Fatty acid supplements alter biological signatures in children with Autism Spectrum Disorder

Principal Investigator: Sarah A. Keim, PhD

Co-Principal Investigator: Lynette K. Rogers, PhD

CONFIDENTIAL

Version 5

Protocol Version 1 was submitted to the FDA in November 2016 and at initial grant submission. Protocol Version 2 was submitted to NIH Nov 2017. No participants were enrolled under Protocol Version 1,2,3,4.

22 May 2018

STATEMENT OF COMPLIANCE

The study will be carried out in accordance with Good Clinical Practice (GCP) as required by the following:

- United States (US) Code of Federal Regulations (CFR) applicable to clinical studies (45 CFR Part 46; 21 CFR Part 50, 21 CFR Part 56, and 21 CFR Part 312)
- ICH E6; 62 Federal Register 25691 (1997)
- NIH Clinical Terms of Award

All key personnel (all individuals responsible for the design and conduct of this study) have completed Human Subjects Protection Training.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable US federal regulations and ICH guidelines.

Site Investigator:*

Signed: _____ Date: _____

Name
Title

**The protocol should be signed by the local investigator who is responsible for the study implementation at his/her specific site; i.e., if Investigational New Drug study, the individual who signs the Form FDA 1572.*

TABLE OF CONTENTS

PROTOCOL SUMMARY

Title: Fatty acid supplements alter biological signatures in children with Autism Spectrum Disorder

Background: Fatty acid supplements are among the most popular complementary products consumed by children with Autism Spectrum Disorder (ASD). Their use is driven by anecdotal evidence of benefit and a few small RCTs that demonstrated improvements in some externalizing behaviors in this population.[1-3] Overall, there remains scant evidence for efficacy in treating the core symptoms of ASD like social-communication deficits and restricted and repetitive behaviors. Furthermore, very little is known about the biological signatures that are affected. Over-the-counter (OTC) fatty acid supplements are plentiful and consist of varying percentages of gamma-linoleic acid (GLA, an omega-6 fatty acid), eicosapentaenoic acid (EPA, an omega-3), and docosahexaenoic acid (DHA, omega-3) and often include vitamins or plant extracts as well. Existing RCTs have tested varying combinations of fatty acids with conflicting results that likely stem from this variation. Because there are no approved medications to treat the core symptoms of ASD, well-intentioned parents are reaching for any product that promises improvement in their child, but they are giving these supplements without guidance as to their function, mechanism of action, or whether they are harmful or helpful in symptom management.

For the proposed project, we will focus on preschool and early school aged children because of the extensive literature indicating that a wide range of ASD therapies are maximally beneficial if they begin as early as in life as possible after diagnosis. This may be because neuroplasticity decreases significantly as children age, accompanied by the fact that DHA accretion in the developing brain displays a corresponding decline in rate with age.[4] Along similar lines, it has recently been reported that fatty acid bioavailability and treatment effects in children may depend partially on body size (smaller children exhibit higher fatty acid levels for a given dose than larger children). Many pediatric medications are dosed based on body weight; however, this concept has been virtually never applied to fatty acid supplementation. Aside from considerations about body size, only rarely are fatty acid supplements subjected to dose-ranging trials where a variety of doses are examined simultaneously to identify the most efficacious and tolerable. At most, a few studies have tested 2 doses vs placebo. These gaps in the literature represent a barrier to informing patients and clinicians about optimal dosing for a given child, with the end result that some children likely take a dose too small to benefit.

Population: A randomized, fully-blind, placebo-controlled trial of 66 children ages 2-6 years with recently-diagnosed ASD. The trial will involve 90 days of treatment with one of 3 different doses (25 mg, 50 mg, or 75 mg twice per day, using child weight) of Omega 3-6 or (equal volume) placebo and will include 4 study visits.

Study Objectives: 1) Optimize safety and bioavailability of Omega 3-6 in a unique population of children with ASD; 2) Evaluate the effect of Omega 3-6 supplementation on biological signatures of inflammation.

1 KEY ROLES

Key Personnel:

Principal Investigator: Sarah A. Keim, PhD, MA, MS

Co-Principal Investigator: Lynette K. Rogers, PhD

Study Doctor / Co-Investigator: Daniel L. Coury, MD

Institution:

The Research Institute at Nationwide Children's Hospital
Center for Biobehavioral Health
700 Children's Drive
Columbus, OH 43205
Phone: 614-722-3182
Fax: 614-722-3544

2 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

2.1 Introduction

Although at least 1 in 68 U.S. children has been diagnosed with Autism Spectrum Disorder (ASD), no approved medications exist to treat the core symptoms. Atypical antipsychotics are commonly prescribed for irritability with ASD, but the side effects are significant, common, and of particular concern for very young children.[5] Behavioral interventions can offer families approaches to live with the atypical behaviors and social deficits of ASD, but they are not cures. Because of the dearth of effective treatments, between 52% and 95% of families commonly try complementary therapies including fatty acid supplements, based largely on anecdotal evidence. To date, the results of efficacy trials testing fatty acid supplementation for ASD are mixed for several reasons including variation in the particular combination and dose of fatty acids tested and study design shortcomings including lack of blinding or placebo control. Another major reason for the lack of clarity is the very limited extent to which studies have evaluated potential biological signatures of interest in order to shed light on the pathways through which fatty acids may exert their effect. This proposal will determine a primary pathway through which Omega 3-6 supplementation can benefit ASD symptoms, by studying a leading candidate set of biological signatures and addressing related gaps in the literature that preclude successful efficacy trials.

Fatty acid supplements are among the most popular complementary products consumed by children with ASD, driven by anecdotal evidence of benefit and a few small RCTs that demonstrated improvements in externalizing behaviors. Because no approved drugs exist to treat the core symptoms of ASD, well-intentioned parents are reaching for any product that may help their child. The implications are that fatty acid supplements are given to children without any evidence base as to their function, mechanism of action, or benefits versus harms. Overall, there remains scant evidence for efficacy of fatty acids in treating core ASD symptoms from well-designed and adequately-powered RCTs, and very little is known about the mechanism(s) of action. Over-the-counter (OTC) fatty acid supplements are plentiful and consist of varying percentages of oleic acid (n-9), gamma-linolenic acid (GLA, n-6), eicosapentaenoic acid (EPA), and/or docosahexaenoic acid (DHA) and often include vitamins or other plant extracts. Existing RCTs have tested varying combinations and doses of fatty acids, and conflicting results likely stem from this variation. Recent evidence from our group and others suggest a combination of fish and borage oils providing supplementation of DHA, EPA and GLA (“Omega 3-6”) is particularly promising compared to other formulations. Based on these foundations, a critical need exists to evaluate Omega 3-6 for optimal dosing and changes in a biological signature that are related to ASD symptoms, before a rigorous, full-scale efficacy trial can be launched.

Our long-term goal is to identify effective treatments for ASD. The objective of the study is to optimize safety and bioavailability of Omega 3-6 in a unique population of children with ASD and evaluate the effect of Omega 3-6 supplementation on biological signatures of inflammation. A later study to be described in a future protocol will relate these changes to core ASD symptoms. Our overall hypothesis for the line of research is that Omega 3-6 will alter biological signatures related to inflammation in a manner that correlates to ASD-related behaviors. Our hypotheses were formulated based on data from our Preemie Tots study and other published data which suggest that the inflammatory markers, IL-1β, IL-2, and IFNγ are consistently elevated in children with ASD and decreases in these markers correlate with ASD symptom improvement. The *rationale for the proposed research* is that its successful completion will provide the infrastructure and endpoints necessary to support a future efficacy trial to give parents and clinicians unequivocal evidence for fatty acid supplementation for ASD.

2.2 Biological Pathways and Mechanisms

Fatty acids (FA) have diverse and complex biological functions. Long chain polyunsaturated fatty acids of the omega-3 series have been extensively studied and demonstrate anti-inflammatory properties affecting multiple pathways. The omega-3 DHA comes from fatty fish or algal sources, is the primary neuro-active fatty acid in the brain, and is a key player in neurotransmitter function, synaptogenesis, gene expression, membrane fluidity, neurogenesis, neuroplasticity, and anti-inflammation.[6-11] EPA, also from fish oil, is often included in omega-3 fatty acid supplements and has triglyceride lowering properties and anti-inflammatory properties similar to DHA. GLA is a shorter-chain fatty acid of the omega-6 series derived from plant sources including borage and evening primrose. GLA is a precursor to fatty acids such as arachidonic acid (AA) which is essential for lipid signaling and hormone synthesis. Biologically available GLA is quickly converted to dihomo-γ-linolenic acid (DGLA), which now has been shown to have substantial anti-inflammatory properties unique among longer chain n-6 fatty acids.[12] As omega-3 and omega-6 fatty acids share common desaturation and elongation enzymatic pathways, combinations of these fatty acids can effectively compete for these enzymes creating a physiologic balance rather than a shift toward one pathway or the other. The mechanisms associated with the anti-inflammatory effects of long chain fatty acids are still not completely understood but include direct binding to g-coupled protein receptor 120 (GCPR120) or peroxisome proliferator-activated receptor gamma (PPARγ) to inhibit MAPK or NFkB activation, production of alternative lipid products such as resolvins and protectins, and changes in membrane composition that alters ligand binding to cytokine or toll-like receptors. Children with ASD have been shown to exhibit altered fatty acid metabolism; several investigations have shown decreased levels of DHA, EPA, and AA in these children. The reason for these decreases is not completely clear but may be due to a combination of diet differences, altered absorption, defects in fatty acid synthesis because of polymorphisms in fatty acid desaturase genes (FADS2) or other metabolic alterations.[13-15] Since all of these FAs contribute to important biological functions, influence anti-inflammatory pathways, and enhance anti-inflammatory mechanisms, we speculate that decreases or deficiencies in these FAs directly contribute to the increased inflammation observed in ASD patients.

