Nutritional Intervention to Prevent (NIP) Type 1 Diabetes Pilot Trial

The NIP Diabetes Pilot Trial (Protocol TN-06)

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Sponsored by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the National Institute of Allergy and Infectious Diseases (NIAID), the National Institute of Child Health and Human Development (NICHD), the National Center for Research Resources (NCRR), the Juvenile Diabetes Research Foundation International (JDRF), and the American Diabetes Association (ADA)

Preface

The protocol for the Type 1 Diabetes Protocol TN-06 Nutritional Intervention to Prevent Type 1 Diabetes Pilot – the NIP Diabetes Pilot Trial describes the background, design and organization of the study. The protocol is distributed and maintained by the TrialNet Coordinating Center at the University of South Florida over the course of the study through new releases of the entire protocol, or issuance of updates either in the form of revisions of complete chapters or pages thereof, or in the form of supplemental protocol memoranda.

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1 Introduction

1.1 Study Overview

Title	NUTRITIONAL INTERVENTION TO PREVENT TYPE 1 DIABETES PILOT TRIAL: THE NIP DIABETES PILOT TRIAL
Conducted By	TrialNet
Protocol Chair	H. Peter Chase, MD
Accrual Objective	90 participants enrolled at 9 clinical sites over one year
Study Design	The study is a two-arm, multicenter, randomized, double-masked, controlled clinical trial.
Treatment Description	Docosahexaenoic acid (DHA) is an omega-3, polyunsaturated, 22-carbon fatty acid that is present in abundance in certain fish (such as tuna and bluefish) and marine animal oils. The DHA for this pilot trial is derived from microalgae. Pregnant and nursing mothers randomly assigned to DHA will receive 800mg/day by capsule. Infants will receive 150mg/day via infant formula and/or capsules to be mixed in with food, and toddlers ages 1-3 years will receive 400mg/day in a suitable form for toddler consumption.
Study Duration	The total duration of the pilot study is approximately 2 years (1 year accrual and 1 year follow-up) with the possibility of an additional 2 years of follow-up if transition time is needed for enrollment into a full-scale study. Children will be followed every six months until either (1) the decision is made to stop the NIP Diabetes Pilot Trial, (2) the development of 2 persistent positive autoantibodies present at 2 consecutive visits, or (3) the development of T1D.
Objective	Five objectives will be assessed for the Pilot Study: (1) Recruitment (2) Treatment Compliance (3) Visit Compliance (4) At least a 20% higher level of plasma and/or red blood cell membrane phospholipid DHA can be achieved in the experimental treatment group in comparison with the control group (5) At least a 20% lower level of the major inflammatory cytokine, IL1-beta, will be achieved in the experimental treatment group in comparison with the control group.
Primary Outcomes	The primary outcome for the pilot study will be to assess the feasibility of implementing a full-scale study.
Major Eligibility Criteria	***Pregnant mothers in their third trimester are eligible if they:*** • Have T1D or the child's father, or a full or half-sibling of the child has T1D • Are 18 years of age or older • Have diabetes and have never had a known HbA1c greater than 9% at anytime during pregnancy • Will not use omega-3 fatty acid supplementation or provide supplementation to the infant independently during the study

Major Eligibility Criteria	***Infants are eligible for enrollment if they:*** • Are less than six months of age at the time of enrollment • Have a mother, father or a full or half-sibling with T1D • Have a HLA DR3 or DR4 allele OR have another 1st or 2nd degree relative with T1D AND they do not have a protective HLA allele (DQB1*0602 or DRB1*0403).

1.2 Study Abstract

1.2.1 Rationale for the Study

Type 1 Diabetes (T1D) is a chronic and potentially disabling disease that represents a major public health and clinical concern. The number of patients being diagnosed with T1D is increasing each year and is approaching an epidemic level in some countries that track this information [1]. Unfortunately, the increase in T1D is the greatest in children under age five years [2]. T1D is caused by organ specific autoimmunity resulting from a gene-environment interaction. The genetics are fairly well delineated, with a high-risk association with the human leukocyte antigen (HLA) DR3 and DR4. Nearly half of the children who develop diabetes in the initial 5-10 years of life have the DR3/DR4 combination, and 95% of all T1D patients have either the DR3 or the DR4 haplotype. However, longitudinal studies have shown that when one identical twin develops diabetes, the second twin has only a 25 to 50% chance of developing the disease. Thus, there are one or more elusive "environmental" factors that we still do not understand. These are likely intertwined with the development of an autoimmune reaction against the islet cells in the pancreas. The appearance of islet autoantibodies in the blood is the first easily detectable sign in the human of an abnormality leading to diabetes. Islet autoantibodies may be detected years before the onset of diabetes and provide a marker of the disease process. This chronic disorder results from an autoimmune destruction of the insulin-producing pancreatic beta cells. Because remission of islet autoantibodies is rare in patients having more than one confirmed positive autoantibody [3], an intervention that would prevent the initial autoimmune attack is desirable. This proposal is innovative in that we are attempting to prevent beta cell autoimmunity rather than to reverse autoimmunity. As recently stated, “we are more likely to win this particular war by gaining the insight needed to negotiate with the immune system than by seeking to bomb it into submission.”[4].

The costs of caring for diabetes and its complications are currently greater than $100 billion a year. Having an intervention, which is extremely safe and will involve only agents already available as food supplements (and already available in some infant formulas), would be of incalculable value. It is unlikely that other approaches (other than nutritional interventions) would be feasible in pregnancy and/or infancy to try to prevent the development of autoimmunity.

This clinical trial is a feasibility study referred to throughout this protocol as the *Nutritional Intervention to Prevent Type 1 Diabetes Pilot Trial (NIP Diabetes Pilot Trial)*. The data, with respect to meeting recruitment goals as well as efficacy of the nutritional supplement, could serve as the basis for a larger trial if the results are sufficiently positive. In the full-scale trial, we propose to conduct a prospective, double-blinded dietary intervention trial using docosahexaenoic acid (DHA) or control to determine whether there is a reduction in the rate of children developing multiple autoantibodies. This would serve to validate important observational studies regarding a reduction in the rate of T1D.

1.2.2 Design of the Feasibility Study

The study is a multi-center study, two-arm, randomized, double-masked, controlled clinical trial. Participants may enter through two pathways. The first pathway enrolls pregnant women in their 3rd trimester. Their infants continue in the study if they are determined to be eligible based on HLA typing after birth. The second pathway enrolls infants up to six months of age. It is estimated that approximately half of the participants will be screened prenatally and the other half in the first six months of post-natal life.

Depending on their mode of intake, participants will receive study substance (DHA supplementation or control) either indirectly through the placenta or breast milk, directly in the form of infant formula, the contents of capsules mixed in with food, or in a form suitable for toddler consumption. Comparisons will be made between the groups receiving DHA supplementation versus the control group.

1.2.3 Objectives of the Pilot Feasibility Trial

Five objectives will be assessed for the pilot feasibility trial:

1. Recruitment: Nine clinical sites will enroll an average of ten participants per site in one year.
2. Treatment Compliance: At least 90% of families will continue to take the study substance as instructed.
3. Visit Compliance: At least 95% of families will continue to attend follow-up visits.
4. At least a 20% higher level of plasma and/or red blood cell membrane phospholipid DHA can be achieved in the experimental treatment group in comparison with the control group.
5. At least a 20% lower level of the major inflammatory cytokine, IL1-beta, will be achieved in the experimental treatment group in comparison with the control group.

The final analysis of these objectives will be conducted when the last participant randomized has been followed for 1 year.

1.2.4 Primary Hypothesis and Primary Outcome of the Full Scale Trial

The objectives for the feasibility study are stated above. If these objectives are fulfilled, a full-scale study will be considered for implementation. The primary outcome for a full-scale study will be to determine if nutritional supplementation with omega-3 fatty acids during the last trimester of the mother's pregnancy and/or the first three years of life for children who are at high risk of T1D will prevent the development of islet autoimmunity.

1.3 Study Protocol

This protocol describes the background, design, and organization of the Nutritional Intervention to Prevent Type 1 Diabetes Pilot Trial. The protocol was written by Dr. H. Peter Chase, the Protocol Chair of the TrialNet NIP Protocol Committee, the TrialNet Chairman's Office and the TrialNet Coordinating Center at the University of South Florida Biostatistics Center. Significant changes that occur to this protocol during the course of the trial require the formal approval of the TrialNet Steering Committee. The study protocol, along with the required informed consent forms, will be approved by each participating institution's Institutional Review Board (IRB).

1.4 Participating Sites

Participating TrialNet clinical sites must have an appropriate assurance, such as a Federal-Wide Assurance (FWA) or an Unaffiliated Investigators Agreement (UIA), with the Office for Human Research Protections (OHRP), since they are actively engaged in research and provide informed consent. The protocol and consent forms will be approved by Institutional Review Boards (IRBs) at each of the participating clinical sites. HIPAA regulations will be followed by each participating institution in accordance with each institution's requirements.

2 Background

2.1 Clinical Problem and Rationale for Study

We hypothesize that docosahexaenoic acid (DHA) supplementation will prevent autoantibody development in children at genetic risk for T1D. We propose that the mechanism of this protection is due to DHA mediated suppression of the inflammatory response, e.g. IL-1β, TNF-α and COX2 in a manner similar to that provided by cytokines IL4 and IL10.

This hypothesis is based upon epidemiologic or observational data as well as animal and human studies about the inflammatory state in individuals at risk for T1D and the effects of omega-3 fatty acids on these markers. Specifically, we suggest that participants at risk for T1D have an increased proinflammatory environment. Furthermore, we propose that DHA administration will result in inhibition of inflammation-related factors and block the early events associated with the subsequent development of autoimmunity in genetically at-risk participants.

