-inflammatory mediators and indirectly influence the production of tumor necrosis factor (TNF)-α. Conversely, omega-3 fatty acids are more closely linked to suppression of inflammation and production of anti-inflammatory or pro-resolution molecules. Specifically, DHA has been shown to suppress NFkB activation which results in decreased inflammatory responses and increased production of anti-inflammatory mediators such as interleukin (IL)-10 [6]. The maternal murine model supplemented with DHA promotes less inflammatory mediators in the lung of the pup and results in significantly better survival.[7] Deficits in nutrient sources of omega-3 long chain fatty acids, specifically DHA, may influence the infant's immune balance and contribute to development of disease [8, 9] [10] [11, 12].

Infants fed diets supplemented with DHA have higher levels of DHA in red blood cell phospholipids [13], and have demonstrated improved functional outcomes [14, 15]. While dietary sources of DHA may ultimately qualify as semi-essential for the preterm infant, DHA is currently lacking in the intravenous nutrition used in neonatal intensive care units (NICU) [16]. Current estimates are that 67 mg/day of DHA is accumulated *in utero* during the last trimester [2] The American Academy of Pediatrics recommends that preterm infant feeding goals mimic intrauterine nutrient intake as the most logical approach to achieving appropriate nutritional levels [17]. However, stable isotope studies indicate a preterm intake of only 4 mg/day [18], far below intrauterine nutrient intake. Human milk is the preferred enteral nutrition for the preterm infant and contains vital anti-infective properties but milk DHA concentrations vary among mothers, dependent on maternal dietary intake [19-22]. Previous studies have demonstrated that fatty acid supplementation improves human milk fatty acid composition [23, 24] but ***levels of human milk DHA that would approximate in utero accretion for the human milk-fed preterm infant have never been targeted***.

Epidemiologic studies have demonstrated that maternal intakes of 1 gram of DHA correspond to 1 mole weight % in human milk [25]. Dietary intake of DHA up to 3 grams per day are considered GRAS by the FDA [26]. A diet of human milk with 1% DHA at 150 ml/kg would better achieve intrauterine intake of 67 mg/day. Animal studies have shown reduced rates of necrotizing enterocolitis (NEC) when fed omega-3 fatty acid supplemented diets [27]. A dietary strategy that seeks to approximate intrauterine accretion values of DHA by improving the mother's diet could therefore promote improved nutrient availability and more appropriate immune response in preterm infants. In our previous 2 years we have demonstrated that the current milk composition of DHA is less than optimal for the preterm infant [28] and that a RCT of 1 gram maternal dietary DHA intake vs. a placebo soy oil resulted in enhanced DHA milk composition equal to intrauterine concentration provided to the NICU infant (Valentine, 2011 manuscript in review)- figure 2.

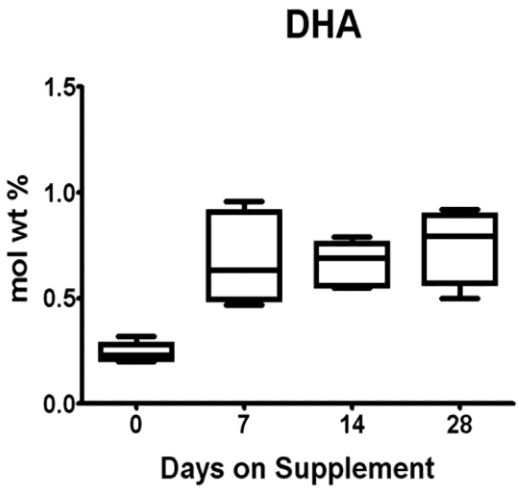


Figure 2: Milk concentration of DHA in DHA supplemented women, Valentine C, Breastfeeding Medicine, 2012

Although the mechanisms by which DHA decreases inflammation are not understood, the anti-inflammatory properties of long chain fatty acids include changes in membrane fluidity, effects on signaling pathways resulting in modified gene transcription, and enhanced production of anti-inflammatory lipid mediators due to the availability of DHA as a substrate [29, 30]. Receptor for Advanced Glycation End Products (RAGE) is described as a "Damage Associated Molecular Pattern" receptor and as such is able to engage classes of unrelated molecules using tertiary structure for ligand recognition [31]. RAGE exists in multiple forms; a membrane bound form (mRAGE), a splice-variant soluble form (svRAGE), and a cleaved form (sRAGE) generated by protease activity from mRAGE. In the context of lung injury, the source of cleaved sRAGE protein is likely type I cells or alveolar macrophages, and greater levels of sRAGE in BAL or plasma are linked to the severity of lung injury [31] specifically capillary leak and fluid clearance [32].We hypothesize that DHA will suppress the production of inflammatory cytokines and chemokines in both the mother and the preterm infant through decreasing RAGE expression and signaling.