2.3 Advantages of Fatty Acid Combination Therapies

DHA has been the most widely investigated fatty acid for treatment of mental disorders and has demonstrated efficacy in patients with depressive or schizophrenic symptoms. In addition, DHA reduces the risk of mild or significant mental delay in extremely preterm infants.[16] However, DHA alone appears to exhibit little or no benefit in treating aberrant behavioral symptoms.[17] Adding EPA to DHA ("EPA+DHA," usually in the form of fish oil) has been evaluated in 4 RCTs for benefits to individuals with ASD. One small trial (n=13) showed benefit to language and maladaptive behaviors,[3] 2 other small trials reported benefits limited to hyperactivity,[2,18] and the most recent trial reported worsening externalizing behaviors.[19] EPA+DHA has been shown to be moderately efficacious in reducing symptoms of Attention Deficit Hyperactivity Disorder (ADHD) in large RCTs (meta-analysis standardized mean difference=0.31, 95% CI: 0.16, 0.47).[20] The combination of Omega 3-6 holds significant promise to be more efficacious than EPA+DHA for ASD for several reasons. Direct comparison of the evidence for Omega 3-6 vs EPA+DHA for treating ADHD in a recent meta-analysis reported larger effect sizes and more consistent benefit for omega 3-6 than for EPA+DHA (standard difference in means -0.31 favoring omega 3-6-9 (CI: -0.46, -0.16)).[21] Among the largest of these studies, Richardson et al. found a >0.5-standard deviation reduction in global Conners' Teacher Rating Scales scores among children randomized to Omega 3-6 for 3 months compared to placebo, and Sinn et al. found medium-strong effects of supplementation on parent ratings of ADHD symptoms.[22,23] However, only 2 placebo-controlled RCTs have tested Omega 3-6 formulations for developmental or behavioral outcomes other than ADHD: one study demonstrated benefits for school-age children with Developmental Coordination Disorder on reading and spelling

achievement and across multiple behavioral domains, and our Preemie Tots study demonstrated reduced autistic behaviors, compared to a placebo.[22]

2.4 Inflammation in Patients with ASD

ASD is characterized by pervasive deficits in social communication accompanied by stereotypic behaviors. The severity of these symptoms varies widely in ASD and can be accompanied by significant cognitive impairment. The etiology of ASD remains unclear, but it has both genetic and environmental contributors which impact inflammatory pathways. Inflammation has been well characterized in patients with ASD with increases in inflammatory cytokines and chemokines both in the circulation and CSF. **Interleukin (IL)-1β** is an acute response cytokine produced by immune cells and acts through targeting cognate receptors present on the cell membrane in tissues.[24] IL-1β can cross the blood-brain–barrier, inducing increased permeability or be produced by astrocytes and microglia in the brain. Increases in IL-1β in neurological tissues, causes increased exocytosis of several neurotransmitters, resulting in increased excitability, anxiety, and activation of the HPA.[25,26] **Interleukin (IL)-2** has been extensively studied in the context of brain development and T-cell fate.[27] IL-2 acts in a trophic manner during development and loss of IL-2 causes deficits in learning and memory processing in animal studies. Alternatively, IL-2 regulates immunological homeostasis, self-tolerance and T cell development and function implying dysregulation may play a significant role in neurological auto-immune diseases.[24,27] **Interferon gamma (IFNγ)** is produced primarily by epithelial cells and is the acute phase cytokine produced in response to viral infections. Besides its effects in mediating viral immunity, IFNγ also modulates adaptive immunity through skewing T cell responses to an inflammatory Th1 phenotype.[28-30] A recent meta-analyses of clinical studies in ASD patients revealed that IL-1β and IFNγ were both elevated in virtually every study included (IL-2 was not evaluated) and we observed similar increases in IL-1β and IFNγ in our Preemie Tots study.[31] Of most importance, we observed lower levels of IL-1β, IFNγ, and IL-2 in the children that were supplemented with the Omega 3-6 formulation.

2.5 Evidence for the Need for Early Intervention and Tailored Dosing

For the proposed project, we will focus on young children ages 2 to 6 because of the extensive literature indicating that a wide range of ASD therapies are maximally beneficial if they begin as early in life as possible after diagnosis. This may be because neuroplasticity decreases significantly as children age, accompanied by the fact that DHA accretion in the developing brain displays a corresponding decline in rate with age.[4] Along similar lines, it has recently been reported that fatty acid bioavailability and treatment effects in children may depend partially on body size (smaller children exhibit higher fatty acid levels for a given dose than larger children). Many pediatric medications are dosed based on body weight; however, this concept has been virtually never applied to fatty acid supplementation. Aside from considerations about body size, only rarely are fatty acid supplements subjected to dose-ranging trials where a variety of doses are examined simultaneously to identify the most efficacious and tolerable. At most, a few studies have tested 2 doses vs. placebo. These gaps in the literature represent a barrier to informing patients and clinicians about optimal dosing for a given child, with the end result that some children likely take a dose too small to benefit.

2.6 Consideration of Comorbidities Common in ASD

In addition to the social-communication and behavioral features of ASD, many individuals with ASD have other comorbidities that impact overall health and daily functioning including at least 34% have gastrointestinal problems like constipation, 34% are overweight and 18% are obese. Theoretically, these conditions may modify the effect of fatty acid supplementation on core ASD symptoms by inhibiting gastrointestinal absorption of the supplement or by sequestering a portion of ingested fatty

acids in adipose tissue rather having them biologically available. A full-scale efficacy trial will likely need to consider these possible treatment modifiers when establishing inclusion/exclusion criteria, setting a target sample size, and selecting dose(s).

3 STUDY AIMS

The aims of this project are to:

1) Optimize safety and bioavailability of Omega 3-6 in a unique population of children with ASD.

2) Evaluate the effect of Omega 3-6 on the biological signatures of inflammation (IL-1β, IL-2, IFNγ).

3.1 Study Outcome Measures

Primary Endpoint (Aim 1): Group differences in bioavailability (each fatty acid as a percent of total erythrocyte fatty acids at the end of the trial) and safety (number of and types of adverse events).

Primary Endpoint (Aim 2): Changes in the biological signatures (IL-1β, IL-2, IFNγ) from baseline to the end of the trial.

4 STUDY DESIGN

4.1 Study Participants

This single-site study will be conducted at Nationwide Children's Hospital (NCH). We will recruit from among patients who have received an ASD diagnosis at NCH within the prior 6 months. We expect large numbers of available and interested participants because NCH diagnoses over 800 children with ASD each year, and recruitment into past treatment studies in this patient population has been very successful. Children evaluated at NCH for ASD undergo a comprehensive 4-6 hour evaluation with a developmental pediatrician, psychologist, and speech pathologist covering all DSM-5 defined ASD criteria, cognitive ability, adaptive behavior, and other behaviors commonly comorbid with ASD like hyperactivity, based on trained clinician evaluation and parent report. Children who are given the ASD diagnosis including a score on the gold standard Autism Diagnostic Observation Schedule-2 (ADOS-2) in the "autism" range which is on the severe end of the spectrum, and who meet the other eligibility criteria for the study will be recruited. We focus on children in this range to reduce the heterogeneity of the sample and to maximize the probability of benefit.

After diagnosis, children will be evaluated for trial eligibility based on medical record review and parent interview. Parents will be contacted via letter, phone, and/or email to discuss participation.

> **Inclusion criteria:** age 2-6 years, ASD diagnosis at NCH within prior 6 months with an ADOS-2 score in the "autism" (severe) range, English is primary language.
>
> **Exclusion criteria:** fatty acid supplements in the past 6 months, consume fatty fish >3 times/week, still breast or formula feeding; quadriparesis; deafness; seizure disorder, blindness; bleeding disorder; autoimmune disorders including Type I diabetes; Fragile X, Rett, Angleman Syndromes; Tuberous Sclerosis; feeding problems precluding consumption of the supplement; ingredient allergy; planned surgeries scheduled within the timeframe of trial participation.

Additionally, and as part of the informed consent process, Study Staff will ensure that parents have a good understanding of study procedures and are able to comply with study procedures for the entire length of the study.

5 STUDY ENROLLMENT, TREATMENT ASSIGNMENT, AND WITHDRAW

5.1 Enrollment

The target sample size for this dose-ranging study is 66 children. The study team will be alerted by the electronic medical record system when an autism diagnosis is made at NCH for a potentially eligible child. The system is updated nightly and study staff will be electronically notified of new diagnoses biweekly. Before contacting potential participants for recruitment purposes, the medical record will be used to prescreen potentially eligible participants. During this process, study staff will examine the ASD diagnostic evaluation to determine if the child meets the inclusion criteria and is not excluded due to any exclusion criteria. The medical record will be screened for eligibility information, but eligibility will also be confirmed with a legal guardian prior to enrollment.