2.1.1 Role of Prostaglandins and Inflammatory Cytokines in T1D

A considerable amount of data suggests that T1D is a T-cell mediated disease [5] and that proinflammatory cytokines such as IL1 and INF-γ, associated with Th1 cells, accelerate and anti-inflammatory cytokines such as IL4 and IL10 and TGF-β associated with Th2 cells, protect against disease [6,7,8]. For example, both autoantibody positive participants at risk for T1D and children with T1D have been reported to have lower levels of the anti-inflammatory cytokines IL4 and TGF-beta [9]. It has been proposed that IL4 and IL-10 suppresses Prostaglandin synthase (PGS2 synthase). PGS2 is more commonly referred to as COX2 (cyclooxygenase 2). PGS2 is one of the Membrane-Associated Proteins in the Eicosanoid and Glutathione metabolism (MAPEG) family of microsomal enzymes and constitutes a novel inducible enzyme involved in inflammation and pyretic responses. Thus, reduced levels of IL4 and and/or an inability to produce a response by IL-10 would be expected to result in an increase in PGS2, other inflammatory mediators and a proinflammatory environment.

Indeed, several reports have described an increase in PGS2 production in children with diabetes [10, 11]. Moreover, Clare-Salzler, Litherland and colleagues identified that abnormal PGS2 expression defines an antigen presenting cell defect for T1D. In individuals identified as at risk for T1D by the presence of islet autoantibodies, they found that aberrant expression of PGS2 increased their risk of developing T1D most profoundly in participants with high risk alleles DR4 and DQB1*0302. The same group demonstrated that abnormal PGS2 expression preceded detectable autoantibodies in very young infants with a genetic risk and a family history of diabetes, thus suggesting that elevation of PGS2 begins before the development of autoimmunity.

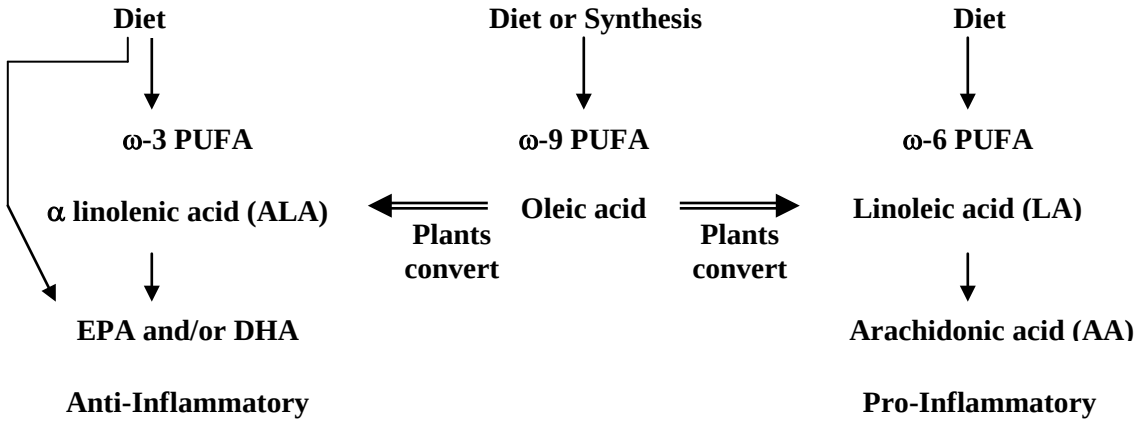
C-reactive protein (CRP) is another marker associated with inflammation that has recently been studied in infants at risk for T1D. In the DAISY study of genetically at risk infants, antibody positive children who progressed to T1D were significantly more likely to have elevated CRP levels than the children whose autoantibodies were transient [12].

2.1.2 Omega-3 Fatty Acids

Cell membranes require unsaturated fatty acids to maintain structure and function. Desaturase enzymes present in mammals add a double bond to form a mono-unsaturated fatty acid, oleic acid

(18:1 n-9), from stearate. Animals, unlike plants, can insert additional double bonds to create two classes of polyunsaturated fatty acids (PUFA), n-6 and n-3. Plants convert oleic acid to linoleic acid (LA) (18:2 n-6) and desaturation of oleic produces α-linolenic (ALA) (18:3 n-3). Marine algae also produce EPA (20:5 n-3) and DHA (22:6 n-3) that are eventually transferred through the food chain to fish whose oils are high in ω-3 fatty acids. Mammals cannot synthesize LA (the predominant PUFA in Western diets) and ALA; therefore these fatty acids are "essential" and must be ingested. Mammals, unlike plants, cannot interconvert n-9, n-6 and n-3 fatty acids, but do further convert LA to other n-6 fatty acids, (e.g., arachidonic acid (20:4 n-6)) and ALA to n-3 EPA and DHA.

Figure 1



2.1.3 Omega-3 Fatty Acids and Inflammation

Omega-3 fatty acids reduce inflammatory cytokines and inflammatory prostaglandins, both of which may be related to the initial inflammation in islet cells. The mechanism of omega-3 fatty acids reducing inflammation has been shown secondary to limiting proinflammatory cytokines and the activity of the PGS2 enzyme [11]. Therefore, it was proposed that the increased PGS2 expression and cytokine production observed in children at high-risk for developing T1D may be reduced through omega-3 fatty acid supplementation [13]. Arachidonic acid (AA), DHA, and eicosapentaenoic acid (EPA) inhibit PGS2 with similar potency, which is greater than that seen with other fatty acid analogues.

Endres et al [14] found that the syntheses of interleukin-1 (IL-1alpha and IL-1 beta) and of tumor necrosis factor (TNF), cytokines with potent inflammatory activities, were reduced by dietary supplementation with n-3 fatty acids. The anti-inflammatory effect of these n-3 fatty acids may be mediated in part by their inhibitory effect on the production of IL-1 and TNF.

2.1.4 Omega-3 Fatty Acids and T1D

The incidence of T1D is increasing, particularly in very young children. We hypothesize that the lack of omega-3 fatty acids in the diet has contributed to this increase. Just 100 years ago, consumption of n-6 to n-3 fatty acids in the diet was at a 1:1 ratio. In 1999, the ratio was reported to be approximately 30:1 [15]. Because of the warnings to eliminate fish during pregnancy, the ratio is likely even greater during pregnancy. This results in increased production of inflammatory prostaglandins and cytokines during this critical time of fetal development. This trend towards substitution of the inflammation producing n-6 fatty acids at the expense of the anti-inflammatory n-

3 fatty acids may be contributing to the increase in the number of cases of T1D diagnosed each year.

More direct evidence also suggests a relationship between omega-3 fatty acids and T1D. Decsi and colleagues demonstrated that 40 children with T1D had significantly lower DHA levels and a lower n-3: n-6 ratio than the control children, which may partially explain the altered prostanoid and inflammatory cytokine production. They conclude that the reduced availability of long-chain polyunsaturates in children with T1D suggests the need for enhanced dietary supply [16].

Observations have been made that children who have received omega-3 fatty acid supplementation have a lower risk of T1D. Children born to women from Norwegian fishing villages had a significantly decreased risk of getting diabetes compared with the children of women who lived in cities away from the coast [17]. Using a case-control design, Stene et al found that cod liver oil, either taken by the mother during pregnancy or by the child during the first year of life, was associated with a decreased risk of developing T1D [18]. They concluded that either the vitamin D or the eicosapentaenoic acid (EPA) or the docosahexaenoic acid (DHA) or all three cod liver oil components have protective effects against developing T1D. In a larger and more recent study, Stene and colleagues in Norway found that supplementation with cod liver oil during the first year of life was associated with a significantly lower risk of T1D before age 15, odds ratio (OR) of 0.7 ($p<0.001$) [19]. This is consistent with the hypothesis that omega-3 fatty acids (or vitamin D) have a protective effect. It now appears that the protective effect may occur during pregnancy or during infancy or both.

2.1.5 Animal Data: Reduction of T1D by Administration of Omega-3 Fatty Acids

Supplementation with omega-3 fatty acids in animal models of T1D has provided supportive information regarding early supplementation. In an alloxan-induced diabetes male Wistar rat model, Suresh et al were able to prevent diabetes by oral supplementation of polyunsaturated fatty acids including DHA [20]. In the Biobreeding (BB) rat, there is contradictory data. One study demonstrated that supplementation with fish oils during the period of insulitis led to a cytokine shift towards Th2 in the gut-associated immune system, and IL-10 was higher [21]. However, another group fed fish oils to BB/W rats and did not find a decrease in the incidence of diabetes compared with rats fed corn oil [22]. However, the diets were not instituted until 25-35 days after birth, and immunologic changes had likely already taken place. Linn et al provided further support for earlier supplementation. Their male streptozocin (STZ) mice were on a fish oil diet eight weeks before STZ injection [23]. After the STZ injection, the fish-oil fed mice had reduced numbers of class II antigen-expressing cells in pancreatic islets followed by decreased insulitis. Fish oil significantly decreased the number of FC receptor-negative dendritic cells in cytospin preparations of islets isolated from diabetic mice. IL1-like activity of peritoneal exudate cell supernatants isolated from mice on fish oil was reduced, providing a beneficial effect of fish oil on the immune component of this mouse model. In the NOD mouse model, reducing macrophage PGE2 production in vivo by dietary fatty acid manipulation or indomethacin to block PGS1 and PGS2 activity significantly reduces diabetes incidence by 70% and 50% respectively (X.T. Xie, unpublished data).

