St. Jude
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A DOUBLE-BLIND, RANDOMIZED, PLACEBO-CONTROLLED STUDY OF ANTIBODY INDUCTION BY VITAMIN SUPPLEMENTATION AT THE TIME OF INFLUENZA VIRUS VACCINATIONS IN CHILDREN

Principal Investigator
Nehali Patel, MD
Department of Infectious Diseases

Faculty Sponsor
Julia Hurwitz, PhD
Department of Infectious Diseases

Sub-Investigators
Ronald Dallas, PhD[1]
Li Tang, PhD[2]
Yilun Sun, MS[2]
Rhiannon Penkert, PhD[1]

[1]Department of Infectious Diseases
[2]Department of Biostatistics

St. Jude Children's Research Hospital
262 Danny Thomas Place
Memphis, Tennessee 38105-3678
Telephone: (901) 595-3300

Protocol MNEMONIC and Title: FLUVIT - A double-blind, randomized, placebo controlled study of antibody induction by vitamin supplementation at the time of influenza virus vaccinations in children

Principal Investigator: Nehali Patel, MD

Brief Overview: Respiratory virus infections are responsible for more than 4.5 million deaths each year worldwide. Among these, up to 0.5 million deaths are caused by influenza viruses. Children are particularly vulnerable to respiratory virus infections, and extensive campaigns now target their improved healthcare. Vaccination is the single best strategy for the prevention of infectious diseases. Despite the clear benefit of respiratory virus vaccines, efficacy rates can be disappointing.

Vitamin A and D deficiencies are each associated with impaired innate and humoral immune responses and vulnerability to respiratory tract infectious diseases. Rates of vitamin A and D deficiencies or insufficiencies have skyrocketed in the United States, especially in the mid-south region. Animal studies have demonstrated that in vitamin deficient mice, antibody immune responses are poor toward influenza virus vaccine and are improved if a vitamin supplement is administered at the time of vaccination. The central hypothesis of this protocol is that vitamin supplements will enhance antibody responses toward an influenza virus vaccine in children. Children will be randomized using a stratified permuted block method to receive an influenza virus vaccine with vitamin A+D supplement or placebo. Participants will be stratified based on retinol binding protein (RBP) levels at screening, using a cut-off indicative of vitamin A insuffiency (<22,000 ng/ml). Co enrolled sibling participants will be first stratified by RBP levels, then siblings within a same stratum will be equally assigned to different arms to provide greater assurance of balanced treatment assignment. Children will be tested for vitamin levels and immune responses before and after influenza virus vaccinations to determine if vitamin supplementation improves the vaccine-induced antibody response.

Intervention: Children will be randomized to receive influenza virus vaccine plus vitamin A and D supplements (intervention) or Influenza virus vaccine plus matched placebo.

Brief Outline of Treatment Plan: Children between the ages of 2 and 8 years will be enrolled. All will receive two doses of an influenza virus vaccine administered at least 28 days apart. Vitamin levels and antibody responses toward the vaccine will be measured on days 0 (baseline levels), 28, and 56. Vitamins A + D will be orally administered at the levels of 20,000 IU and 2,000 IU respectively on the days of vaccinations for the children randomized to receive vitamin supplements. Parents will be asked to fill out diary cards to indicate food intake for children (intervention & control) over the study period along with an optional food frequency questionnaire given on day

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56. Specific measurements on days 28, and 56 will include analyses of vaccine-specific and total antibody, IgA, IgG, and IgA/IgG plus IgA/IgM ratios in sera. Functional activities of antibodies toward the vaccine will also be measured. Data analyses will indicate effect size for the design of a larger, clinical analysis.

Study Design: Stratified permuted block, double-blind randomized controlled trial of influenza virus vaccine + vitamin A & D supplements, vs. influenza virus vaccine + matched placebo.