Understanding this relationship will help determine if maternal dietary supplementation strategies with DHA are optimal or if infant direct supplementation is necessary to improve immune homeostasis.

Study Design: We propose a prospective, randomized, trial of oral DHA supplementation of lactating mothers. Cincinnati Children's Hospital research staff will screen all infants admitted to the Cincinnati University Hospital NICU on a weekly basis. Eligible participants will be identified by the Principle Investigator and the research coordinator. Potential participants (mothers) will be approached regarding the possibility of study participation by study staff trained in the consent process. Eligible participants will be screened and if eligible and interested, will be consented within one week of being admitted to Cincinnati University Hospital NICU. Maternal consent will be obtained for the infant. Consent will be administered by Cincinnati Children's Hospital Medical Center staff trained in the consent process and having completed all required CITI training. Informed consent will be documented by an Informed Consent Process Note. All data and infants biological sample collection will take place during the infants stay in Cincinnati University Hospital NICU. Mother's biological samples will be collected by Cincinnati University Hospital staff, CCHMC study staff and Hoxworth outpatient lab.

After informed consent, lactating mothers will be randomized by The Cincinnati University Hospital Investigational Pharmacy to receive DHA (DHA- Martek Biosciences, now known as DSM Nutritional Lipid- Expecta®) 200 mg, or 1000 mg/day. The randomization schedule has been devised in collaboration with the Biostatistics expert, Dr. Ardythe Morrow, identified in these studies. We have used the Consolidated Standards of Reporting Trials (CONSORT)framework [33].
Participants who meet any exclusion criteria during the course of the study, are non-compliant or are lost to follow-up will be withdrawn. Provided that there are at least 8 mother/baby pairs with evaluable data in each group, withdrawn participants will not be replaced. Anticipated enrollment will take two years.

Inclusion criteria: Lactating mothers at Cincinnati University Hospital with infants born at $\leq$ 29 weeks gestation admitted to The Cincinnati University Hospital NICU

Exclusion criteria:

- Infants with the presence of congenital anomalies (trisomy 13, 18, 21, urethral, gastrointestinal and cardiac defects) that affect the standard course of care.

- mother's <18 and
- mothers with known allergy to algeal source
- mothers with history of bleeding disorders or taking any other medication that may increase the risk of bleeding
- infants with bleeding disorders or receiving any other medication that may increase the risk of bleeding

Study Procedures:

Maternal Dietary intake and breastmilk samples will be collected and assessed at initiation of supplementation and at weeks 2, 4, and 8. Maternal breast expression and breastfeeding patterns; anthropometry (weight, height, circumferences); exercise patterns; and smoking habits will be obtained at week 4.

Maternal blood and buccal swabs (saliva) will be collected and assessed at baseline and at weeks 4, and 8.

Infant Dietary intake, blood samplesbuccal swabs (saliva), and stool samples will be collected and assessed at baseline and at weeks 2, 4, and 8. – see Figure 3.

Maternal blood collected will be 5 mL at each collection therefore a total of 15 mL whole blood collected for the study. Infant blood collected will be ~ 1 mL at each collection therefore a total of ~5 mL whole blood collected. The infant blood will be collected during routine nutritional lab collections which are clustered with standard of care.
The dietary and supplemental intake of the mother will be assessed at The Bionutrition Center using NDSR software and the dietary intake of the infant will be calculated by the Neonatal Dietitian. The fatty acid contents of the breastmilk, blood, and stool samples will be measured by gas chromatography in our laboratory using Bligh-Dyer extraction (Chloroform: Methanol (2:1))[34]. Cytokine (IL-10, IL 6, IL-8, IL 1B, TNF a, TGF, adiponectin, leptin) and sRAGE concentrations will be examined in maternal milk, and maternal and infant blood samples using a MSD SECTOR® Imager 2400A. This instrument allows for the analyses of 40 individual samples in duplicate at one time using a 96-well format. Infant growth, clinical outcome, and development data will be collected. Breastmilk samples will be analyzed for fatty acids and certain proteins as is routinely analyzed at Human milk banks of North America and clinically at Cincinnati Children's Hospital Medical Center. Cytokine analysis will be performed on infant stool samples. Saliva samples will be analyzed for genetic polymorphisms. Buccal swabs (saliva) will be analyzed for fatty acids.
An additional teaspoon of fresh milk will be collected at Week 4 and aliquoted into whole, aqueous, and fat layer fractions. Fat layer RNA will be isolated using a Maxwell™16 integrated system (Promega, Madison, WI). RNA quantity (target=1000 ng) and quality (A260:A280 target $\geq$1.9) will be confirmed prior to RNA sequencing. Na:K ratio, as a marker of mammary gland inflammation, will be assayed on the milk aqueous fraction.
All biological samples will be collected within study time frames as feasible.