2.1.6 Vitamin D

As noted above, the observation that cod liver oil was associated with lower risk of T1D suggests the possibility of a synergistic effect of both of the components of cod liver oil (DHA and vitamin D) [18]. A large retrospective cohort study demonstrated an association between vitamin D supplementation in the first year of life and a lower risk of T1D [24]. This association was also demonstrated in a prospective study in which infants received vitamin D supplementation [25]. The

mechanism may be related to the immunosuppressive properties of Vitamin D [26-31] or possibly a direct effect on islet cell insulin secretion. Vitamin D receptor (VDR) gene polymorphisms have recently been associated with susceptibility to T1D in humans [32-35]. Interestingly, Stene et al found an indication for a protective effect with certain polymorphisms, but this was strongest for children who took cod liver oil during the first year of life [36].

Since the American Academy of Pediatrics recommends that all mothers provide vitamin D to nursing infants and vitamin D is currently included in all infant formulas, we expect that the infants in this study will receive vitamin D. Vitamin D levels will be measured during the study.

2.1.7 Possible enrollment into other TrialNet Studies

While we hypothesize that genetically at-risk infants receiving DHA will have reduction in early inflammatory events and subsequent autoimmunity, the pilot study is not powered to detect this difference. Nonetheless, all genetically at-risk infants will be offered prolonged follow-up either through a subsequent full-scale study testing the DHA hypothesis or through the TrialNet Natural History Study of the Development of Type 1 Diabetes, which closely monitors at-risk individuals for diabetes. Thus, all participants may benefit from early detection of T1D prior to the development of symptoms. In the DPT-1 Trial, the majority of participants in whom diabetes was diagnosed were asymptomatic (102 of 139, 73.4%) [37], thus resulting in decreased morbidity associated with T1D onset.

2.2 Review of Clinical Studies

2.2.1 Absorption of DHA

A randomized study with healthy adults (n=32) was conducted to determine if the absorption of DHA from gelatin capsules containing DHA oil derived from algae (DHASCO® oil, Martek Biosciences Corp, Columbia, MD) was as good as DHA oil derived from fish. Study volunteers consumed 600 mg of DHA derived from either DHASCO® capsules or cooked salmon daily for two weeks. DHA levels increased by 28% in the algal DHA group and 21% in the salmon group. EPA levels increased in the fish but not the DHASCO® group since only the fish contains EPA. Plasma phospholipid DHA levels increased significantly in each group. This study indicates that DHA from DHASCO® capsules is absorbed as efficiently as from a natural food source [38].

2.2.2 Affects On Prematurity

Instructions by obstetricians have guided pregnant women away from consuming fish during pregnancy. This is due to recent findings that contaminants such as methylmercury and polychlorinated biphenyl (PCBs) in the oceans may harm the developing fetus [39-41]. However, this lack of fish consumption results in low intake of omega-3 fatty acids, which was thought to contribute to premature delivery. Multiple trials have demonstrated that fish oil supplementation reduced this risk [42-44].

2.2.3 Supplementation In Pregnancy and During Lactation

DHA declines in late pregnancy if expressed as a proportion of total fatty acids, and this has been suggested to be at least partly due to the selective transfer of fatty acids to the developing fetus [45, 46]. Reduced DHA and total n-3 fatty acids in pregnant women are paralleled by increased proinflammatory n-6 fatty acids, docosatetraenoic (DTA) and docosapentaenoic (DPA). In addition, post-partum, the reduction in maternal DHA is greater the longer the duration of breastfeeding.

The daily transfer of DHA from a nursing mother to her infant was calculated to be 50-53 mg/day [47].

Other studies have indicated that DHA may decrease with each subsequent pregnancy, and maternal stores of DHA may be reduced in mothers vs. nulliparas [48, 49]. This data suggests a possible explanation for the observation that an association between maternal age at delivery and T1D is seen among the fourth-born and subsequent children. [50].

It is known that the fetus is dependent on maternal fatty acid intake [51, 52]. Maternal supplementation with long chain omega-3 fatty acids increases concentrations in maternal and umbilical plasma phospholipids [53] and results in higher concentrations of DHA in the blood of the newborn infant.

Several studies have investigated the use of DHA-rich eggs as a method to increase DHA levels of pregnant women during their third trimester [54]. Supplementing the diet of lactating women with DHA administered by DHA-rich eggs, or DHA-enriched oil was demonstrated to be an effective means of increasing the DHA content of human milk and providing additional DHA to the developing infant [55-58],

2.3 Safety of Human DHA Supplementation

2.3.1 Safety of DHA Supplementation in Participants with T1D

Prior to submitting a proposal for an intervention study for children with an increased risk for developing T1D, safety data must be obtained to support that no harm is done in newly diagnosed participants. Safety data are available from several studies. A pilot trial of fish oil has been done in newly diagnosed participants, mean age 25 years, with T1D [58]. The control group received olive oil (a good source of linoleic/arachidonic acids). Both groups had HbA1c levels below the upper limit of normal for the lab (7.5%) when the study was discontinued after nine months. It was thus not possible to determine if there was a long-term beneficial effect from the fish oil. No detrimental effects in the newly diagnosed participants with T1D were detected.

Additional support for the safety of this intervention is widely available. Studies have shown that the use of fish oil is helpful in reducing triglyceride levels and a meta-analysis of the use of fish oil in people with diabetes, primarily to reduce cardiovascular risk, has shown that there is no adverse effect on glycemic control [59]. In fact, the opposite has been reported. Stiefel and colleagues [60] treated 18 T1D patients with low dose n-3 fatty acids (330 mg/day DHA and 630 mg/day of EPA) and found significantly different changes in membrane lipid composition and total n-3 fatty acid as well as a significant decrease of HbA1c without significant changes in the dose of insulin required. Bagdade et al found that patients with T1D have an increase in cholesteryl ester transfer (CET) which increases atherogenicity [61]. They treated nine normolipemic T1D patients for two months with n-3 fatty acids at a dose of 4.6 grams per day. Marine lipids helped modestly decrease triglyceride levels with no change in glycemic control.

2.3.2 Safety of DHA Supplementation In Other Autoimmune Disorder Studies

Observations about omega-3 supplementation have been made in other autoimmune disorder studies. As recently reviewed, fish oil has been used as an anti-inflammatory agent in other chronic autoimmune disorders such as rheumatoid arthritis, systemic lupus erythematosus and Crohn's disease [62]. No significant adverse effects have been reported in multiple studies in other autoimmune diseases, and in some cases biological and/or clinical benefit has been seen [63-71].

2.3.3 Safety of DHA Supplementation in Non-Autoimmune Disease

Further support for the proposed n-3 supplementation comes from several large studies showing positive health and safety outcomes. In studies of cardiovascular disease endpoints, DHA has shown some efficacy [72-76]. DHA was well tolerated in all these studies. Newly diagnosed individuals with T1D given fish oil had mean HbA1c levels in the normal range after nine months of supplementation. Some studies using DHA to treat bipolar disorder have safely used ten times the amount of DHA proposed for use in this study [77].

2.4 Dietary Consumption of DHA

Numerous studies of omega-3 fatty acids have been done in a variety of clinical states including studies involving pregnant and lactating mothers. There are no studies that suggest any adverse effects from supplementing with DHA as a source of n-3 fatty acid. DHA provides important benefits during pregnancy, but because of recent contamination concerns, fish consumption during pregnancy has been decreasing. This decrease in fish consumption during pregnancy has been occurring as the rate of diabetes has been increasing. Farm-raised fish that consume corn or grain do not have the same n-3 content as fish that consume algae or other marine sources of food. Fish and other n-3 fatty acids were previously a primary source of DHA during pregnancy and to the developing infant during lactation. The modern diet has a ratio of pro-inflammatory n-6 fatty acids to anti-inflammatory n-3 fatty acids that has changed significantly over only a few decades. The impact of this change may be most pronounced during fetal development and infancy. The FDA has granted GRAS (Generally Recognized as Safe) status to DHA and it has been approved for addition to infant formulas. Several infant formulas containing DHA are now commercially available in the U.S.

2.5 Biochemical Islet Autoantibody Development in T1D Genetic Risk Individuals

The outcomes of interest for this protocol are the development of T1D and the presence of biochemical islet autoantibodies. There is a significant increase in the risk of diabetes with the number of biochemical islet autoantibodies present, comparing zero, one, two, and three autoantibodies ($P<0.0001$, log-rank test) by Cox regression analysis [78]. This is independent of which biochemical islet cell autoantibodies and age. For relatives of T1D patients having two or more of these biochemical islet autoantibodies present, the risk of diabetes within 3 years was 39% (95% CI, 27-52) and the risk within 5 years was 68% (95% CI, 52-84). Relatives with all three biochemical islet autoantibodies had a risk of developing T1D within 5 years estimated to be 100% [79].

3 Study Design

3.1 Overview

The study is a multi-center, two-arm, randomized, double-masked, controlled clinical trial. The total length of this pilot feasibility trial will be two years (one year of recruitment and one year of follow-up to assess feasibility) with the possibility of up to another 2 years of follow-up if transition time is needed for enrollment into a full-scale NIP study.