Table. Subject Allocation by Intervention vs. Control

Group	Influenza virus vaccine Dose	Vitamin A Dose	Vitamin D Dose	N
Intervention	per manufacturer's specification	20,000 IU	2,000 IU	40
Control	per manufacturer's specification	Placebo	Placebo	40

Sample Size: Approximately 100 participants enrolled over 2 flu seasons.

Data Management: The Department of Infectious Diseases will design forms for data collection. The Department of Biostatistics will provide statistical analysis.

Human Subjects: The risks to human subjects are considered minimal. Possible risks to the participant include those associated with the licensed influenza virus vaccine product or when blood is drawn. Parents/guardians will be informed that the study is experimental.

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1.0 OBJECTIVES

1.1. Primary Objective

To assess the vaccine-induced and total antibody (including IgG and IgA) response after influenza virus vaccine administration and IgA/IgG plus IgA/IgM ratios at 28 and 56 days in sera

1.2. Secondary Objectives

To assess the functional antibody response toward influenza virus vaccine in the sera

1.3. Exploratory Objectives

To describe the socio-demographic characteristics and diet among children participating in the trial.

To assess the feasibility of the food diary among healthy volunteers as measured by completion of the diary.

To monitor serum Vitamin A and D levels at various time points.

2.0 BACKGROUND AND RATIONALE

2.1. Background

Respiratory virus infections are the leading causes of morbidity and mortality in children (1). The risk of death by respiratory disease is approximately 2:1 in vitamin A deficient children compared to controls (2). Low Vitamin D is similarly associated with increased morbidity and mortality due to respiratory disease (3-6). Vaccination is the best way to prevent childhood infections, but many respiratory virus vaccine candidates have failed in clinical trials. Furthermore, the currently licensed influenza vaccines are not fully protective in children. A recent study by MedImmune reported that approximately 10% to 50% of children between the ages of 2 to 17 years who received the quadrivalent FluMist vaccine had evidence of immune responses towards any one of the influenza virus A and B components, respectively (7).

Nutrition and the immune response. Basic studies have shown that vitamins A and D each play a key role in the differentiation of T cell subsets, the proper migration ("homing") of cells into tissues, and the development of protective antibody responses (3, 8-10). Antibodies, a first line of defense against influenza virus at its point-of-entry (11), is impaired when individuals are vitamin deficient (12). Epidemiologic studies confirm that deficiencies of vitamin A and vitamin D impact the immune response (3-6;13-15).

While vitamins A and D are often studied separately, their activities are cross-regulated. They differentially bind heterodimeric receptor complexes (RAR-RXR, VDR-RXR), which in turn bind DNA sequence motifs in or near promoter regions of immune response genes (3; 16-17). The cross-regulatory capacities of vitamins A and D are often overlooked, perhaps explaining in part why past clinical supplementation studies with one vitamin or the other have yielded conflicting results (15;18-24). No clinical study has comprehensively evaluated the influences of vitamins A and D, independently or together, on responses toward influenza virus vaccines.

This project will test the central hypothesis that vitamin A+D supplementation in children will improve systemic antibody responses toward an influenza virus vaccine. Our studies are expected to demonstrate a logistically feasible method to improve influenza virus vaccine efficacy. Ultimately, our data may instruct new healthcare initiatives to improve vaccination against numerous respiratory pathogens, and thereby reduce morbidity and mortality in children.

2.2. Rationale

Vitamin deficiencies and insufficiencies are widespread among children in the United States (3;25-27). Based on results from our mouse model (please see preliminary data), we hypothesize that children with low vitamin levels will exhibit poor antibody responses toward the influenza virus vaccine, but that antibodies (including IgG and IgA), IgA/IgM, and IgA/IgG1 ratio measurements will be improved among children who receive vitamin A and D supplements at the time of vaccination. To test our hypothesis, we will randomize children who are receiving the influenza virus vaccine into two groups. One group will receive oral vitamin A+D supplements on the days of vaccination while the other group will only receive the influenza virus vaccine. If we find that supplements enhance antibody responses, we will (i) provide an explanation for the low efficacy of respiratory virus vaccines in humans, and (ii) provide evidence that vaccine efficacy can be easily improved. By optimizing the influenza virus vaccine and

other respiratory virus vaccine programs, we may ultimately reduce the morbidity and mortality caused by respiratory infections in children.