5.1.1 Medical Record Data

The following data items will be collected from the child's medical record:

- Results of the comprehensive ASD evaluation conducted at the NCH Child Development Center including scores from developmental and behavioral testing and clinical summary notes
- Related diagnoses including gastrointestinal disorders and other developmental and behavioral diagnoses (e.g., ADHD) and dates of diagnoses
- Demographic and participant characteristics (Date of birth, Sex, Race, Ethnicity, Medical insurance payor, Language, Life status, Multiple birth)
- Percent no shows to appointments
- Food allergies
- Current medication list
- Contact information

5.1.2 Recruitment Mailing

An introductory letter and informational study brochure will be sent to all eligible families after the electronic medical record screening phase. This letter will briefly introduce the study and be signed by Dr. Keim and Dr. Rogers. The introductory letter and study brochure will briefly describe the purpose of the study and will provide contact information for study staff. This informational mailing will request change of address information from the post office to identify families who have recently moved. Although we expect up-to-date contact information to be available in the electronic medical record due to the recent NCH diagnostic appointment, contact information may be verified and/or attempts to locate unreachable families (e.g., those with outdated contact information on file) will be made using a web-based online search service that provides a fast and effective way to look-up or verify contact information about a person, phone number or address of interest. Contact information will be verified using names, addresses, and phone numbers.

5.1.3 Recruitment Contact

Study staff will phone the family approximately one week after mailing the introductory letter and study brochure to further assess eligibility and gauge general interest in participating. Study staff will use the phone script devised for this study as a conversation guide. During this phone call, study staff will provide a description of the study and its requirements. If the family is interested in participating, study staff will describe the study to the legal guardian and ask if the guardian and child are interested in coming into Nationwide Children's Hospital to enroll in the project. During this appointment, study staff will meet with the guardian to provide detailed information about the study and answer any questions the guardian may have. If the parent is interested in enrolling his/her

child and the child meets all eligibility criteria and is not excluded based on the exclusion criteria, study staff will guide the parent through the informed consent process. Participation or refusal to participate in the study will not affect the child's entitlement to clinical care.

This study intends to enroll children a special population and will accordingly adhere to additional protections specified under 45 CFR Part 46 Subpart D – Additional Protections for Children Involved as Subjects in Research (45 CFR Part 46.401-409).

5.1.4 Treatment Assignment

Study staff will develop a detailed Standard Operating Procedure (SOP) for Randomization and Blinding, and Emergency Unblinding Procedures. This SOP will describe how the randomization scheme will be generated and that the scheme will be stored in a locked cabinet.

5.1.5 Randomization and Masking Procedures

Randomization will involve a stratified block design of randomly varying block sizes of 6 and 12 with equal allocation to each of the two study arms. A pseudorandom number generator in the statistical software package R will be used by Dr. Rausch to implement randomization, and he will maintain the randomization sequence such that it is unavailable to other team members. Randomized block sizes will be generated within each of 4 strata formed by crossing 2 stratification variables: age (2-3 vs 4-6 years) and sex. This stratification design minimizes confounding across groups due to the stratification variables. This block design assures a balanced allocation across the 2 study arms. Randomly varying block sizes reduces the chance that study staff can guess the next group assignment and thus minimizes unconscious bias in patient allocation in the RCT. Furthermore, having an experienced statistician not otherwise involved in study operations produce the randomization scheme will help maintain blinding which is critical to the integrity of the trial and the value of the findings. Investigators, staff, parents, and participants will be blind to treatment assignment until all data are collected whereupon only those involved in analysis will be unblinded as needed. One to two staff people (Adherence Aides) will be unblind and have the sole role of counseling families with strategies for protocol compliance (encouraging children to ingest the assigned volume) during scheduled phone calls. In order to perform this duty, they will need to have access to information about assigned dose.

Dr. Rausch will create tamper-resistant opaque envelopes. These will be sequentially numbered with study ID numbers (a sequence prepared for each of the 4 total strata). These will be placed in sequence in 4 labeled and secured boxes, one for each stratum. Each time a participant is recruited the next envelope in order will be drawn from the stratum specific box that corresponds to that participant's stratum. Inside each envelope will be a designation (e.g., one of six letters of the alphabet) for assignment to treatment or placebo and at which dose. This designation will not be revealed to families. The participant will be registered in the electronic medical record system and the study doctor will print and sign the prescription. The participant study ID will be added to this prescription. Study staff will alert the NCH Investigational Drug Service as to the enrollment and randomization of the participant and the participant's weight. IDS will be given the signature page of the consent form and the prescription. IDS will have access to the randomization scheme so they can match the letter designation and ID to the appropriate treatment and dose. IDS will prepare the prescription and the oil will be distributed directly from the pharmacy to the family to prevent unblinding of study staff.

5.1.6 Emergency Unblinding

An emergency unblinding SOP also will be generated. Only the IRB or PI are allowed to authorize early unmasking in the event of emergency. Otherwise, the randomization assignments will be kept secure until closure of the study.

5.1.7 Withdraw

Study participation will be discontinued under the following circumstances:

1. PI or Study Doctor believe it is in the best interest of the participant;

2. Participant's legal guardian requests withdraw from the study;

3. Any clinical AE, laboratory abnormality, intercurrent illness, or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the child, as determined by the PI and Study Doctor; or

4. Development of any exclusion criteria may be cause for discontinuation.

5. Study instructions are not followed, participation in the study may also be stopped

Withdrawal from the study will not affect the receipt of clinical care at NCH. If the study is halted for any reason, participants will be instructed to discontinue taking the assigned treatment and return the unused portion to study staff. Participants who choose to discontinue taking the assigned treatment during the study will continue to be followed for assessment of the outcomes, and their discontinuation will be recorded in the study database. Participants who continue to participate but are found to have low adherence to the assigned treatment oil or placebo oil will not be withdrawn and will continue to be followed.

5.1.8 Handling of Withdrawals

The study will continue to collect safety data on any participant discontinued because of an AE or serious adverse event (SAE). If a participant stops taking the assigned oil, the participant will be asked to continue scheduled evaluations.

5.1.9 Termination of Study

Because interventions based on products with similar composition have not found harm to young children, it is not anticipated that the study would need to be terminated due to development of toxicities or adverse events due to the intervention. However, the study will be terminated if the PI, Study Doctor, IMC, or IRB conclude based on their findings that termination is in the best interests of the participants.

6 STUDY PROCEDURES AND SCHEDULE OF EVENTS

Participation will last for 90 days. The study will involve three in-person study visits at 0, 45 and 90 days and one remote "e-visit" at 25 days. Visit 1 will involve informed consent, randomization, gathering anthropometric data, parent completed questionnaires, distribution and explanation of the study diaries, a 3 mL blood collection, and distribution of the treatment oil/placebo oil. This visit will last approximately 90 minutes. Day 25 e-visit will review the study diaries to date, assess compliance, barriers to participation, and adverse events. Visits 2 and 3 will last approximately 60 minutes. They will involve returning all oil bottles distributed at the prior in-person visit, review of study diaries, assessment of compliance and adverse events, parent completed questionnaires, and a 3 mL blood collection. In addition, study staff will check in with families at four other time points (Day 5 +/-3 days, Day 35 +/-7 days, Day 60+/-7 days, and Day 80 +/-7 days) to assess compliance, adverse events, and barriers to participation. We will ask families for updated contact information at the end of each study visit, as well as to provide up to three additional contacts (e.g., friends/family) who we may contact if their contact information becomes outdated.

6.1 Visit 1 (Day 0)

The enrollment visit will consist of the following activities:

Informed Consent: See Informed Consent section.

Randomization: After consent, families will be randomized. See Randomization and Masking Procedures.

Blood Collection: To measure baseline fatty acid levels, a trained pediatric phlebotomist will draw 3 mL whole blood into a purple top tube.

Anthropometrics: Child weight and height will be measured using SECA scales and stadiometer to inform IDS in calculating the assigned dose. If child cannot be accurately weighed, staff will use most recent weight found in medical record system (EPIC).

Parent Perception of Treatment Group: Parents will be asked what treatment group they believe their child will be in.

Barriers to Participation: The family's intentions to complete the study, attend the next study visit, and give their child the assigned oil on a daily basis throughout study duration.

Parent Interview: A parent interview will gather baseline child and family characteristics (sex, age, socioeconomic index) to characterize the sample and verify comparability across groups. In addition, parents will complete the Baylor fatty acid-specific food frequency questionnaire as an interview to measure usual dietary intake of long-chain fatty acids. Finally, parents are asked to report on current medications, upcoming surgeries, as well as use of complementary and behavioral therapies or programs.

Supplement Dispension: Study staff will counsel families on instructions for taking the daily treatment oil/placebo oil, how to record each dose in a provided daily study diary, and reporting adverse events. IDS will dispense (4) 8 oz. bottles of assigned oil, which will be ample for the majority of participants. If child weighs more than 32 kg, an extra bottle will be dispensed at Visit 1 and Visit 2. IDS will dispense the assigned oil directly to the family and will further instruct on taking the assigned oil.