3.2 Inclusion Criteria

Potential participants must meet the following inclusion criteria:

Pregnant mothers are eligible for enrollment into this study if they:

1. have T1D or the child's father, or a full or half-sibling of the child has T1D
2. are 18 years of age or older
3. are in their third trimester of pregnancy (i.e. gestation is 24 weeks or longer)
4. have given written informed consent and HIPAA authorization
5. are willing to be assigned to either treatment group

Infants are eligible for enrollment into this study if they:

1. are less than or equal to six months (i.e. ≤ 182 days from the date of birth) of age on the date of randomization
2. are found to be at risk for type 1 diabetes because they have a mother, father or full or half-sibling with T1D AND
   - have a HLA DR3 or DR4 allele OR
   - have another 1st or 2nd degree relative with T1D (multiplex family)
3. have a parent or legal guardian who has given written informed consent and HIPAA authorization
4. have a parent(s) or legal guardian(s) who are willing for their baby to be assigned to either treatment group

3.3 Exclusion Criteria

Potential participants must **not** meet any of the following exclusion criteria:

Pregnant mothers are NOT eligible for enrollment into this study if they:

1. have any condition the investigator believes will put the mother or her fetus at an unacceptable medical risk for participation in this study
2. have a known complication of pregnancy causing an increased risk for the mother or fetus prior to entry into the study

3. have previously had multiple (2 or more) pre-term births (<36 weeks)
4. will take omega-3 fatty acid supplementation or will provide omega-3 fatty acid supplementation to her infant independently during the study
5. have diabetes and a known HbA1c greater than 9% at anytime during the pregnancy

Infants are NOT eligible for enrollment into this study if they:

1. have any condition the investigator believes will put the infant at an unacceptable medical risk for participation in this study
2. have a mother with a condition the investigator believes will put her or the infant at an unacceptable medical risk for participation in this study
3. have a nursing mother who will take omega-3 fatty acid supplementation or a parent or legal guardian who will provide supplementation to his/her infant independently during the study (if infant entered prenatally, this exclusion criteria is assessed only once when the pregnant mother entered the study)
4. have a protective HLA allele (DQB1*0602 or DRB1*0403)
5. were born prior to 36 weeks gestation or require a pre-term infant formula

3.4 Screening and Enrollment

3.4.1 Pregnant Women

Pregnant women may be referred to the study by their physician or recruited at participating TrialNet study sites. All sites will comply with HIPAA regulations. Screening will consist of a clinic visit to be conducted after the 24th week of gestation for the pregnant woman. The pregnant woman will be asked if she would be interested in participating in the research project. Those indicating interest will be referred to one of the Investigators or Study Coordinators for a description of the study. Essential components of the presentation are that the parent(s) are being invited to participate in research, participation in the study is voluntary, and participation may be ended at the parent(s)' request. TrialNet research personnel will provide the parent(s) with a full description of the study, the inclusion and exclusion criteria, the procedures involved, the study groups and the randomization process, time commitments and the schedule of follow-up visits. A screening data form will be completed.

Pregnant women without diabetes can be enrolled after screening with no laboratory tests required if they meet eligibility criteria. Pregnant women with diabetes will need an HbA1c test as part of eligibility screening prior to randomization.

Supplementation with the study substance (DHA or control) will begin immediately after randomization, and continue at least until the HLA type of the infant is known. At that time, infants will either be enrolled into the study as continuing infants and continue taking the study substance, or be deemed ineligible for continuation in the study. The consented mother will receive a TrialNet identification number, and a baseline data collection form will be completed. To determine HLA eligibility status for the infant, a sample will be obtained either at birth by cord blood collection, or by a heel stick collected 2 to 28 days after the birth.

3.4.2 Infants of mothers enrolled during pregnancy

Infants with mothers who started on supplementation during the third trimester of pregnancy are screened for continued participation in the study. This process requires laboratory tests using cord blood collected from the placenta at birth, or by a heel stick collected 2 to 28 days after the birth of the infant. A screening data collection form will be completed, and the infant's blood sample will be analyzed for HLA determination. The screening process will be completed within approximately four weeks of birth. Infants who have either a single 1st degree relative with T1D and higher risk HLA genes, or infants with one 1st degree relative and another 1st or 2nd degree relative with T1D (a multiplex family) will be enrolled in the study. If the infant has a protective allele, he/she will not be eligible for the study. In the case that multiple infants within the same family are eligible, either simultaneously (e.g. twins), or successively (e.g. siblings), all may be entered into the study. Eligible infants born to mothers entering in the study when pregnant will continue in the study with the same treatment group assignment as the first prenatal infant. Nursing mothers and infants will continue taking the study substance while eligibility is being determined.

3.4.3 Infants whose mothers were previously not enrolled

Eligible infants 5 months of age and younger will be recruited at participating TrialNet study sites. The parent(s) will be asked if they would be interested in participating in the research project. Those indicating interest will be referred to one of the Investigators or Study Coordinators for a description of the study. Essential components of the presentation are that the parent(s) are being invited to participate in research, participation in the study is voluntary, and participation may be ended at the parent(s)' request. TrialNet research personnel will provide the parent(s) with a full description of the study, the inclusion and exclusion criteria, the procedures involved, the study groups and the randomization process, time commitments and the schedule of follow-up visits.

Screening will occur at 5 months of age or younger (less than or equal to 152 days from date of birth) for the infants who would be enrolled after birth. The screening process will consist of an initial screening visit to be conducted at 2 days to 5 months of age. A screening data collection form will be completed, and the infant's blood sample (by a heel stick) will be taken for HLA determination. The screening process for selection of infant participants for enrollment in the study will be completed within approximately four weeks. Infants who have either a single 1st degree relative with T1D and higher risk HLA genes, or infants with one 1st degree relative and another 1st or 2nd degree relative with T1D (a multiplex family) will be enrolled in the study. If the infant has a protective allele, he/she will not be eligible for the study. In the case that multiple infants within the same family are eligible, either simultaneously (e.g. twins), or successively (e.g. siblings), all may be entered into the study.

3.5 Group Assignment, Randomization, and Masking

After the parent(s) have signed the consent form, completed the screening visit(s), (i.e. met all of the inclusion criteria and none of the exclusion criteria), and completed the baseline procedures, the participant will be randomized to one of the two study groups: DHA study substance or control.

The Coordinating Center will specify a randomization schedule for the study sites. Participants will be randomized, in approximately equal numbers, to the two arms of the study. The randomization method will be stratified by TrialNet study site. This approach ensures that the treatment groups will be approximately balanced within each site.

In the event that multiple siblings are entered from a single family, only the first family member will count towards the recruitment goal of 90 participants. The treatment randomly assigned to the first family member will also be assigned to all other family members who enter. This is due to statistical considerations since family members do not represent independent observations.

The study will be double-masked, in that the participant and the clinical sites and personnel involved in participant care will be masked to the mother and infant's treatment group assignment. The Coordinating Center will maintain the list of participant randomization assignments.

Participants who do not meet all of the inclusion criteria or who have one of the exclusion criteria will not be eligible to continue in this study, but will be offered participation in another TrialNet study, if one is available.

3.6 Study Substance

The product used in this study is a gelatin capsule containing DHASCO®-S oil or a neutral vegetable control oil. The DHASCO®-S oil is derived from microalgae in vats (no mercury or pesticide contaminants). The expectation is that there will be similar absorption of DHA contained in the capsules versus DHA oil derived from fish. The FDA has granted GRAS status to DHASCO®-S and it has been approved for addition to infant formulas. Several infant formulas containing DHASCO®-S are now commercially available in the U.S. The DHASCO®-S oil is emulsified in a cow's milk-based infant formula that is available commercially. Table 1 describes the amount of DHA per capsule and 1 oz study infant formula for the experimental treatment and control group. The amount of DHA in the infant formula given to the control group is comparable to that found in some commercial formulas.

Table 1. Approximate Amount of DHA in Capsule and Study Formula

	Per Gel Capsule	Per 1 Oz Study Formula
Experimental treatment group:	200 mg DHA	10.2 mg DHA
Control group:	0 mg DHA	3.4 mg DHA

3.7 Treatment Groups

3.7.1 Description of Treatment Groups and Treatment Modalities

The sample of 90 eligible participants will be randomly assigned in approximately equal numbers to the two groups (experimental treatment or control group). About half (forty-five) of the participants will receive the docosahexaenoic acid (DHA) study substance daily, and half (forty-five) will receive the control.

Depending on their mode of intake participants will receive the study substance either indirectly through the placenta or breast milk, or directly in the form of infant formula or the contents of capsules mixed in with food, or in a form suitable for toddler consumption. Comparisons will be made between groups receiving DHA supplementation (experimental treatment) versus the control as described in Tables 1 and 2. Group assignment will be random and masked.

The study substance will be given to the infant in one of the following ways for a minimum duration of 12 months from the date of birth (if entered prenatally), or date of randomization (if entered post-partum). The study substance is given in one of the following ways (Tables 2 and 3).

During pregnancy:

1. Mothers enrolled during pregnancy take 4 capsules a day until the infant's HLA type is determined.

[After birth, if the infant does NOT have the higher-risk genes (and did not pre-qualify by having multiple family members with T1D) or has a protective allele, the family will be withdrawn from the study.]

After the HLA type is known from birth until 1 year of age:

Nursing mothers will be provided with study formula or capsules to use if nursing is discontinued or the infant is weaned.

1. For mothers that are nursing:
    a. The mother takes 4 capsules/day until she stops nursing or until baby is 12 months old.
    b. At 6 months old the infant is given 1 capsule/day and the nursing mother continues to take 4 capsules/day. Assume that the infant will start transitioning to foods at this time.
2. For infants that are taking study substance:
    a. At 0 to 6 months old the infant is given the study formula.
    b. At 6 to 12 months old, if the infant is taking more than 32 oz/day of the study formula, no additional study capsules will be mixed in the food.
    c. At 6 to 12 months old if the infant is taking less than 32 oz/day but more than 16 oz/day of the study formula on average over the previous week, the parent will give the content of 1 study capsule per day to the infant by mixing in food.
    d. At 6 to 12 months old if the infant is taking less than 16 oz/day of the study formula on average over the previous week, the parent will give contents of 2 study capsules per day to the infant by mixing in food.
3. For infants partially breastfeeding and taking infant formula (study formula):
    a. The nursing mother follows the schedule as if she is exclusively nursing.
    b. The infant follows the schedule as if he/she is exclusively taking the study formula according to infant age.