Stool will also have bacteria and yeast measured by using 16S ribosomal markers.

Growth and Hospitalization

Growth measures are standard of care at University NICU and include weekly weight, length, and head circumference measurements and will be extracted from the participant's charts. At discharge we will obtain body composition on the infant using a validated isolette that has non-invasive, volume and mass measurement capability. Cincinnati University Hospital course records that could be pertinent to the study outcomes will be collected from both mother and infant. Compliance will be assessed as part of diet record evaluations; rate of compliance will not be assessed

Statistical Plan: All data analyses will employ intention to treat principles. In our primary analysis, ANOVA will be used to compare DHA levels at four weeks in the breast milk of mothers assigned to the different concentrations of DHA supplements. As in any prospective study, If we experience substantial loss to follow-up (e.g., > 10% of subjects), we will fit a linear mixed model [35] We will use two sample t tests to compare infant blood DHA concentrations and TNF-alpha and IL-10 levels at 8 weeks of infants randomized to DHA-supplemented milk. The comparison of DHA concentrations will be performed at a significance level of 0.05 while the comparison of TNF-alpha and IL-10 levels will be performed at a significance level of 0.025 to adjust for the two outcomes. All analyses will be performed using SAS (SAS Inc., Cary, NC). Our sample size determination to detect a two-fold difference in the DHA levels at four weeks in expressed milk of mothers assigned DHA treatments, we will need data on a total of 16 mothers at week 4 (8 per group). This sample size calculation was based on 90% power, a two-sided significance level of 0.025, and a coefficient of variation (CV) of 30%; the CV is based on a previous study [36]. Assuming a conservative 20% loss to follow-up before week 4, we will enroll enough mother-infant pairs to achieve our desired sample size of 16 at 4 weeks. It is expected that evaluable data at week 4 from at least sixteen of the mother/baby pairs (8 pairs per group) will be obtained. The University Hospital Investigational Pharmacy will randomize participants per the Consolidated Standards of Reporting Trials (CONSORT) framework. Secondary outcomes will use linear mixed models and Bonferroni correction.

Among participants with adequate quality milk fat layer RNA at Week 4, five pairs will be randomly selected from each group for sequencing (matched on gestational week of delivery, milk DHA concentration at baseline, and Week 4 breast milk output level (exceeds, at, or below infant's needs). The remaining will be set aside for future studies. RNA integrity (RIN $\geq$7) will be confirmed at the microarray core and then sent to the gene discovery core for RNA-Seq library preparation (Illumina TruSeq RNA kit) and sequencing (Illumina Hi-Seq2500). Libraries will be multiplexed in batches of 10 with at least 30 million 150bp paired-end reads per sample. Sequence reads will be trimmed for quality and then aligned to the human genome using TopHat. We will use Cufflinks to determine gene expression levels, normalized to Fragments Per Kilobase of transcript per Million mapped reads (fpkm). We will identify differentially expressed genes using DESeq and intersect this gene list with known cytokine signaling genes. We will also conduct Gene Set Enrichment Analysis. Significant biomarker genes will be assayed for all participants using TaqMan™ qPCR. Power: N=5 pairs per group enables 90% power (α=0.05) to detect 1.5-fold differences between DHA versus placebo for moderate to high abundance genes (FPKM $\geq$ 10) and 3.0-fold differences for low abundance genes (FPKM ~1.0).

Safety: All records will be kept according to IRB and regulatory standards. All PHI will be stored in a password protected database. All biological sample data will be kept in a separate database from PHI and study identifiers. Written research records, participant contact information, and signed consent forms will be kept in a locked file cabinet near the study coordinator's work station. All data will be entered from source documents into a Redcap database using a password protected, secure server. Individually identifiable information will not be included in the data set or in any reports.
Laboratory samples will be de-identified and stored at Cincinnati Children's Hospital Medical Center and Nationwide Children's in Columbus Ohio..
All supplements will be stored in the Investigational pharmacy at Cincinnati University Hospital. FDA Investigator IND was accepted and in addition regards DHA as a generally regarded as safe (GRAS) category with levels < 3 grams per day in the adult diet. Our supplement strategy is well below this level.

Risk/Benefit Analysis: The risk is very negligible since the product has been on the market and women have been taking up to 3 grams per day without adverse effects in cardiovascular prevention trials. The benefit is great to the pregnant and lactating woman to improve health.

Collaboration: Dr. Morrow will collaborate with the original author of this work Christina J. Valentine MD, MS, RD who is now at Mead Johnson Nutrition to complete and write the manuscript. Dr. Henry Akinbi will provide medical oversight as a Sub-Investigator.
Dr. Laurie Nommsen-Rivers will collaborate in collecting the lactation history data and capturing mammary cell RNA from the milk fat layer and conducting transcriptome analysis on the RNA-sequenced samples.