Compensation: At the conclusion of the visit, participants receive $75 in compensation for their time and a Children's Hospital Parking Garage Pass ($2). Children are given a developmentally-appropriate children's book or small toy (value =$5).

Visit scheduling: Study staff will schedule for in-person visits 2 and 3.

6.2 E-Visit (Day 25 +/- 7 days phone contact)

This visit will be a remote "E-Visit" via phone and will include the following activities:

Parent Perception of Treatment Group and Adherence: Parents will be asked what treatment group they perceive their child to be in and to estimate what proportion of doses they administered out of the desired number and what proportion of the assigned oil they estimate their child usually consumed. Study staff will review study diaries use with the parent and will probe for any problems encountered that reflect acceptability, tolerability or compliance. Study staff will request that the caregiver take photos of each study diary page used thus far. Caregiver will email or text study staff with these photos.

Adverse Events: Parents will be asked if the child has any health or behavior changes since the last communication. We will use a modified version of the *Safety Monitoring Uniform Report Form* (*SMURF*) to document adverse events.

Barriers to Participation: The family's intentions to complete the study, attend the next study visit, and give their child the assigned oil on a daily basis throughout study duration; as well as any anticipated barriers to study participation are assessed during each contact with the family.

To simplify our recruitment and retention materials, participants will see that there are 3 in-person visits and 5 follow up phone calls. This "E-Visit Day 25" phone call will be one of the five follow-up contacts.

6.3 Visit 2 (Day 45 +/- 14 days)

This visit will consist of the following activities:

The following activities will follow the same procedures as for Visit 1: Blood Collection, Parent Interview, Parent Perception of Treatment Group and Adherence, Adverse Events, Barriers to Participation.

Supplement Return: Families will return all previously dispensed oil bottles to IDS for calculating of used/unused portion.

Supplement Dispension: IDS will dispense (4) 8 oz bottles of assigned oil (extra bottle will be dispensed if child is over 32 kg). IDS will dispense the assigned oil directly to the family and will further instruct on taking the assigned oil. Completed study diary pages will be scanned and diary returned. New diary dispensed as necessary.

Compensation: Compensation will be the same as for Visit 1. In addition, families will receive $2 for every study diary returned at the study visit (1 diary page covers a 2-week period).

6.4 Visit 3 (Day 90 +/- 14 days)

The final visit will consist of the following activities:

The following activities will follow the same procedures as for Visit 2: Blood Collection, Parent Interview, Parent Perception of Treatment Group and Adherence, Adverse Events, Supplement Return.

Anthropometrics: Child weight and height will be measured using SECA scales and stadiometer.

Supplement Return: Families will return any empty assigned oil bottles and any partially used or unopened assigned oil bottles to IDS.

Compensation: Compensation will be the same as for Visit 2, except if families completed all 4 study visits (1 of which is the E-Visit), they will receive an additional $20 incentive. Families will receive $2 for every study diary returned at the study visit (1 diary page covers a 2-week period).

6.5 Additional Routine Communication (throughout participation)

In addition to the study visits, study staff will routinely check in with families at least 5 times (more if a family requires additional support) spaced between the visits to assess adherence, adverse events, and barriers to participation. These checks may take place in person, over the phone, or through email or text, depending on family preference and ease of communicating.

Families will be reminded of scheduled/upcoming appointments via phone, email and/or text (based on family preference). Attempts will be made via phone, email, text, and/or mail to contact families who do not show for their scheduled visits. During these communications, both remotely and in person, parents may be asked to estimate what proportion of doses they administered out of the desired number and what proportion of the assigned oil they estimate their child usually consumed. Interim study diary data will be collected in the form of photos (of each completed study diary page) as often as possible from caregiver via email or text. This will assist in keeping families engaged in study diary use and will provide data if study diaries cannot be returned at in-person visits. Study staff will work with the family to increase adherence where possible. We will use the modified *Safety Monitoring Uniform Report Form* (*SMURF*) to document adverse events. Children who do not return for the 2 in-person follow up visits will be considered lost to follow-up, but attempts will be made to contact them via phone at these pre-prescribed intervals.

6.6 Rationale for Data Collection

Data are collected to:

1) Assess demographic and other participant characteristics, thereby allowing a check of the randomization procedure and to report on the composition of the sample;

2) Assess the outcomes of interest; and

3) Determine the feasibility of and aims for the next stage of research.

7 LABORATORY EVALUATIONS

7.1 Specimen Preparation, Handling, and Shipping

Instructions for the preparation, handling, and storage of specimens are explained clearly in the study's SOP, including required temperatures, aliquots of specimens, where they will be stored, and how they will be labeled. Samples will be stored at -80^{o}C until analysis in Dr. Lynette Rogers' laboratory in the Center for Perinatal Research. All laboratory analyses of biospecimens will be conducted by Dr Rogers. Serum and RBC samples will be collected from patients at 3 time points to determine bioavailability of the supplement in this specific population and to measure the effects of treatment on biological signatures.

Fatty acids will be measured in patient red blood cells by standard protocols using gas chromatography and will include: capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0), palmitoleic (16:1n-7), steric (18:0), oleic (18:1 n-9), vaccenic (18:1n-7), linoleic acid (18:2 n-6), α-linolenic acid (18:3 n-3) , γ-linolenic acid (18:3 n-6), dihomo γ-linolenic acid (DGLA, 20:3 n-6) arachidonic (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5 n-3), and docosahexaenoic acid (DHA, 22:6 n-3). High and low controls will be run with each batch analysis and a run will be considered non-valid if the controls are greater than 2 standard deviations from the mean values for DHA, EPA, and GLA. A non-valid analysis will be discarded and the samples will be re-analyzed. Biological signatures will include the cytokines IL-1β, IL-2, IFNγ. These inflammatory cytokines will be measured using an ELISA based technology offered by Meso Scale Discovery.

Any remaining specimens will be maintained in a secured freezer in the Center for Perinatal Research, or Center for Biobehavioral Health (depending on space availability) at NCH for future research use. Only the investigators for this study will have access to these specimens and permission for future use. The consent form for the study will state that leftover specimens may be used for studies of ASD, child development or nutrition conducted only by the investigators for the present study.

7.2 Biohazards

Blood samples will be obtained from study participants at each visit. This service will be provided by the Outpatient Lab at Nationwide Children's Hospital. As a back-up, Clinical Research Services nursing staff can perform this duty. All personnel will be trained in the handling of biohazardous samples and are experienced in working with children in these situations. Blood samples will be separated prepared for storage by personnel trained in handling hazardous biological samples. All procedures will follow Good Clinical Practice and federal, state, and institutional regulations and guidelines.

8 INVESTIGATIONAL PRODUCT

8.1 Acquisition

Nordic Naturals Complete Omega™ liquid (treatment oil) will be provided by Nordic Naturals (Watsonville, CA). Bulk canola oil will be purchased from Welch, Holme and Clark Co., Inc. (Newark, NJ). The PI will request an initial supply of the treatment oil and placebo oil to be delivered to IDS prior to the study commencing. Additional products will be requested as needed. All treatment oil will come from the same manufacturing batch. A sufficient supply of the oils will be maintained in IDS throughout the study.

8.2 Formulation, Packaging, and Labeling

The treatment oil will be provided by Nordic Naturals in blue glass bottles. It is available over-the-counter. The Nordic Naturals Complete Omega™ liquid contains in each 1 tsp (5ml):

Supplement Facts

Serving Size: **1 Teaspoon (5 mL)**

Amount Per Serving		% Daily Value*
Calories	45	
Calories from fat	45	
Total Fat	5.0 g	8%
Saturated Fat	1.0 g	5%
Trans Fat	0 g	†
Omega-3s	1270 mg	†
EPA (Eicosapentaenoic Acid)	610 mg	†
DHA (Docosahexaenoic Acid)	405 mg	†
Other Omega-3s	255 mg	†
Omega-6s	500 mg	†
GLA (Gamma-Linolenic Acid)	170 mg	†
Omega-9s	500 mg	†
OA (Oleic Acid)	400 mg	†

* Percent Daily Values are based on a 2,000 calorie diet.
† Daily Value not established.
Less than 5 mg of Cholesterol per serving.

Ingredients: purified deep sea fish oil (from anchovies and sardines), borage seed oil (*borago officinalis*), natural lemon flavor, d-alpha tocopherol, rosemary extract (a natural preservative).

No gluten, milk derivatives, or artificial colors or flavors.

Welch, Holme and Clark Co., Inc. will provide the canola oil placebo. Although fatty acid composition varies slightly with each lot of the canola oil, the distributor provides average fatty acid composition per 2.5 ml (2.3g) daily serving. Specifically, each 2.5 ml serving of the canola oil placebo contains 124 mg of palmitic acid, 39 mg stearic acid, 513 mg linoleic acid, 225 mg linolenic acid, and 1346 mg oleic acid. The bulk canola oil placebo will be shipped from the distributor in 5-gallon shipments to NCH IDS who will mix the oil with lemon oil at 1:66.6 (1.5 mL of lemon oil per 100 mL of canola oil). In this way, the treatment oil and placebo oil will be indistinguishable in terms of appearance, smell and taste. IDS will bottle and cap the placebo oil in identical bottles and caps as the treatment oil.