After 1 year of age through study end:

1. Transition all infants at 12 months old to mixing the contents of 2 capsules/day in food or the equivalent provided in a suitable form for toddler consumption.
2. The nursing mother stops DHA/control capsule supplementation.

Table 2. Approximate Amount of Nutrient Intake and Assessment

Subject	DHA Supplementation group
Pregnant Mother	800 mg/day
Nursing Mother	800 mg/day
Nursing Infant	≅ 120-400 mg/day (via the mother)
Formula-fed Infant[1]	≅ 120-400 mg/day
Toddlers aged 1-3 yrs	≅ 400 mg/day

[1]The control formula will contain about one-third of the amount of DHA as the intervention formula.

Table 3. Summary of Study Substance Intake for Mother and Baby

	Baby's Age				
	3rd trimester to Birth	**Birth to 4 mos**	**4 to 6 mos**	**6 to 12 mos**	**12 to 36 months of age or end of study**
Mother					
Pregnant	4 caps/day	NA	NA	NA	NA
Exclusively nursing	NA	4 caps/day	4 caps/day	4 caps/day	0
Partially nursing and providing study formula	NA	4 caps/day	4 caps/day	4 caps/day	0
Baby					
Nursing	NA	0	0	1 cap/day	2 caps/day
Partially nursing and taking study formula or Exclusively taking study formula	NA	Study Formula	Study Formula	32 oz of Study Formula or 16 to 32 oz of Study Formula plus 1 cap/day or ≤16 oz of Study Formula plus 2 cap/day	2 caps/day

3.8 Study Assessments

All study participants will follow a given schedule of study visits and procedures. All study assessment visits will be timed in relation to the date of birth of the infant.

3.8.1 Study Assessments for the Pilot Feasibility Trial

During the course of the study, participants will frequently undergo assessments of their immunologic and inflammatory status and overall health and well being as per the protocol Schedule of Assessments (see Appendix A). Laboratory parameters of the enrolled infant will be obtained at screening and every 6 months of age thereafter for a minimum of one year and a maximum of 4 years. The assessments performed during the first year will be utilized to determine the feasibility of a full-scale trial. The duration of additional follow-up will depend upon the need for a transition period from the pilot study to a full-scale study. The assessments will be the same for each 6 month visit and will include blood tests measuring levels of blood glucose, vitamin D, fatty acid levels, inflammatory mediators (including inflammatory cytokines (IL1-beta), prostanoids, eicosanoids, docasanoids, C-reactive protein, and COX2 enzyme) and islet cell autoantibodies (see Appendix B for amounts of blood drawn). Follow-up will only be terminated if (1) the decision is made to stop the NIP Diabetes Pilot Trial, (2) the development of 2 persistent positive autoantibodies present at 2 consecutive visits, or (3) the development of T1D. Children in the pilot study who have confirmed two autoantibodies will be told of this outcome and will no longer receive the study substance. They may enroll in the TrialNet Natural History Study for the Development of Type 1 Diabetes for regular close monitoring for development of diabetes.

3.8.2 Transition time (continued follow-up)

There is a possibility that children without 2 confirmed autoantibodies or T1D developing during follow-up will have continued follow-up for up to an additional 2 years if transition time is needed to enroll into a full-scale NIP Diabetes study. Parents will be re-consented for a full-scale study at that time. Although the study substance will be discontinued after the child's 36-month visit, the child will continue to attend follow-up visits every 6 months as described in Section 4. If a full-scale study is not undertaken, the child may be eligible to enroll in the TrialNet Natural History Study of the Development of Type 1 Diabetes for continued monitoring for diabetes risk depending on the autoantibody status. The double-masking will be maintained throughout this period.

3.8.3 Stored Samples

Some residual samples obtained from the infants may remain after the scheduled assays are performed. These samples will be stored at the TrialNet Core Laboratories for potential use in future TrialNet approved studies related to diabetes. This may include umbilical cord blood, mother's blood, and/or breast milk. If blood volumes allow, additional samples from the child will be collected and stored at the NIDDK Repository at the 24-month visit. Storage of samples is discussed further in Section 9.4.

3.8.4 Quality Assurance

During the study, duplicate collections of blood samples for assays will be obtained in a small sample of participants for the purpose of external quality surveillance of the performance of the central laboratories. These additional collections will be taken from mothers. Blood volumes will not exceed 3 ml/kg at a single visit.

3.9 Description of the Full Scale Study Design

If a full-scale NIP Diabetes Study is determined to be feasible based on the objectives of the pilot study, participants enrolled into this NIP Diabetes Pilot Trial may enroll into a full-scale study and continue to receive the study substance until the development of two persistent autoantibodies, T1D, 36 months of age, or the termination of the study. It is planned that the child will have 3 years of follow-up post treatment, with one additional contact with the child's parents at about the time of his or her 9th birthday. The development of two or more persistent autoantibodies or T1D will be the primary outcome.

4 Management of Participants

4.1 Visit Schedules, Tests, and Procedures at Each Follow-up Visit

Screening and enrollment are described in Section 3.4. Detailed study activities follows in Section 4.2.

Overview of Follow-up Study Visits

All mothers will have a follow-up study visit every 3 months.

Nursing mothers be asked to bring a sample of breast milk to the clinic for fatty acids analysis when the infant is 3, 6, 9, and 12 months old. See Section 4.3 for details regarding these study visits.

Infants will come to follow-up study visits when they are 6, 12, 18, 24 months old and every 6 months thereafter. At each of these visits, the infant will have a limited physical examination and a blood collection for analysis. See Section 4.3 for details regarding these study visits.

Parents will be asked about any adverse events that may have occurred in the interval between visits.

4.2 Screening and Enrollment Visit Schedule

4.2.1 Entry Point A: Screening and Enrollment During Pregnancy

4.2.1.1 Pregnant Woman (Prenatal Infant) Screening and Enrollment:

Informed Consent Process

Specimen Tests:

- If woman has diabetes (type 1, type 2, or gestational): local HbA1c to determine eligibility
- Baseline islet autoantibodies, RBC fatty acid levels, inflammatory mediators levels, Vitamin D level, and CRP level
- Residual specimens for storage (if available)

Data Collection Procedures:

- Assessment of concomitant medications including vitamin and supplementation record, medical history, family history and food frequency questionnaire

If the pregnant woman meets eligibility criteria to participate:

- Unborn infant(s) randomized to the experimental treatment or control group
- Study capsules dispensed.
- Cord blood kit provided to parent with instructions to collect and ship

4.2.1.2 Infant Screening (Infant of Participating Pregnant Woman*)*

a. Day of Infant Delivery:

Specimen Tests (if cord blood available):

- HLA to determine eligibility
- Baseline islet autoantibodies, RBC fatty acid levels, inflammatory mediator levels, Vitamin D level and CRP level.
- Residual specimens for storage (if available)

b. Infant (2 to 28 days of age):

Informed Consent Process

Specimen Tests:

Infant:

If cord blood not collected at birth, blood is collected by heel stick for the following:

- HLA to determine eligibility
- Islet autoantibodies
- Residual specimens for storage (if available)

Mother:

- Islet autoantibodies
- Residual specimens for storage (if available)

Data Collection Procedures:

- Limited physical examination, pregnancy and birth history, medical history of infant, medication history, assessment of concomitant medications including vitamin and supplementation record, food frequency questionnaire, family history, and adverse events assessments.

4.2.1.3 Infant Enrollment (approximately 4 weeks after blood collected for HLA typing):

Other Procedures:

- Results of infant's HLA testing discussed with parent(s). Parents are only told if genes are higher or lower risk for type 1 diabetes.
- Authorization to release medical records related to birth
- Study capsules and/or formula collected/dispensed.

Mother Specimen Tests:

- RBC fatty acid levels, inflammatory mediator levels, Vitamin D level, and CRP
- If nursing, breast milk fatty acid levels
- Residual specimens for storage (if available)

Data Collection Procedures:

- Medical history, pregnancy history, birth history, a food frequency questionnaire, assessment of concomitant medications including vitamin and supplementation record, an immunization history, a food introduction history, and adverse events assessments.

4.2.2 Entry Point B: Screening After Birth (for mothers not enrolled during pregnancy)

4.2.2.1 Infant Screening (2 days to 5 months of age):

Informed Consent Process

Specimen Tests:

Infant:

- HLA to determine eligibility
- Islet autoantibodies
- Residual specimens for storage (if available)

Mother:

- Islet autoantibodies
- Residual specimens for storage (if available)

Data Collection Procedures:

- Limited physical examination, pregnancy and birth history, medical history of infant, medication history, assessment of concomitant medications including vitamin and supplementation record, food frequency questionnaire, and family history

4.2.2.2 Infant Enrollment (approximately 4 weeks after infant screening):

Other Procedures:

- Results of infant's HLA testing discussed with parent(s). Parents are only told if genes are higher or lower risk for type 1 diabetes.
- Authorization to release medical records related to birth
- Infant randomized to experimental treatment or control group
- Study capsules and/or formula collected/dispensed.

Nursing Mother Specimen Tests:

- RBC fatty acid levels, breast milk fatty acid levels, inflammatory mediator levels, Vitamin D level, and CRP
- Residual specimens for storage (if available)

Data Collection Procedures:

- Medical history, pregnancy history, birth history, a food frequency questionnaire, assessment of concomitant medications including vitamin and supplementation record**,** an immunization history, a food introduction history, and adverse events assessments.

4.3 Visit Schedule After Enrollment:

4.3.1 3 Months of Age Visit (mother only)

Nursing Mother Specimen Tests:

- Breast milk fatty acid levels
- Residual specimens for storage (if available)

Data Collection Procedures:

- Follow-up data collection (includes medical history, immunization history, assessment of concomitant medications including vitamin and supplementation record, nursing mother food frequency questionnaire, infant food introduction history, and adverse events assessments)

Other Procedures:

- Study capsules and infant formula collected/dispensed.