Vitamin deficiencies and insufficiencies. Memphis, TN, the site of St. Jude Children's Research Hospital (St. Jude), is a mid-south city with many families of low socioeconomic status and high vulnerability to infectious disease. A published study of adults in a general internal medicine practice at the University of Tennessee demonstrated very low vitamin levels in Memphis (25). We also examined (i) individuals from Memphian families who had at least one influenza virus-infected member, and (ii) individuals from the Tennessee Blood Services for levels of vitamin A using the surrogate retinol-binding protein [RBP, (28)]. RBP is generally present at a 1:1 molar ratio with retinol. We also measured vitamin D levels. As shown in Figure 1, we found that approximately ½ individuals exhibited deficiencies or insufficiencies in vitamin A based on IOM standards (vitamin A deficiencies= ≤0.7μM or ≤15,400 ng/ml RBP; insufficiencies = >0.7 to ≤1.05 μM or >15,400 to <22,000 ng/ml RBP). Most individuals were deficient in vitamin D [25(OH)D] based on IOM standards (<20 ng/ml (http://ods.od.nih.gov/ factsheets/VitaminD-HealthPorfessional)). No one in the study exhibited sufficiency for vitamin D, based on reports that certain tissues require >30 ng/ml, an opinion reflected by the practice guidelines of the US Endocrine Society (30-31).

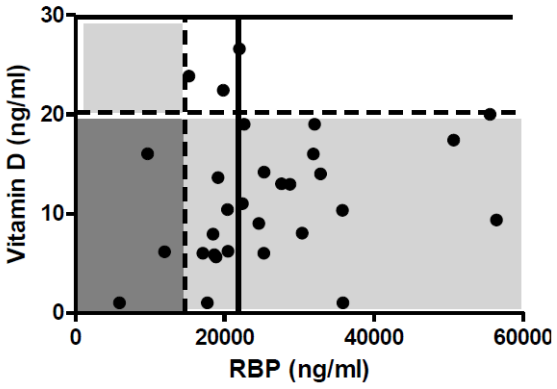


Figure 1, Low vitamin levels in Memphis, TN. Samples from households in which at least one individual was influenza virus infected (32) and from the Tennessee Blood Services were tested for human RBP4 (Quantikine ELISA Kit, Panel) and for vitamin D in the Pathology Department at St. Jude using a Roche Elecsys Vitamin D assay that measures 25-hydroxylated metabolites of cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2). Black and dotted lines indicate cut-offs for vitamin insufficiencies and deficiencies respectively.

Pre-clinical studies of vitamin A and D deficiencies and the immune response toward viral vaccines.

To study the impact of vitamin deficiencies on respiratory virus vaccine immunogenicity, we established vitamin deficient mouse models. Briefly, C57BL/6 pregnant mice were placed on vitamin deficient or control diets at 4-5 days gestation (Harlan diets). Pups were born and maintained on the diets until they reached adulthood and throughout the course of experiments. Vitamin D deficient mice were housed under LED lights.

Analyses were first conducted to monitor CD8+ T cell recruitment to the respiratory tract airway following immunization with low dose (250 EID50) Sendai virus (SeV, a parainfluenza virus type I of mice) in vitamin deficient mice. The frequencies of virus-specific CD8+ T cells in the airways were significantly reduced compared to controls when there were deficiencies in vitamins A or D [(33) and unpublished data].

Experiments were then focused on the study of respiratory virus vaccines and antibody responses in vitamin deficient mice. We found that vaccine-specific IgA antibody forming cells and IgA responses in the upper respiratory tract were significantly reduced in animals with vitamin A deficiencies (34), and were further reduced in animals with vitamin A+D deficiencies. This demonstrated a failed first line of immune defense at the pathogen's point-of-entry (11-12).