8.3 Dispension

Each participant who is 32 kg or less will receive four 8 oz bottles of assigned oil at Visits 1 and 2. An additional bottle will be dispensed at T1 and T2 if participant is over 32 kg. At the initial dispension, a prescription will be delivered to IDS, signed by the Study Doctor. At this point, IDS will select the appropriate product, determine the appropriate dose based on child's weight and group randomized, and place a label conducive to Ohio BOP and FDA regulations is placed on each bottle of the treatment oil or placebo oil before being dispensed. Additionally, the amount of product dispensed to a participant is recorded in an overall master log, maintained by IDS. No information on the bottles will indicate which product is contained in them upon dispension from IDS.

In the case of a participant requiring additional supplies of assigned oil (spillage, losing a bottle), interim dispensions can occur via shipping product to the family's home. One 8 oz. bottle (or more if necessary) will be provided in these instances.

8.4 Product Storage and Stability

The treatment oil and placebo oil will be kept in a dry, secured environment, controlled and monitored for the appropriate temperature. The expiration dates on the products will be monitored by IDS to ensure product freshness. The treatment oil and placebo oil will be kept refrigerated to maximize freshness. All families will be instructed to keep their bottles refrigerated.

8.5 Dosage, Preparation and Administration

Children are randomized to receive 25 mg, 50 mg, or 75 mg of Omega 3-6 (treatment group) or equal volume of placebo oil to be administered twice per day by mouth for 90 days.

8.6 Modification of Investigational Product

No circumstances are known for which the dose should be modified.

8.7 Accountability Procedures for the Investigational Product

IDS will monitor the receipt of the treatment oil and placebo oil from the manufacturers, dispension of the treatment oil and placebo oil to participants, the return of the treatment oil and placebo oil from participants, and the appropriate disposal of any unused assigned oils.

8.8 Assessment of Subject Compliance with the Investigational Product

Parents will be provided with a series of paper diaries and instructions to record whether the child consumed the assigned oils each day and what proportion they consumed each day as well as any acceptability or tolerability issues. Set of study diaries will be dispensed at Visit 1. Pages of this diary will be scanned and diary will be returned. A new set of study diaries can be dispensed at follow up visit or by mail as needed. Photos of any completed study diary pages will be requested from caregivers during follow up contact. Caregivers will text or email photos of these pages to study staff. See Study Procedures and Schedule of Events for procedures for assessment of compliance.

8.9 Concomitant Medications/Treatments

There are no restrictions on what drugs or treatments may be concomitant with the study intervention.

9 RISKS AND BENEFITS

9.1 Risks

All of the research related procedures, surveys, and questionnaires, are designed to meet the definition of "minimal risk" in the federal regulations [§45 CFR 46.102(i)] and to be reviewed by IRBs under §45 CFR 46.404 "Research not involving greater than minimal risk." Minimal risk as defined in the federal regulations means "that the probability and magnitude of harm or discomfort anticipated in the research are not greater, in and of themselves, than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests." In addition, the Study Staff is committed to minimizing risks even when the risks are minimal.

The informed consent process will take place in private. Questionnaires will be structured to avoid creating discomfort for the research participants; and participants will be reminded at each data collection encounter that participation is voluntary, that they have the right to withdraw from the research project at any time, and they may refuse to answer or may skip any question.

Experienced pediatric phlebotomists staff will perform biospecimen collections that involve minimal discomfort or pain. All staff involved in collection of biospecimens will be credentialed accordingly. The physical risks of drawing blood by placing a needle in a vein may cause pain, lightheadedness, bleeding, bruising, or swelling at the puncture site. Infection is a rare possibility. No alternatives to blood collection exist to reliably assess fatty acid and inflammatory status in humans.

The risks of collecting and storing linked clinical data and biospecimens are primarily psycho-social. Developing coding strategies to mitigate these risks is a fundamental ethical requirement of the study. Potential harm could result from a breach of confidentiality. One of the primary concerns is that employment and insurance discrimination might result from exposure of information about health history, genetic makeup, or familial predisposition to disease. The risk is minor, especially since unique participant identifiers will not be stored with biospecimens.

The Nordic Naturals Complete Omega™ liquid product contains a combination of fish and borage oils. These oils include several LCPUFAs including eicosapentaeonic acid (EPA) and gamma-linolenic acid (GLA). Although giving EPA to preterm infants (i.e., children much younger than those eligible in this study) has been found to pose some risk related to poorer growth in a single-site study that has not been replicated,[32] numerous other infancy and post-infancy studies using EPA have not reported adverse effects. Because EPA also can increase bleeding time, albeit generally clinically insignificantly, children with existing bleeding disorders will be excluded from participation. Children with planned surgeries will be excluded.

The presence of borage oil in the supplement contributes the GLA content. Most of the safety information available about GLA comes from studies that used evening primrose oil, another major source of GLA. Animal studies have shown no toxic or carcinogenic effects.[33] Previous human studies have involved over 4,000 individuals, and no significant adverse effects have been reported. There have been two case reports of excessive intake of borage oil or other GLA-containing supplements causing worsening seizures in epileptics, but there have been no published cases or studies since these reports were published in 1981.[34] Although the likelihood of this occurring in the present study is very low, children with a seizure disorder will be excluded from the study. A 2004 study of 238 preterm neonates evaluated an infant formula with fish and borage oil compared to a vegetable oil formula fed to infants for 9 months.[35] Daily intake amounts varied based on volume consumed, but the fish and borage oil group received up to an estimated 26mg per day of GLA. This is the equivalent of between 3 and 17 mg/kg/day depending on the child's body composition

and age. Although Fewtrell and colleagues[35] stated adverse events were present in both formula groups, the fish and borage oil group experienced more days on ventilator, more days on oxygen, and more days with umbilical catheter. Although these adverse events were reported, many pre-existed randomization. The infants in that study were already NICU patients with multiple co-morbidities present before randomization. Because of the older age of the participants in this study, adverse events such as those reported by Fewtrell and colleagues are not anticipated.[35]

The placebo for this study will be canola oil with a natural lemon oil flavoring added. Canola oil is a commonly consumed food present in many prepared food products. Whether it is possible to have an allergy to canola/rapeseed is controversial, but very rarely some people may have a non-allergic reaction. Nevertheless, we will exclude any children whose parent states they have canola/rapeseed allergy or sensitivity. Lemon oil is Generally Recognized as Safe (GRAS) by the FDA.

The study staff will maintain confidentiality of the data in its databases. Data will be summarized in aggregate for reporting purposes. Data submitted to the study does include participant identifiers, as defined by the U.S. Health Insurance Portability and Accountability Act of 1996 (HIPAA). The study will follow all necessary measures to assure the participant data stored in the database are protected and secure from unauthorized access.

The knowledge to be gained from this investigation outweighs these minimal risks. This study has the potential to inform future dietary recommendations for children, especially those diagnosed with ASD and associated comorbidities.

9.2 Benefits

Omega fatty acid supplementation may benefit participating children by reducing their ASD symptomology, and comorbidities. It is not known if these benefits exist. Participation in this study will aid in acquiring new knowledge that might help other children with an ASD diagnosis and their families in the future.

There are no other known benefits expected from participation in this study.

10 ASSESSMENT OF SAFETY

10.1 Methods and Timing for Assessing, Recording, and Analyzing Safety Parameters

Safety oversight will be under the direction of the Principal Investigators (PIs), Study Doctor, and the Independent Study Safety Monitor. All adverse events will be reviewed to rule out the study intervention as a potential cause. All Adverse Events will be recorded on a form designated for that purpose, labeled with a participant ID and no personal identifiers. For any adverse events required to be reported to the IRB or the FDA, and for review purposes by the Independent Monitoring Committee (IMC), only the coded ID number will be included in the documentation as needed, and no identifiers. The IMC will review adverse events anonymously. If the child's medical record must be accessed to provide additional details as to the event or its treatment, study staff will provide abstracted information that is de-identified. Annual reports of findings will be submitted to the FDA in line with IND regulations.

10.2 Adverse Events

Adverse Event (AE): ICH E6 defines an AE as any untoward medical occurrence in a patient or clinical investigation participant administered a pharmaceutical product regardless of its causal relationship to the study treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of investigational product. The occurrence of an AE may come to the attention of study personnel during study visits and interviews of a study recipient presenting for medical care, or upon review by a study monitor.

All AEs including local and systemic reactions not meeting the criteria for "serious adverse events" should be captured on the appropriate CRF. Information to be collected includes event description, time of onset, Study Doctor's assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis, which would include MD, PA, Nurse Practitioner, DO, or DDS), and time of resolution/stabilization of the event. All AEs occurring during study participation must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is present at the time that the participant is screened should be considered as baseline and not reported as an AE. However, if the medical condition deteriorates at any time during the study, it should be recorded as an AE.

All AEs must be graded for severity and relationship to study product.

Severity of Event: All AEs will be assessed by the Study Doctor using a protocol defined grading system. For events not included in the protocol defined grading system, the following guidelines will be used to quantify intensity.

- Mild: events require minimal or no treatment and do not interfere with the participant's daily activities.
- Moderate: events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe: events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.

- Life threatening: any adverse drug experience that places the participant, in the view of the PI, at immediate risk of death from the reaction as it occurred (i.e., it does not include a reaction that had it occurred in a more severe form, might have caused death).