4.3.2 6 Months of Age Visit (mother and infant)

Specimen Tests:

Infant:

- Blood glucose (local meter), islet autoantibodies, RBC fatty acid levels, inflammatory mediator levels, Vitamin D level and CRP level.
- Residual specimens for storage (if available)

Nursing Mother:

- Breast milk fatty acid levels
- Residual specimen for storage (if available)

Data Collection Procedures:

- Limited physical examination, medical history, medication history, immunization history, infant food introduction history**,** nursing mother food frequency questionnaire, assessment of concomitant medications including vitamin and supplementation record, and adverse events assessments.

Other Procedures:

- Study capsules and/or infant formula collected/dispensed.

4.3.3 9 Months of Age Visit (mother only)

Nursing Mother Specimen Tests:

- Breast milk fatty acid levels
- Residual specimens for storage (if available)

Data Collection Procedures:

- Follow-up data collection (includes infant medical history, immunization history, assessment of concomitant medications including vitamin and supplementation record, nursing mother food frequency questionnaire, infant food introduction history, and adverse events assessments)

Other Procedures:

- Study capsules and infant formula collected/dispensed.

4.3.4 12 Months of Age Visit (mother and infant)

Specimen Tests:

Infant:

- Blood glucose (local meter), tetanus antibody levels and WBCs for proliferation response, islet autoantibodies, RBC fatty acid levels, inflammatory mediator levels, Vitamin D level and CRP level.
- Residual specimens for storage (if available)

Nursing Mother:

- Breast milk fatty acid levels
- Residual specimens for storage (if available)

Data Collection Procedures:

- Limited physical examination, medical history, medication history, immunization history, infant food introduction history**,** nursing mother food frequency questionnaire, assessment of concomitant medications including vitamin and supplementation record, and adverse events assessments.

Other Procedures:

- Study capsules and/or a suitable form for toddler consumption collected/dispensed.
- Infant formula collected.

4.3.5 15 Months of Age Visit (mother only)

Data Collection Procedures:

- Follow-up data collection (includes infant medical history, immunization history, assessment of concomitant medications including vitamin and supplementation record, infant food introduction history, and adverse events assessments)

Other Procedures:

- Study capsules and/or a suitable form for toddler consumption collected/dispensed

4.3.6 18 Months of Age Visit (mother and infant)

Infant Specimen Tests:

- Blood glucose (local meter), islet autoantibodies, RBC fatty acid levels, inflammatory mediator levels, Vitamin D level and CRP level.
- Residual specimens for storage (if available)

Data collection procedures:

- Limited physical examination, medical history, medication history, immunization history, infant food introduction history**,** assessment of concomitant medications including vitamin and supplementation record, and adverse events assessments.

Other procedures:

- Study capsules and/or a suitable form for toddler consumption collected/dispensed.

4.3.7 21 Months of Age Visit (mother only)

Data collection procedures:

- Follow-up data collection (includes medical history, immunization history, assessment of concomitant medications including vitamin and supplementation record, infant food introduction history, and adverse events assessments)

Other procedures:

- Study capsules and/or a suitable form for toddler consumption collected/dispensed

4.3.8 24 months of age visit (mother and infant)

Infant Specimen Tests:

- Blood glucose (local meter), islet autoantibodies, RBC fatty acid levels, inflammatory mediator levels, Vitamin D level and CRP level.
- Blood sample collected for storage (if blood volumes allow)
- Residual specimens for storage (if available)

Data collection procedures:

- Limited physical examination, medical history, medication history, immunization history, infant food introduction history**,** assessment of concomitant medications including vitamin and supplementation record, and adverse events assessments.

Other procedures:

- Study capsules and/or a suitable form for toddler consumption collected/dispensed.

4.3.9 After 24 Months of Age, every 6 months for child (for possible transition time into a full-scale NIP Diabetes study)

Specimen Tests:

Infant:

- Blood glucose (local meter), islet autoantibodies, RBC fatty acid levels, inflammatory mediator levels, Vitamin D level and CRP level.
- Residual specimens for storage (if available)

Data collection procedures:

- Limited physical examination, medical history, medication history, immunization history, infant food introduction history**,** assessment of concomitant medications including vitamin and supplementation record, and adverse events assessments.

Other procedures:

- Study capsules and/or a suitable form for toddler consumption collected/dispensed (up to 36 months of age).

4.4 Compliance and Adherence

Analysis of fatty acids from blood and breast milk samples will be the primary method to assess participant compliance with daily doses of the study substance. The baseline and follow-up data collection forms capture the number/amount of capsules/formula dispensed and collected. TrialNet research personnel will maintain records of the study substance dispensation and collection.

Measurements of blood/breast milk fatty acids will be used as a second level of compliance monitoring.

4.5 Biochemical Islet Autoantibody Development

If a child develops two positive biochemical islet autoantibodies on or after the 12 month visit, the child and family will be asked to return within 1-2 months for a confirmatory blood test. If the same 2 autoantibodies are present at both visits, the child will be withdrawn from the study, and may be eligible to enroll in the TrialNet Natural History Study of the Development of Type 1 Diabetes.

4.6 Withdrawal from Treatment, Continued Follow-up

Analyses for this study will be conducted in accordance with the intent-to-treat principle. Once randomized into the study, a participant will undergo all scheduled follow-up assessments and will remain in the assigned treatment group until the end of follow-up or participation in the study, regardless of whether the mother or child is continuing to receive study substance. Withdrawal from the study and its scheduled follow-up assessments should only occur if the parent(s) withdraws consent or the infant develops two persistent positive autoantibodies which are

confirmed on or after the 12 month visit (see Section 4.5).

If the mother decides to discontinue treatment for her or her child for any reason, this will be documented on the appropriate form and the participant will be encouraged to continue with all scheduled follow-up visits. TrialNet research personnel will make every effort to keep mothers and children in the study even if the participant is no longer taking the study substance, or is not fully compliant with the treatment schedule. If a visit is missed, TrialNet research personnel will contact the participant to reschedule and encourage the participant to come back for their follow-up evaluations.

4.7 Re-entry into the Trial

Should the family decide to discontinue the study substance and not return for follow-up visits, the family will be encouraged to bring their child back for follow-up and can resume taking the study substance. If the parent(s) decides to bring their child back for follow-up assessments at a later date, he or she will be allowed and encouraged to do so.

5 Statistical Considerations

Analysis of study data will be conducted to address the primary objectives of the pilot feasibility trial, after all participants have completed the twelve-month visit. These objectives are stated in Section 1.2.3. An interim analysis will be conducted after 6 months of follow-up in the cohort for the Data and Safety Monitoring Board. Analyses will also be conducted to address other stated objectives, and other interrelationships among elements of study data of interest to the investigators and of relevance to the objectives of the study. Such analyses may also entail the use of data from other studies in combination with data from this study. Likewise, data from this study may be used in combination with data from another study to address objectives of that study. Analyses by gender and race/ethnicity, as appropriate, are also planned.

5.1 Statistical Analysis

The statistical analyses to be employed to address each of the study objectives are:

1. *Recruitment: Nine clinical sites will enroll an average of ten participants per site in one year (post approval by their IRBs).* The rate of accrual will be estimated using the mean rate from all sites, with a 95% confidence interval based on an underlying Poisson distribution [80].
2. *Treatment Compliance: At least 90% of families will continue to take the study substance as instructed.* The simple proportion of treatment compliance among all families and the 95% confidence limits will be computed.
3. *Visit Compliance: At least 95% of families will continue to attend follow-up visits.* The simple proportion of visit compliance among all families will be computed and the 95% confidence limits, separately for the month 6 and month 12 visits.
4. *At least a 20% higher level of plasma and/or red blood cell membrane phospholipid DHA can be achieved in the experimental treatment group in comparison with the control group.* The comparison between the two treatment arms will be based on a t-test of treatment effect in an Analysis of Covariance (ANCOVA) model adjusted for the baseline level of each measure [81].
5. *At least a 20% lower level of the major inflammatory cytokine, IL1-beta, will be achieved in the experimental treatment group in comparison with the control group.* The comparison between the two treatment arms will be based on a t-test of treatment effect in an Analysis of Covariance (ANCOVA) model adjusted for the baseline level of each measure [82].

All analyses will be stratified according to whether the participant was entered into the study prenatally (i.e. during pregnancy) or was entered after birth. This adjustment will be conducted using an additional factor in a regression model or through a stratified-adjusted analysis.

Families that contribute multiple participants to a treatment group require special methods. When possible, statistical methods that consider the family as the unit of analysis will be employed, in which all family members will contribute. Where such methods are not feasible, only the first participant entered from each family will be included in the analysis.

5.2 Sample Size Calculations

The sample size of 90 participants was selected based on the feasibility objective of the nine clinical sites recruiting an average of 10 participants in one year. The statistical properties of the study with this sample size to address each objective are:

Objective 1. A sample size of 90 participants would allow the estimation of the rate of accrual to within $\pm$ 11.1%.

Objective 2. This sample size would allow the estimation of a treatment compliance of 0.90 with logit-based confidence limits of 0.819, 0.947.

Objective 3. This sample size would allow the estimation of visit compliance with logit-based confidence limits of 0.880, 0.980.

Objectives 4-5. This sample size will provide 85% power to detect a difference of 0.63 standard deviation units between groups with a test at the 0.05 level two-sided.