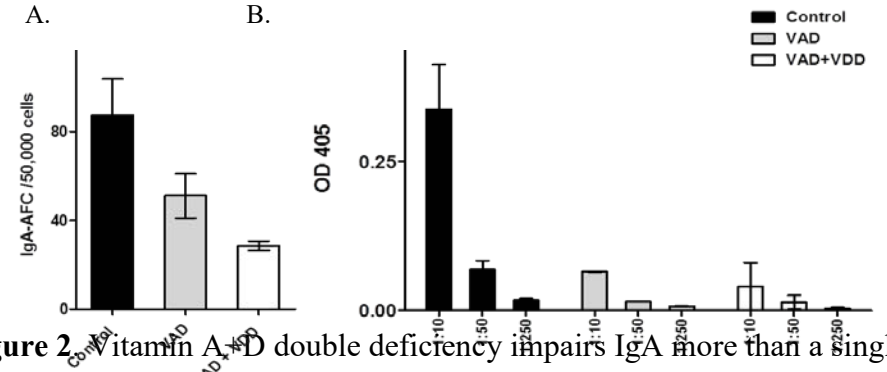


Figure 2. Vitamin A+D double deficiency impairs IgA more than a single vitamin deficiency. Mice were reared on a diet deficient in vitamin A (VAD) or both vitamins A+D (VAD+VDD). As adults, mice were vaccinated with a cold-adapted influenza virus vaccine. After one month, mice were evaluated for IgA-producing AFCs (panel A) or IgA in nasal washes (NW) diluted 1:10, 1:50 and 1:250 prior to assay (panel B). Averages are shown.

To determine if failed immune responses could be corrected, we vaccinated mice with influenza virus vaccine and supplemented mice with vitamins on days 0, 3, and 7 relative to vaccination (35). As shown in Figure 3, the IgA responses were extremely low in animals that were deficient for vitamins A+D (clear bars; VAD + VDD) compared to controls (black bars). IgA responses were partially corrected in double deficient animals when they received either vitamin A or vitamin D. However, the best correction was when animals received both vitamins A and D (hatched bars; +Vit A and D).

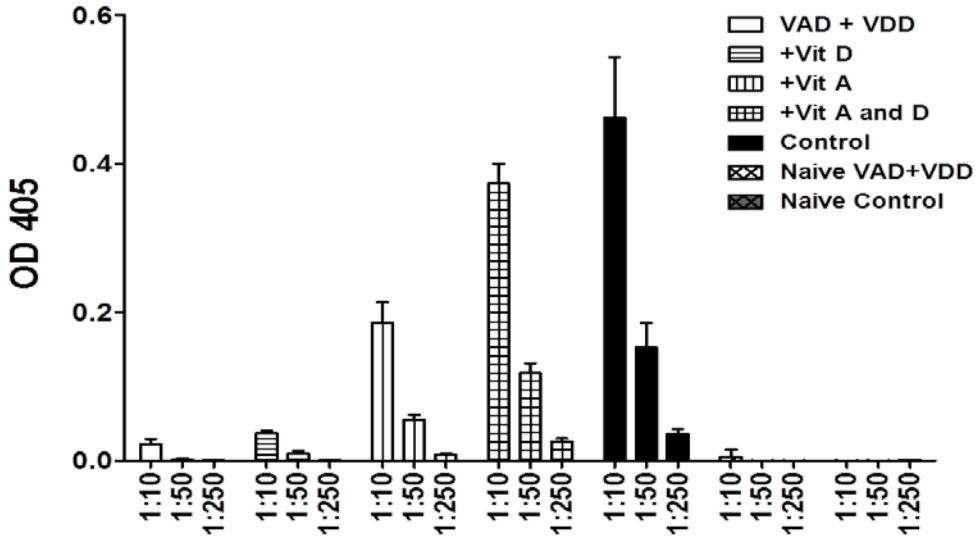


Figure 3. Oral vitamin supplements correct IgA responses in double deficient mice. Vitamin A (retinyl palmitate, 600 IU) and vitamin D (cholecalciferol, 40 IU) were administered to mice deficient in vitamins A and D on days 0, 3, and 7 relative to vaccination with a cold-adapted influenza virus vaccine. Mucosal IgA responses were examined 1 month later. IgA responses were examined in nasal washes. Shown are averages for each mouse set, with sample dilutions of 1:10. 1:50, and 1:250.