Changes in the severity of an AE should be documented to allow an assessment of the duration of the event at each level of intensity to be performed. Adverse events characterized as intermittent require documentation of onset and duration of each episode.

Relationship to Study Products: The Study Doctor's assessment of an AE's relationship to study intervention is part of the documentation process, but it is not a factor in determining what is or is not reported in the study. If there is any doubt as to whether a clinical observation is an AE, the event should be reported. All AEs must have their relationship to study product assessed using the terms: related or not related. In a clinical trial, the study product must always be suspect. To help assess, the following guidelines are used.

- Related (Possible, Probable, Definite)
    a. The event is known to occur with the study intervention.
    b. There is a temporal relationship between the intervention and event onset.
    c. The event abates when the intervention is discontinued.
    d. The event reappears upon a re-challenge with the intervention.
- Not Related (Unlikely, Not Related)
    a. There is no temporal relationship between the intervention and event onset.
    b. An alternate etiology has been established.

10.3 Unanticipated Problems

The Office for Human Research Protections (OHRP) considers unanticipated problems involving risks to subjects or others to include, in general, any incident, experience, or outcome that meets **all** of the following criteria:

- Unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;
- Related or possibly related to participation in the research ("possibly related" means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

10.3.1 Reporting Unanticipated Problems

Incidents or events that meet the OHRP criteria for unanticipated problems will be reported using an unanticipated problem report form. The unanticipated problem report form will include the following information when reporting an adverse event, or any other incident, experience, or outcome as an unanticipated problem to the IRB:

- Appropriate identifying information for the research protocol, such as the title, investigator's name, and the IRB project number;
- A detailed description of the adverse event, incident, experience, or outcome;
- An explanation of the basis for determining that the adverse event, incident, experience, or outcome represents an unanticipated problem;
- A description of any changes to the protocol or other corrective actions that have been taken or are proposed in response to the unanticipated problem.

To satisfy the requirement for prompt reporting, unanticipated problems will be reported using the following timeline:

- Unanticipated problems that are serious adverse events will be reported to the IRB, Independent Safety Monitor(s), and NCCIH within 7 days of the investigator becoming aware of the event.
- Any other unanticipated problem will be reported to the IRB, Independent Safety Monitor(s), and NCCIH within 14 days of the investigator becoming aware of the problem.

All unanticipated problems should be reported to appropriate institutional officials (as required by an institution's written reporting procedures), the supporting agency head (or designee), and OHRP within one month of the IRB's receipt of the report of the problem from the investigator.

10.4 Serious Adverse Events

Serious Adverse Event (SAE): An SAE is defined as an AE that meets one of the following conditions:

- Death during the period of protocol defined surveillance.
- Life-threatening event (defined as a subject at immediate risk of death at the time of the event).
- An event requiring inpatient hospitalization or prolongation of existing hospitalization during the period of protocol defined surveillance.
- Results in congenital anomaly or birth defect.
- Results in a persistent or significant disability/incapacity.
- Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious adverse experience when, based upon appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above. Examples of such medical events include allergic bronchospasm requiring intensive treatment in an

emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.

The Study PI and Independent Monitoring Committee will be responsible for determining whether an SAE is expected or unexpected. An adverse event will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the intervention.

All SAEs will be:

- Recorded on the appropriate SAE CRF.
- Followed through resolution by a Study Doctor.
- Reviewed and evaluated by a Study Doctor.

10.4.1 Reporting SAE's

Any AE considered serious by the PI or Study Doctor or which meets the aforementioned criteria must be submitted on an SAE form to the NCH IRB within 3 business days of discovery. All SAEs will be followed until satisfactory resolution or until the Study Doctor deems the event to be chronic or the patient to be stable.

Annual reports of findings will be submitted to the FDA in line with IND regulations.

SAEs that are unanticipated, serious, and possibly related to the study intervention will be reported to the Independent Monitors(s), IRB, FDA, and NCCIH in accordance with requirements. For the IND:

- 7-day IND Safety Report (unexpected fatal or life-threatening AEs related to the intervention); a copy of the report sent to the FDA will be submitted to the NCCIH Program Officer and Independent Safety Monitor(s) within 24 hours of FDA notification.
- 15-day IND Safety Report (any other serious and unexpected AE related to the intervention); a copy of the report submitted to the FDA will be submitted to the NCCIH Program Officer and Independent Safety Monitor(s) within 24 hours of FDA notification.
- All other AEs documented during the course of the trial will be reported to NCCIH on an annual basis by way of inclusion in the annual report and in the annual AE summary which will be provided to NCCIH and to the Independent Monitors. The Independent Safety Monitor(s) Report will state that all AEs have been reviewed.

10.5 Procedures in the Event of Abnormal Laboratory Test Values/Clinical Findings

Collection of laboratory data should be limited to those laboratory parameters that are relevant to safety, study outcome measures, and/or clinical outcome. No known fatty acid value is considered to be abnormal or to present a safety concern. As a result, it is not anticipated that any abnormal laboratory values will be found. However, any abnormal clinical findings will be reported to the child's physicians.

10.6 Type and Duration of Follow-up of Subjects after Adverse Events

Unanticipated problems will be recorded in the data collection system throughout the study. The PI will record all reportable events with start dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. At each study visit, the investigator will inquire about the occurrence of AE/SAEs since the last visit. Events will be followed for outcome information until resolution or stabilization.

10.7 Halting Rules

If it is found that the study intervention is related to any serious adverse events, enrollment would be temporarily suspended until a safety review is convened, the objective of which is a decision as to whether the study (or intervention for an individual or study cohort) should continue per protocol, proceed with caution, be further investigated, be discontinued, or be modified and then proceed. Suspension of enrollment (for a particular group or for the entire study) is another potential outcome of a safety review.

Subsequent review of serious, unexpected, and related AEs by the PI, Study Doctor, the Independent Study Safety Monitor, the IRB, or the FDA or relevant local regulatory authorities may also result in suspension of further trial interventions/administration of study product. The FDA retains the authority to suspend additional enrollment and study interventions/administration of study product for the entire study, as applicable.

Based on the large number of previous studies that have utilized a similar intervention in children and the widespread use of the study products in the U.S. consumer marketplace, there are no expectations for halting the study enrollment or intervention due to safety concerns. However, it will be left to the judgment of the PI with the advice of the Study Doctor and Safety Monitor whether a pattern of adverse events or other circumstances warrant halting.

10.8 Safety Oversight

Safety oversight will be under the direction of the PIs and Independent Study Safety Monitor. All adverse events will be reviewed to rule out the study intervention as a potential cause.

11 STATISTICAL CONSIDERATIONS

11.1 Study hypotheses

This study will test the hypothesis that Omega 3-6 will produce a clinically significant reduction (improvement) in plasma levels of IL-1β, IL-2, and/or IFNγ and assess bioavailability and safety, of this product.

11.2 Sample Size Considerations

Our power analysis is based on a comparison on pre-post change between all the placebo group participants compared to all treatment group participants receiving any Omega 3-6. We use a Type I error rate of .05 to test our primary aim that at least one of our inflammatory markers will demonstrate improvements from baseline to posttest compared to the placebo group.

For the purposes of examining power, we assume an independent samples t-test on pre-post change with ≥80% power to obtain a sufficient per group sample size (n), equal across groups. Consequently, the standardized mean difference (δ) is the effect size of interest. We base our estimate of the intervention effect size on our Preemie Tots study where δs of .75, .77, and 1.04 were obtained for the inflammatory markers of IL-1β, IL-2, and IFNγ, respectively. We propose $\delta > .5$ as part of our go/no-go decision for proceeding to the next phase of research (a second trial). This effect size (effect on inflammation) is clinically meaningful, based on the meta-analysis by Masi which compared children with ASD to typically-developing children and observed differences in cytokine levels of $\delta = 0.6$ and 1.0 for 2 of our biomarkers of interest.[41] Importantly, the dose used in Preemie Tots is comparable to the smallest dose treatment group here; consequently, if a dose effect does indeed exist across the treatment groups, we expect the effect sizes we observe will be even larger in the present study when compared to the placebo group. Given these effect sizes and conservatively accounting for attrition rates of 10%, n=33 for each of the placebo and treatment groups will allow us to detect treatment effects with at least 80% power.[36]

11.3 Planned Interim Analyses (if applicable)

No interim analysis is planned.

11.3.1 Safety Review

Based on the large number of previous studies that have utilized the same intervention in similar populations and the widespread use of the study products in the U.S. consumer marketplace, there are no expectations for halting the study enrollment or intervention due to safety concerns. However, it will be left to the judgment of the Principal Investigator with the advice of the IMC whether a pattern of adverse events or other circumstances warrant halting. See the section "Assessment of Safety" for details about safety monitoring.

11.3.2 Immunogenicity or Efficacy Review

None

11.4 Final Analysis Plan

Aim 1

Aim 1 Primary Analysis: To examine group differences in bioavailability and safety, we will compare the entire treatment group to the entire placebo group, and at the different dosing levels within treatment, by: 1) comparing mean and median %FA for each fatty acid at the end of the trial and 2) comparing the total number of adverse events that occurred in each group and also by adverse event type (e.g., gastrointestinal; severe vs. not).