6 ADVERSE EVENT REPORTING AND SAFETY MONITORING

6.1 Adverse Event Definitions

6.1.1 Adverse Event

An adverse event is any occurrence or worsening of an undesirable or unintended sign, symptom or disease whether or not associated with the treatment and study procedures.

Throughout the study, the investigator must record adverse events on the appropriate adverse event form, regardless of the severity. The investigator should treat participants with adverse events appropriately and observe them at suitable intervals until the events resolve or stabilize.

Adverse events may be discovered through:

- observation of the participant;
- questioning the participant;
- unsolicited complaint by the participant

In questioning the participant the questioning should be conducted in an objective manner.

6.1.2 Serious Adverse Event

For this trial, an adverse event associated with the treatment or study procedures that suggests a significant hazard, contraindication, side effect or precaution (as described below) is to be reported as a serious adverse event (SAE).

A serious adverse event (experience) or reaction is any untoward medical occurrence that:

- results in death,
- is life-threatening,
- requires inpatient hospitalization or prolongation of existing hospitalization,
- results in persistent or significant disability/incapacity, or
- is a congenital anomaly/birth defect.

Important medical events that may not result in death, be life threatening, or require hospitalization may be considered serious adverse events when, based upon appropriate medical judgment, they may jeopardize the patient and may require medical or surgical intervention to prevent one of the outcomes listed above.

6.1.3 Unexpected Adverse Event

An adverse event is considered unexpected when the nature (specificity) or severity of the event is not consistent with the risks described in the protocol or informed consent document for a particular protocol required intervention.

6.1.4 Grading Event Severity

TrialNet has adopted usage of the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE) and/or study-specific criteria for classification to describe the severity of adverse events.

6.2 Adverse Event Reporting and Monitoring

Adverse events will be reported to the TrialNet Coordinating Center in accordance with the TrialNet Adverse Event Monitoring Plan (see Manual of Operations). The investigator will make a determination as to the relation to therapy and severity of event. Events will be assessed and reported in accordance with the ICH Guideline For Good Clinical Practice and per the guidance of the DHHS Office for Human Research Protections (OHRP).

The adverse event case report form for the protocol must be completed for all adverse events (AE). For reporting serious adverse events (SAE), the TrialNet MedWatch Form should also be completed and faxed to the TNCC *within 24 hours of when the site was notified of the event.* This will be reviewed by the TrialNet Medical Monitor, the TrialNet Safety Committee, and the DSMB as appropriate. Deaths must be reported immediately. Event outcome and other follow-up information regarding the treatment and resolution of the event will be obtained and reported when available, if not known at the time the event is reported. The follow-up information should contain sufficient detail to allow for a complete medical assessment of the case and an independent determination of possible causality.

Adverse events will be assessed and adjudicated, if required by the TrialNet Medical Monitor. The DSMB will conduct regular safety reviews approximately every three to six months (and, as needed) of adverse events by treatment group assignment. Serious adverse events as well as adverse events leading to study discontinuation will be reviewed by the DSMB.

7 PARTICIPANT SAFETY

7.1 Expected Side Effects and Adverse Events

7.1.1 Risks due to DHA and Infant Formula

There are no known risks of DHA to the fetus. DHA has been shown to reduce the risk of pre-term delivery. The risk of pre-term delivery was reduced from 33% to 21% when provided fish oil supplementation of 2.7 g to 6.1 g of n-3 fatty acids/day [42-44].

DHA is already provided in some new infant formulas. There may be some minor digestive system discomforts from the DHA, such as burping and reflux. The control group will receive control oil from the capsules or formulas. There could be reactions to the infant formula itself (not from the DHA). These could include severe digestive system symptoms, eczema or other rashes, or other signs of food allergy. As with all infant formulas, there is a small risk of poor weight gain and growth or temporary decrease in growth.

7.1.2 Risks due to Blood Draws and Heel Sticks

There may be some pain when the needle goes into the skin. In about one out of every 10 individuals, a small amount of bleeding under the skin will produce a bruise. There is also a small risk of more serious problems, including the temporary clotting of the vein, infection of the site or significant blood loss.

7.2 Rules and Procedures for Unmasking Randomized Participants

The management of minor symptoms will be the same regardless of a participant's treatment group assignment, and TrialNet research personnel will not need to be unmasked. The participant's treatment group assignment will not be unmasked until the end of the study. If a situation arises in which it is essential for TrialNet research personnel to be unmasked in order to appropriately treat a study participant, the NIP Principal Investigator at the clinical site will contact the Coordinating Center within 24 hours of the event.

8 ETHICAL CONSIDERATIONS AND COMPLIANCE WITH GOOD CLINICAL PRACTICE

8.1 Statement of Compliance

This study will be conducted in compliance with the protocol and current Good Clinical Practices (GCP), adopting the principles of the Declaration of Helsinki, and all applicable regulatory requirements.

Prior to study initiation, the protocol and the informed consent documents will be reviewed and approved by an appropriate Independent Ethics Committee (IEC) or Institutional Review Board (IRB). Any amendments to the protocol or consent materials must also be approved before they are implemented.

8.2 Participating Centers

Participating TrialNet clinical sites must have an appropriate assurance, such as a Federal-wide Assurance (FWA) or an Unaffiliated Investigators Agreement (UIA), with the Office for Human Research Protections (OHRP), since they are actively engaged in research and provide informed consent. The protocol and consent forms will be approved by Institutional Review Boards at each of the participating clinical sites. HIPAA regulations will be followed by each participating institution in accordance with each institution's requirements.

The investigator is required to keep accurate records to ensure the conduct of the study is fully documented. The investigator is required to ensure that all case report forms are legibly completed for every participant entered in the trial.

The investigational sites participating in this study will maintain the highest degree of confidentiality permitted for the clinical and research information obtained from participants participating in this study. Medical and research records should be maintained at each site in the strictest confidence. However, as a part of the quality assurance and legal responsibilities of an investigation, the investigational site must permit authorized representatives of the sponsor(s) and regulatory agencies to examine (and when required by applicable law, to copy) records for the purposes of quality assurance reviews, audits and evaluation of the study safety and progress. Authorized representatives as noted above are bound to maintain the strict confidentiality of medical and research information that may be linked to identify individuals. The investigational site will normally be notified in advance of auditing visits.

8.3 Informed Consent

The informed consent form is a means of providing information regarding the study to a prospective participant and allows for an informed decision about participation in the study. All participants (or their legally acceptable representative) must read, sign and date a consent form prior to participation in the study, and/or undergoing any study-specific procedures.

The informed consent form must be updated or revised whenever important new safety information is available, for certain protocol amendments, and/or whenever any new information becomes available that may affect a patients' participation in the study.

Participants will be given a written consent form by qualified study personnel (the Trial or Study Coordinator and/or Investigator or other designee). These research personnel will understand the research study, and will complete any necessary courses required by their Institutional Review Board prior to implementing the consent process. The consent form for this clinical study will be reviewed with participants and the participant will be given time to review the written consent form and ask questions. Qualified personnel, as listed above, will obtain written consent prior to the initiation of study procedures. The participant will be given a copy of his or her signed consent form.

8.4 Study Participant Confidentiality

Study records with the study participant's information for internal use at the clinical sites will be secured at the study site during the study. At the end of the study, all records will continue to be kept in a secure location. Study participant data, which is for reporting purposes, will be stored at the University of South Florida Coordinating Center. Case report forms sent to the Coordinating Center will identify participants by a unique TrialNet Identification Number. The data entry system at the Coordinating Center is a secured, password protected computer system. At the end of the study, all study databases will be archived at the Coordinating Center, and the data collection forms will be electronically scanned and saved in electronic format for long-term storage. All paper copies of the forms will ultimately be destroyed after the data is transferred.

HLA genotyping is for research purposes only. Genetic material may be stored for future use with the permission of the study participant. The results of these future analyses will not be made known to the participant.

8.5 Disclosure of HLA results

Participants will be told if their infants have lower-risk HLA genes. If there is one family member with type 1 diabetes, participants will be told if the baby has higher-risk HLA genes.

8.6 Risks and Benefits

The risks of this study are presented in the informed consent form and are described in Chapter 7. There is no guaranteed benefit to participants for their participation in the study. However, infants may benefit from early diagnosis of type 1 diabetes.

9 STUDY ADMINISTRATION

9.1 Organizational Structure

This study is part of Type 1 Diabetes TrialNet, which is funded by the National Institutes of Health through R01 grant awards. Funding will cover the costs of administration and laboratory tests associated with this study during the participant's period of follow-up.

9.2 Groups and Committees

9.2.1 TrialNet Coordinating Center

The TrialNet Coordinating Center (TNCC) will provide overall leadership to the TrialNet study group to include protocol and manual preparation, development of statistical design for each study, and analysis of study results. The TNCC will also coordinate interactions among the participating TrialNet clinical centers, test laboratories including TrialNet core laboratories and other subcontract laboratories, NIDDK, and other sponsoring agencies.

9.2.2 Clinical Sites

Each Principal Investigator at the participating TrialNet clinical site will oversee all operations. The clinical sites will forward all laboratory and data collection form information to the University of South Florida Coordinating Center for analysis. Conference calls and site visits, as needed, will facilitate evaluation of the trial management.

9.2.3 TrialNet Laboratories

TrialNet core laboratories will be utilized to perform tests and assays for this trial. In addition, local laboratories will be used. All laboratory results will be forwarded to the TrialNet Coordinating Center at the University of South Florida for analysis.

9.2.4 Clinical Site Monitoring

In order to conduct this study with established research principles and ICH-GCP guidelines, there may be site visits conducted during the study to evaluate study conduct. All sites will be monitored by the Coordinating Center and appropriate TrialNet committees for patient enrollment, compliance with protocol procedures, completeness and accuracy of data entered on the case report forms (CRFs), and the occurrence and reporting of adverse events (AEs) and serious adverse events (SAEs).