Potential Risks, Discomforts, Inconveniences and Precautions
Minimal pain or bruising may occur at the blood draw site. This occurrence is minimized by having blood drawn by trained and experienced nurses and phlebotomists.
All infant blood will be drawn during standard of care routine nutritional labs therefore eliminating an additional venipuncture**.**

Because of the longevity of the product on the market and its use in infant formulas we do not anticipate any unknown or unforeseeable risks associated with this study.

The mothers are sent home with an AE form to collect any adverse events that occur from their first dose of the investigational agent until the end of the study. This form will be checked at all study time points by study staff.
Since infants in this study are premature, it is anticipated that adverse changes in clinical status will occur related to their prematurity. The following common, serious neonatal morbidities as defined in the NRN Generic Data Base (GDB) will also be collected. These include data on in-hospital growth, the incidence and severity of intraventricular hemorrhage, seizures, patent ductus arteriosus (PDA) and its treatment, nosocomial sepsis (and organisms), hearing impairment, and pneumothorax.

Data And Safety Monitoring Plan

Independent review of the ongoing data, patient reports, and adverse events will be reviewed routinely every quarter to determine on going safety. Dr. Kurt Schibler, The Medical Director of Clinical research will be our member on the DSMB.

All serious adverse events that are determined to be related to the study intervention and that are unexpected will be reported in an expedited fashion to the FDA, IRB and study DSMB. Serious adverse events that are deemed related will be reported within 7 calendar days of the sponsor's initial receipt of the information. Serious adverse events that are deemed related but not fatal or life-threatening will be reported within 15 calendar days.

On-going Reporting: Serious adverse events not meeting the criteria for expedited reporting and all other adverse events will be reported annually per federal and institutional regulations and guidelines and to the study member of the DSMB per the Data and Safety Monitoring Plan.

Pregnancy and fetal special circumstances will be given through the IRB review. The informed consent is reviewed by the IRB. Quarterly data surveillance for safety will be done by a Neonatologist in the Division of Neonatology and Pulmonary Biology, Kurt Schibler, MD.

Current financial support:

Divisional Funds are the primary source of funding

The PI in the study does receive 8% salary support from a collaboration with:

R01AT006880-01 (PI: LK Rogers) 7/1/2011- 6/30/2015 NCCAM/ODS
DHA Attenuates Inflammatory Responses through Altering RAGE Signaling
The goal of this grant is to identify the mechanisms associated with decreased inflammation in response to DHA supplementation, specifically through RAGE-mediated signaling. Funding is also provided by **The Division of Neonatology/ Perinatal Research Institute at Cincinnati Children's Hospital, Ohio**. No extra cost outside of standard of practice will be incurred to the patient.

Based on admissions at University Hospital, the anticipated time frame for the study will be 1 year and then a following year for data analysis and manuscript preparation.

Figure 3. Study time table for recruitment, sample, and data collection

0 baseline {within first week of life}	***2 week***	***4 week***	***8 week/Discharge***
*Enroll & Consent **Maternal** Milk - fatty acids cytokines Maternal blood – fatty acids Diet analysis Buccal swabs (saliva) **Infant** blood - fatty acids, cytokines, and sRAGE Nutrition labs Growth measures: Weight, length, head circumference Diet analysis Stool from diaper Buccal swabs (saliva)	**Maternal** Milk - fatty acids, cytokines, Diet analysis **Infant** blood - fatty acids and cytokines labs, sRAGE Stool from diaper Buccal swabs (saliva) Growth measures	**Maternal** - Milk fatty acids, cytokines, , RNA, Na:K ratio Maternal blood – fatty acids Diet analysis Exercise and Smoking habits Breast milk expression and breastfeeding patterns and concerns Anthropometry (weight, height and circumferences) Buccal swabs (saliva) **Infant** blood fatty acids and cytokines labs, sRAGE Stool from diaper Buccal swabs (saliva) Growth measures	**Maternal** - Milk fatty acids, cytokines, Maternal blood – fatty acids Diet analysis Buccal swabs (saliva) **Infant** blood - fatty acids and cytokines labs, sRAGE Buccal swabs (saliva) Stool from diaper Growth measures Body composition using the Pea Pod ® air displacement isolette

Summary and Significance:

Premature birth is responsible for 75% of perinatal death [37]. Inflammatory conditions such as necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), and retinopathy of prematurity (ROP) in the preterm infant are a tremendous source of morbidity and mortality. DHA is a key regulator of inflammation. Breast milk is the ideal delivery vehicle for DHA based upon the ability of the breast to provide balanced fatty acid composition as well as anti-infective molecules. Further understanding of the mechanisms could ensure our nutritional strategy is optimal.

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