Aim 1 Secondary analysis: Demonstrate that children receiving Omega 3-6 will exhibit a clinically relevant absolute increase in RBC EPA or DHA of at least 0.5 mol%, between baseline and the end of the trial (i.e., an increase of approx. 20% for DHA, 67% for EPA depending on baseline status).

Aim 1 Secondary analysis (exploratory): Explore the effect of dose on changes in RBC EPA and DHA by examining differences in the increase in RBC EPA and DHA by assigned dose among children assigned to Omega 3-6.

Aim 2

Aim 2 Primary Analysis: To formally examine the efficacy of the intervention, the primary analysis will be to compare the treatment group, where anyone assigned to Omega 3-6 is considered a member, to the placebo group where anyone assigned to placebo is a member, on pre-post change in each inflammatory marker.

Aim 2 Secondary analysis (exploratory): Explore the effect of dose by comparing each Omega 3-6 dose to the placebo group (all children assigned to placebo) on pre-post change in each inflammatory marker.

The primary approach for the analyses is intent-to-treat: individuals will be kept in the group to which they were randomized, regardless of protocol violations or drop-out. Every effort will be made to minimize missing data. Analyses will explore plausible missing data mechanisms, and baseline predictors of missingness will be investigated. Also, when possible maximum likelihood estimation (MLE) will be employed where predictor variables associated with missingness will be investigated in sensitivity analyses. MLE generally provides valid inferences when the missing data mechanism is missing at random[37] and is more appropriate than many commonly employed approaches to data imputation.[38]

We will examine group differences descriptively on bioavailability and safety data for the entire placebo group, the entire treatment group, and (secondarily, exploratory) at the different dosing levels within treatment. Specifically, for the Primary Analysis in Aim 1, we will use the independent samples t-test and the Wilcoxon rank-sum test to examine treatment vs. placebo group differences on the %FA for each fatty acid at the end of the trial, the total number of adverse events, and number adverse events by event type. This approach will allow us to examine both parametric and non-parametric approaches to group differences on each of these measures of bioavailability or safety.

For the Secondary Analysis in Aim 1, we will calculate the increase for each of RBC EPA and DHA to determine if a clinically relevant value is obtained. For the Exploratory component of our Secondary Analysis in Aim 1, we will employ a repeated-measures mixed effects model to examine change in each of RBC EPA and DHA as a function of dose within the treatment group only.

For the Primary Analysis in Aim 2 to formally examine the efficacy of the intervention we will compare the treatment group where anyone given Omega 3-6 is considered a member to the placebo group when anyone given a placebo dose is considered a member on pre-post change in each inflammatory marker. The following is an illustration of our statistical model for repeated measures (RM), similar to analysis of covariance (ANCOVA) but based on maximum likelihood estimation, that will be employed for these analyses[39]: $y_{it} = \beta_0 + \beta_1 Time_t + \beta_2 Time_t \times Trt_i + \varepsilon_{it}$, where implicitly it is assumed that there is no main effect of Trt_i, y_{it} is the value for a given inflammatory marker for individual *i* at time *t*, $Time_t$ is coded as 0 for the pretest and 1 for the 90-day post-treatment, and the primary effect of interest is β_2, which corresponds to the difference between the groups on change in the inflammatory marker of interest. In this RM model, the baseline measure of the individual's inflammatory marker is constrained to have equal means across groups by assuming there is no main effect for Trt_i. This constraint yields a statistical test for β_2 analogous to the test of the treatment group main effect in an ANCOVA covarying the pretest measure of the outcome variable. The main advantage of the RM model is that it can be estimated in SAS PROC MIXED using maximum likelihood estimation on all available data by treating $Time_t$ as a repeated measures factor with 2 levels, whereas an ANCOVA model would require listwise deletion for missing data.[38]

Importantly, for the Secondary/Exploratory Analysis in Aim 2, we will investigate the dose effect by using dosage given to a participant as quantitative and categorical predictors within separate models in place of the treatment effect variable, Trt_i, using the same type of model used in the previous analysis. Because of the exploratory nature of our hypotheses concerning the parametric form of the relationship between dose and the response in each inflammatory marker, separate analyses using quantitative and categorical versions of the dosing variable will allow us to fully explicate the dose-response relationship. We will conduct exploratory analyses by child sex to examine sex as a modifier.

12 SOURCE DOCUMENTS AND ACCESS TO SOURCE DATA/DOCUMENTS

The study will maintain appropriate medical and research records for this trial, in compliance with ICH E6, Section 4.9 and regulatory and institutional requirements for the protection of confidentiality of subjects. Only authorized Study Staff and NCH IRB staff will have access to the documents.

Source data are all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Examples of these original documents and data records include, but are not limited to, caregiver completed questionnaires/interviews, hospital records, clinical and office charts, laboratory notes, memoranda, subjects' memory aid or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, and subject files and records kept at the pharmacy, at the laboratories, and medico-technical departments involved in the clinical trial. It is acceptable to use CRFs as source documents.

13 QUALITY CONTROL AND QUALITY ASSURANCE

The quality management program for this study will consist of a system of quality checks on all data collection procedures in addition to a system of training.

All study staff that will have contact with study participants will be trained in accordance with the gold standard for each data collection method. At periodic intervals the quality of the data collections being performed will be evaluated by direct observations by the PI or a designated subject matter expert. All paper-based data collection will be evaluated for protocol compliance, completeness, and accuracy by the PI via direct observation of Study Staff and checks of the completed forms. Data entry quality will be assessed by a random check of 10% of the data entries. Laboratory quality control and assurance procedures will follow SOP already in place in the laboratory and used during previous studies.

14 ETHICS/PROTECTION OF HUMAN SUBJECTS

14.1 Ethical Standard

The investigator will ensure that this study is conducted in full conformity with the principles set forth in The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research of the US National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research (April 18, 1979) and codified in 45 CFR Part 46 and/or the ICH E6; 62 Federal Regulations 25691 (1997).

14.2 Institutional Review Board (IRB)

The Nationwide Children's Hospital IRB will review and approve the protocol for this study including the associated informed consent documents and recruitment materials. Any amendments to the protocol or consent materials will also be approved before they are placed into use.

14.3 Informed Consent

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continuing throughout the individual's study participation. Discussion of risks and possible benefits will be conducted with the participants and their families. Consent forms describing, in detail, the study interventions/products, study procedures, and risks are given to the family and written documentation of informed consent is required prior to starting intervention/administering study product. Consent forms will be IRB-approved and the parent will be asked to read and review the document. Upon reviewing the document, Study Staff will explain the research study and answer any questions that may arise. The parent will sign the informed consent document prior to randomization and any procedures being done specifically for the study. The parent will have the opportunity to discuss the study with his/her family or think about it prior to agreeing to participate. The participant may withdraw consent at any time. A copy of the informed consent document will be given to the family for their records. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

14.3.1 Informed Consent/Assent Process (in Case of a Minor)

Children may participate in this study under the written informed consent provided by a parent or legal guardian.

Children of a parent who is cognitively impaired or mentally ill are not eligible if the parent is not able to understand fully the research project and to grant informed consent.

14.4 Exclusion of Women, Minorities, and Children (Special Populations)

The study will not exclude any special populations.

14.5 Subject Confidentiality

Subject confidentiality is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their agents. This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participating subjects.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party.

An Independent Study Safety Monitor or other authorized representatives may inspect all documents and records required to be maintained, including but not limited to, data collection records and pharmacy records for the subjects in this study. Records will be retained indefinitely.

14.6 Study Discontinuation

In the event that the study is discontinued, participants may discontinue use of the treatment oil and placebo oil safely. There is no known risk to discontinuation.

14.7 Future Use of Stored Specimens

Any residual specimens will be maintained after the study is complete and will be available for future analysis and use, per the participant informed consent document. Specimens may be analyzed for future studies of child nutrition, neurodevelopment and behavior and growth. Specimens will be maintained in a secure location in the Center for Perinatal Research or Center for Biobehavioral Health at NCH and will be labeled with a numeric code and no identifying information. This is noted on the consent form and additional consent to bank leftover biospecimens in this manner will be obtained from participating families. Only the investigators for this study will have access to these specimens and permission for future use.

15 DATA HANDLING AND RECORD KEEPING

The PI is responsible to ensure the accuracy, completeness, legibility, and timeliness of the data reported. All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data. Dark ink is required to ensure clarity of reproduced copies. When making changes or corrections, cross out the original entry with a single line, and initial and date the change.

Copies of the CRF will be used as source documents and maintained for recording data for each participant enrolled in the study. Data reported in the CRF derived from source documents should be consistent with the source documents or the discrepancies should be explained.

15.1 Data Management Responsibilities

All source documents and laboratory reports will be reviewed by the investigator team and data entry staff, who will ensure that they are accurate and complete. Adverse events must be graded, assessed for severity and causality, and reviewed by the PI or designee.

Data collection is the responsibility of the Study Staff under the supervision of the PI. During the study, the investigator must maintain complete and accurate documentation for the study.

The Study Staff is also responsible for data management, quality review, analysis, and reporting of the study data under the direction of the investigator team.

15.2 Data Capture Methods

For the purposes of this pilot study, data capture will be via paper and electronic methods (e.g., Red Cap). The data entry system will include password protection and internal quality checks, such as automatic range checks and data validation, to identify data that appear inconsistent, incomplete, or inaccurate. Clinical data will be entered directly from the source documents.