9.2.5 Data and Safety Monitoring Board (DSMB)

The DSMB will meet approximately every 6 months to review efficacy issues and adverse events prepared by the Coordinating Center. All adverse events will be recorded on the adverse event forms, which will be sent to the local IRBs, per their reporting requirements, and to the Coordinating Center. The DSMB will independently evaluate whether the adverse events constitute grounds to discontinue the study.

9.3 Partnering with Industry

Martek Biosciences Corporation and Mead Johnson Nutritionals are providing the study formulations for the DHA and control for this study. Martek Biosciences Corporation is providing the capsules and Mead Johnson Nutritionals is providing the formula.

9.4 Sample and Data Storage

Samples to be stored for research purposes will be located at the NIDDK Repository and at TrialNet site(s). While TrialNet is active, the use of the samples will be restricted to TrialNet researchers unless researchers from outside of TrialNet obtain approval from the TrialNet Steering Committee and the NIDDK to utilize the samples. The samples will be coded with unique study numbers, but TrialNet researchers will be able to identify samples if it is necessary to contact participants for reasons of health or for notification to them about future studies. Approval from the TrialNet Steering Committee and the NIDDK would be required before such linkage could occur. Researchers from outside of TrialNet will not be permitted to identify samples.

Data collected for this study will be sent to the TrialNet Coordinating Center at the University of South Florida. After the study is completed, de-identified data will be stored at the NIDDK Repository, under the supervision of the NIDDK/NIH, for use by researchers outside of TrialNet.

When TrialNet is completed, samples will continue to be stored at the NIDDK Repository Sites. Since the stored data will be fully de-identified upon the completion of TrialNet, it will no longer be possible to identify samples. Thus, whereas a sample can be destroyed upon a participant's request during the existence of the TrialNet, it can no longer be destroyed once TrialNet is completed. However, there will still be the potential to link data derived from the samples with data that had been derived from TrialNet studies. Once TrialNet is completed, researchers will only obtain access to samples through grant proposals approved by the NIDDK. The NIDDK will convene an external panel of experts to review requests for access to samples.

9.5 Preservation of the Integrity of the Study

The scientific integrity of the trial dictates that results be reported on a study-wide basis; thus, an individual center will not report the data collected from its center alone. All presentations and publications using TrialNet trial data must protect the main objectives of the trial. Data that could be perceived as threatening the masking will not be presented prior to release of the primary study outcomes. Approval as to the timing of presentations of data and the meetings at which they might be presented will be given by the TrialNet Steering Committee. Study results should be discussed with the news media only upon authorization of the Steering Committee, and never before the results are presented. Any written statements about this study that are shared with national media should be approved by the Steering Committee before release.

9.6 Participant Reimbursement and Compensation

Participants will be compensated for each visit attended in the study.

10 STUDY TIMELINE

The pilot feasibility trial will have a duration of 2 years: one year for enrollment and one year for follow-up. It is expected that it could take up to an additional 2 years before a full-scale study is initiated. This additional period will be necessary for the analysis of the pilot data, the approval of a full-scale study protocol by TrialNet, and ultimately IRB approval of a full-scale protocol. As discussed above, the children could continue to receive the study substance according to the randomization group for up to 36 months of age in this transition period.

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Appendix A. Schedule of Assessments

Timeline	Pregnant Woman during 3rd trimester Enters (A)			New Child Enters (B)	Follow-up Visits									
	Pregnant Woman Screening/ Enrollment	Infant Screening		Infant Screening 2 days to 5 mos of age	Infant Enrollment (4 wks after HLA blood collection)	Infant Age in Months								
		At Birth	2 to 28 days			3	6	9	12	15	18	21	24	q 6 mos
Informed Consent and HIPAA	P			Parent(s), I										
Medical History	P		I, N	I, N	I, M	N	I, N	N	I, N		I		I	I
Limited Physical Examination			I	I			I		I		I		I	I
Local HbA1c Screening[5]	P													
Local Blood Glucose Testing[4]							I		I		I		I	I
HLA Typing		I[cb]	I[2]	I[2]										
Islet Autoantibodies	P	I[cb]	I[2], M[A]	I[2], M			I		I[3]		I[3]		I[3]	I[3]
Tetanus antibody and cellular response									I					
Fatty Acids Analysis (RBC)	P	I[cb]			N[B], M[A]		I		I		I		I	I
Fatty Acids Analysis (breast milk)					N[B], M[A]	N	N	N	N					

P: Mother who entered her infant prenatally (she entered during pregnancy)
M: Mother (includes non-nursing and nursing mothers)
I: Infant
N: Nursing mother
Schedule of Assessments does not reflect when mother or infant attends the visit but what information is collected regarding mother and/or infant. Infants are not required to attend the infant enrollment visit, the 3, 9, 15, 21 month old visits, or every 6 months thereafter.
[A] Entry A: The mother entered during pregnancy
[B] Entry B: The infant entered after delivery
[cb] HLA typing on umbilical cord blood using an cord blood collection kit
[2] HLA typing and islet autoantibodies by heel stick for infants if umbilical cord blood was not obtained
[3] If 2 or more autoantibodies are present on or after the 12 month visit specimen, 1 to 2 months later a confirmation blood draw will be done. If the same 2 autoantibodies are present at both visits, the child will discontinue the study substance and withdraw from the study.
[4] If infant's blood glucose level is above 150 mg/dL the mother will be asked to do further tests, approx two hours after eating, and to provide these values to the local site.
[5] Local HbA1c screening will be done on pregnant mother if she has diabetes (Type 1, 2, or gestational)

Timeline (continued)	Pregnant Woman during 3rd trimester Enters (A)			New Child Enters (B)	Follow-up Visits									
	Pregnant Woman Screening/ Enrollment	Infant Screening		Infant Screening 2 days to 5 mos of age	Infant Enrollment (4 wks after HLA blood collection)	Infant Age in Months								
		At Birth	2 to 28 days			3	6	9	12	15	18	21	24	q 6 mos
Inflammatory mediators	P	I[cb]			N[B], M[A]		I		I		I		I	I
Vitamin D	P	I[cb]			N[B], M[A]		I		I		I		I	I
CRP	P	I[cb]			N[B], M[A]		I		I		I		I	I
Blood sample collected for storage													I	
Concomitant Medication Assessment (including vitamin and supplement)	P		M[A]	I, N	I, N[B], M[A]	I, N	I, N	I, N	I, N	I	I	I	I	I
Food Frequency Questionnaire	P		M[A]	N	N[B], M[A]	N	N	N	N					
Food Introduction History					I	I	I	I	I	I	I	I	I	I
Immunization Record					I		I		I		I		I	I
Provide DHA or Control (capsules or infant formula)	P		I, M[A]		I, N	I, N	I, N	I, N	I, N	I	I	I	I	I*
Adverse Events Assessments			I, M[A]		I, M[A]	I, N	I, N	I, N	I, N	I	I	I	I	I

P: Mother who entered her infant prenatally (she entered during pregnancy)
M: Mother (includes non-nursing and nursing mothers)
I: Infant
N: Nursing mother
***Up to 3 years of age**
[A] Entry A: The mother entered during pregnancy
[B] Entry B: The infant entered after delivery
Schedule of Assessments does not reflect when mother or infant attends the visit but what information is collected regarding mother and/or infant. Infants are not required to attend the infant enrollment visit or visits when they are 3, 9, 15, 21 months old.

Appendix B. Summary and Prioritization of Blood Tests

PARTICIPANT	**Mother[3]**			**Infant**						
PHASE	Pregnant Woman Enrollment	Infant Screening	Follow-up	Screening		Follow-Up				
TIMELINE (In months of age)	3rd trimester of pregnancy	Delivery or during 5 mos after delivery	At infant enrollment: Nursing or Mother who entered during preg	Birth	2 days to 5 mos	6 mos	12 mos	18 mos	24 mos	q 6 mos
METHOD OF BLOOD COLLECTION	Venous	Venous	Venous	Cord Blood	Heel Stick	Venous	Venous	Venous	Venous	Venous
LAB TEST										
HbA1c[1] (local)	1.2 cc									
Random Blood Glucose[2] (local)						0.05 cc	0.05 cc	0.05 cc	0.05 cc	0.05 cc
HLA Typing				4 cc	0.5 cc					
Islet Autoantibodies	6 cc	6 cc		6 cc	0.5 cc	1 cc	1 cc	1 cc	1 cc	1 cc
Tetanus antibody and cellular response							4 cc			
Fatty Acids Analysis (RBC)	2 cc		2 cc	6 cc		2 cc	2 cc	2 cc	2 cc	2 cc
Inflammatory mediators	2 cc		2 cc	4 cc		1 cc	1 cc	1 cc	1 cc	1 cc
Vitamin D	1 cc		1 cc	2 cc		1 cc	1 cc	1 cc	1 cc	1 cc
C-Reactive Protein	1 cc		1 cc	2 cc		1 cc	1 cc	1 cc	1 cc	1 cc
Blood sample collected for storage									6 cc	
TOTAL (in cc)	12 - 13.2 cc	6 cc	6 cc	24 cc	1.0 cc	6.05 cc	10.05 cc	6.05 cc	12.05 cc	6.05 cc

Note: The NIH guideline for blood draws in infants is 3 cc/kg. Priority of draws starts with the first lab test given for each visit

[1] Local HbA1c screening will be done on pregnant mother if she has diabetes (Type 1, 2, or gestational)

[2] If infant's blood glucose level is above 150 mg/dL the mother will be asked to do further tests, approx two hours after eating, and to provide these values to the local site.

[3] An additional specimen will be collected on a small number of mothers for quality assurance purposes.