15.3 Types of Data

Data for this study will include questionnaires, interviews, medical record, and laboratory measures.

15.4 Study Records Retention

Study records will be kept in a secure, locked location in the Center for Biobehavioral Health at NCH and kept per procedures laid out in federal regulations.

15.5 Protocol Deviations

A protocol deviation is any noncompliance with the clinical trial protocol, Good Clinical Practice (GCP), or Manual of Procedures requirements. The noncompliance may be either on the part of the subject, the investigator, or the Study Staff. As a result of deviations, corrective actions are to be implemented promptly.

These practices are consistent with ICH E6:

4.5 Compliance with Protocol, sections 4.5.1, 4.5.2, and 4.5.3
5.1 Quality Assurance and Quality Control, section 5.1.1
5.20 Noncompliance, sections 5.20.1, and 5.20.2.

All deviations from the protocol must be addressed in study subject source documents and must be maintained in the regulatory file, as well as in the subject's source document. Protocol deviations must be sent to the local IRB per their guidelines.

16 PUBLICATION POLICY

Following completion of the study, the PI is expected to publish the results of this research in scientific journals. The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a trials-registration policy as a condition for publication. This policy requires that all clinical trials be registered in a public trials registry such as ClinicalTrials.gov, which is sponsored by the National Library of Medicine. Other biomedical journals are considering adopting similar policies. The PI will register this trial in an acceptable registry.

17 LITERATURE REFERENCES

1. Bent S, Bertoglio K, Ashwood P, Bostrom A, Hendren RL. A pilot randomized controlled trial of omega-3 fatty acids for autism spectrum disorder. *J Autism Dev Disord.* 2011;41(5):545-554.
2. Bent S, Hendren RL, Zandi T, et al. Internet-based, randomized, controlled trial of omega-3 fatty acids for hyperactivity in autism. *J Am Acad Child Adolesc Psychiatry.* 2014;53(6):658-666.
3. Amminger GP, Berger GE, Schafer MR, Klier C, Friedrich MH, Feucht M. Omega-3 fatty acids supplementation in children with autism: a double-blind randomized, placebo-controlled pilot study. *Biol Psychiatry.* 2007;61(4):551-553.
4. Martinez M. Polyunsaturated fatty acids in the developing human brain, red cells and plasma: influence of nutrition and peroxisomal disease. *World Rev Nutr Diet.* 1994;75:70-78.
5. McPheeters ML, Warren Z, Sathe N, et al. A systematic review of medical treatments for children with autism spectrum disorders. *Pediatrics.* 2011;127(5):e1312-1321.
6. Chalon S. Omega-3 fatty acids and monoamine neurotransmission. *Prostaglandins Leukot Essent Fatty Acids.* 2006;75(4-5):259-269.
7. Bazan NG. Cell survival matters: docosahexaenoic acid signaling, neuroprotection and photoreceptors. *Trends in neurosciences.* 2006;29(5):263-271.
8. Kitajka K, Puskas LG, Zvara A, et al. The role of n-3 polyunsaturated fatty acids in brain: modulation of rat brain gene expression by dietary n-3 fatty acids. *Proc Natl Acad Sci U S A.* 2002;99(5):2619-2624.
9. Coti Bertrand P, O'Kusky JR, Innis SM. Maternal dietary (n-3) fatty acid deficiency alters neurogenesis in the embryonic rat brain. *J Nutr.* 2006;136(6):1570-1575.
10. Salem N, Litman B, Kim HY, Gawrisch K. Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids.* 2001;36(9):945-959.
11. Simopoulos AP. Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr.* 2002;21(6):495-505.
12. Sergeant S, Rahbar E, Chilton FH. Gamma-linolenic acid, Dihommo-gamma linolenic, Eicosanoids and Inflammatory Processes. *European journal of pharmacology.* 2016;785:77-86.
13. Brigandi SA, Shao H, Qian SY, Shen YP, Wu BL, Kang JX. Autistic Children Exhibit Decreased Levels of Essential Fatty Acids in Red Blood Cells. *International journal of molecular sciences.* 2015;16(5):10061-10076.
14. Vancassel S, Durand G, Barthelemy C, et al. Plasma fatty acid levels in autistic children. *Prostaglandins Leukot Essent Fatty Acids.* 2001;65(1):1-7.
15. Innis SM. Dietary omega 3 fatty acids and the developing brain. *Brain research.* 2008;1237:35-43.
16. Makrides M, Gibson RA, McPhee AJ, et al. Neurodevelopmental outcomes of preterm infants fed high-dose docosahexaenoic acid: a randomized controlled trial. *JAMA.* 2009;301(2):175-182.
17. Johnson CR, Handen BL, Zimmer M, Sacco K. Polyunsaturated Fatty Acid Supplementation in Young Children with Autism. *JOURNAL OF DEVELOPMENTAL AND PHYSICAL DISABILITIES.* 2010;22(1):1-10.

18. Abdallah MW, Larsen N, Grove J, et al. Amniotic fluid chemokines and autism spectrum disorders: an exploratory study utilizing a Danish Historic Birth Cohort. *Brain, behavior, and immunity.* 2012;26(1):170-176.
19. Mankad D, Dupuis A, Smile S, et al. A randomized, placebo controlled trial of omega-3 fatty acids in the treatment of young children with autism. *Mol Autism.* 2015;6:18.
20. Bloch MH, Qawasmi A. Omega-3 fatty acid supplementation for the treatment of children with attention-deficit/hyperactivity disorder symptomatology: systematic review and meta-analysis. *J Am Acad Child Adolesc Psychiatry.* 2011;50(10):991-1000.
21. Puri BK, Martins JG. Which polyunsaturated fatty acids are active in children with attention-deficit hyperactivity disorder receiving PUFA supplementation? A fatty acid validated meta-regression analysis of randomized controlled trials. *Prostaglandins Leukot Essent Fatty Acids.* 2014;90(5):179-189.
22. Richardson AJ, Montgomery P. The Oxford-Durham study: a randomized, controlled trial of dietary supplementation with fatty acids in children with developmental coordination disorder. *Pediatrics.* 2005;115(5):1360-1366.
23. Sinn N, Bryan J. Effect of supplementation with polyunsaturated fatty acids and micronutrients on learning and behavior problems associated with child ADHD. *Journal of developmental and behavioral pediatrics : JDBP.* 2007;28(2):82-91.
24. Xu N, Li X, Zhong Y. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. *Mediators of inflammation.* 2015;2015:531518.
25. Anisman H, Hayley S, Turrin N, Merali Z. Cytokines as a stressor: implications for depressive illness. *The international journal of neuropsychopharmacology.* 2002;5(4):357-373.
26. Vezzani A, Viviani B. Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. *Neuropharmacology.* 2015;96(Pt A):70-82.
27. Petitto JM, Meola D, Huang Z. Interleukin-2 and the brain: dissecting central versus peripheral contributions using unique mouse models. *Methods in molecular biology.* 2012;934:301-311.
28. Tostes MH, Teixeira HC, Gattaz WF, Brandao MA, Raposo NR. Altered neurotrophin, neuropeptide, cytokines and nitric oxide levels in autism. *Pharmacopsychiatry.* 2012;45(6):241-243.
29. Sweeten TL, Posey DJ, Shankar S, McDougle CJ. High nitric oxide production in autistic disorder: a possible role for interferon-gamma. *Biol Psychiatry.* 2004;55(4):434-437.
30. Lazear HM, Nice TJ, Diamond MS. Interferon-lambda: Immune Functions at Barrier Surfaces and Beyond. *Immunity.* 2015;43(1):15-28.
31. Masi A, Quintana DS, Glozier N, Lloyd AR, Hickie IB, Guastella AJ. Cytokine aberrations in autism spectrum disorder: a systematic review and meta-analysis. *Molecular psychiatry.* 2015;20(4):440-446.
32. Carlson SE, Cooke RJ, Rhodes PG, Peeples JM, Werkman SH. Effect of vegetable and marine oils in preterm infant formulas on blood arachidonic and docosahexaenoic acids. *The Journal of Pediatrics.* 1992;120(4, Part 2):S159-S167.

33. Horrobin DF. Nutritional and medical importance of gamma-linolenic acid. *Prog Lipid Res.* 1992;31(2):163-194.
34. Vaddadi KS. The use of gamma-linolenic acid and linoleic acid to differentiate between temporal lobe epilepsy and schizophrenia. *Prostaglandins Med.* 1981;6(4):375-379.
35. Fewtrell MS, Abbott RA, Kennedy K, et al. Randomized, double-blind trial of long-chain polyunsaturated fatty acid supplementation with fish oil and borage oil in preterm infants. *J Pediatr.* 2004;144(4):471-479.
36. Cohen J. *Statistical power analysis for the behavioral sciences.* 2nd ed. Hillsdale, NJ: Erlbaum; 1988.
37. Rubin D. Inference and missing data. *Biometrika.* 1976;63:581-592.
38. Schafer JG, Graham JW. Missing data: Our view of the state of the art. *Psychological methods.* 2002;7(2):147-177.
39. Winkens B, van Breukelen GJ, Schouten HJ, Berger MP. Randomized clinical trials with a pre- and a post-treatment measurement: repeated measures versus ANCOVA models. *Contemporary clinical trials.* 2007;28(6):713-719.