To answer the question of benefit of vitamin supplementation with intramuscular influenza vaccine, we injected mice i.m. with an inactive H1N1 influenza virus vaccine. Each mouse received two i.m. injections with a 4 week interval. Half of the mice received 600 IU retinyl palmitate on Days 0, 3, and 7 relative to both the priming and boosting doses of vaccine. Animal sera were evaluated for function with a hemagglutination inhibition assay (HAI) against influenza virus. Samples were tested before vaccination, and after both prime and booster injections. As demonstrated in Figure 4, animals that received vitamin supplements generated significantly improved influenza virus-specific antibody responses compared to

controls. Results were striking in that no mice responded to the priming vaccine dose, except for mice that received vitamin supplements. After the booster vaccine, vitamin supplemented mice exhibited significantly improved antibody titers compared to controls. Altogether, data demonstrate that vitamin supplements improve antibody responses when animals are vaccinated either with a replication-competent or inactive influenza virus vaccine, and either by the i.n. or i.m. route.

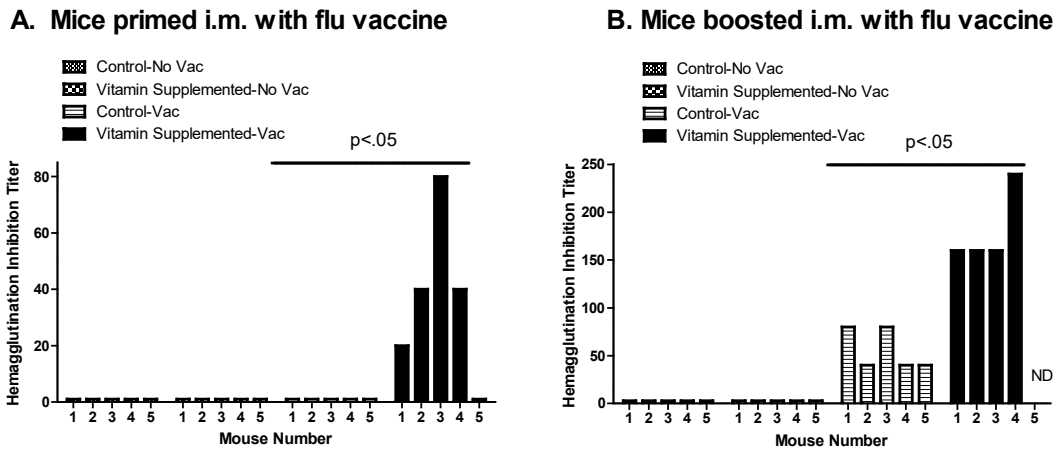


Figure 4. Vitamin supplements improve immune responses to an i.m. influenza virus vaccine. HAI titers are shown for unvaccinated and vaccinated mice. Results from vaccinated, control mice are shown as hatched bars and results from vaccinated, vitamin-supplemented mice are shown as solid bars. Responses are shown after the priming vaccine dose (A), and after the booster (B). In both cases, the vitamin supplements significantly improved antibody activities (Mann Whitney Tests). ND = not done

Human samples were next examined to determine if vitamins A and D associated with antibodies. Samples were available for this study from the Memphian families affected by influenza virus infections and from the Tennessee Blood Service (Memphis, TN). Strong associations were revealed between RBP and isotype patterns. There were significant associations between RBP and IgA, IgA/IgM, IgA/IgG1, and IgG4 (Figure 5). There were also associations between RBP and neutralizing antibody titers toward influenza virus (data not shown). These results are exciting and supportive of our previous findings in vitro and in mice.

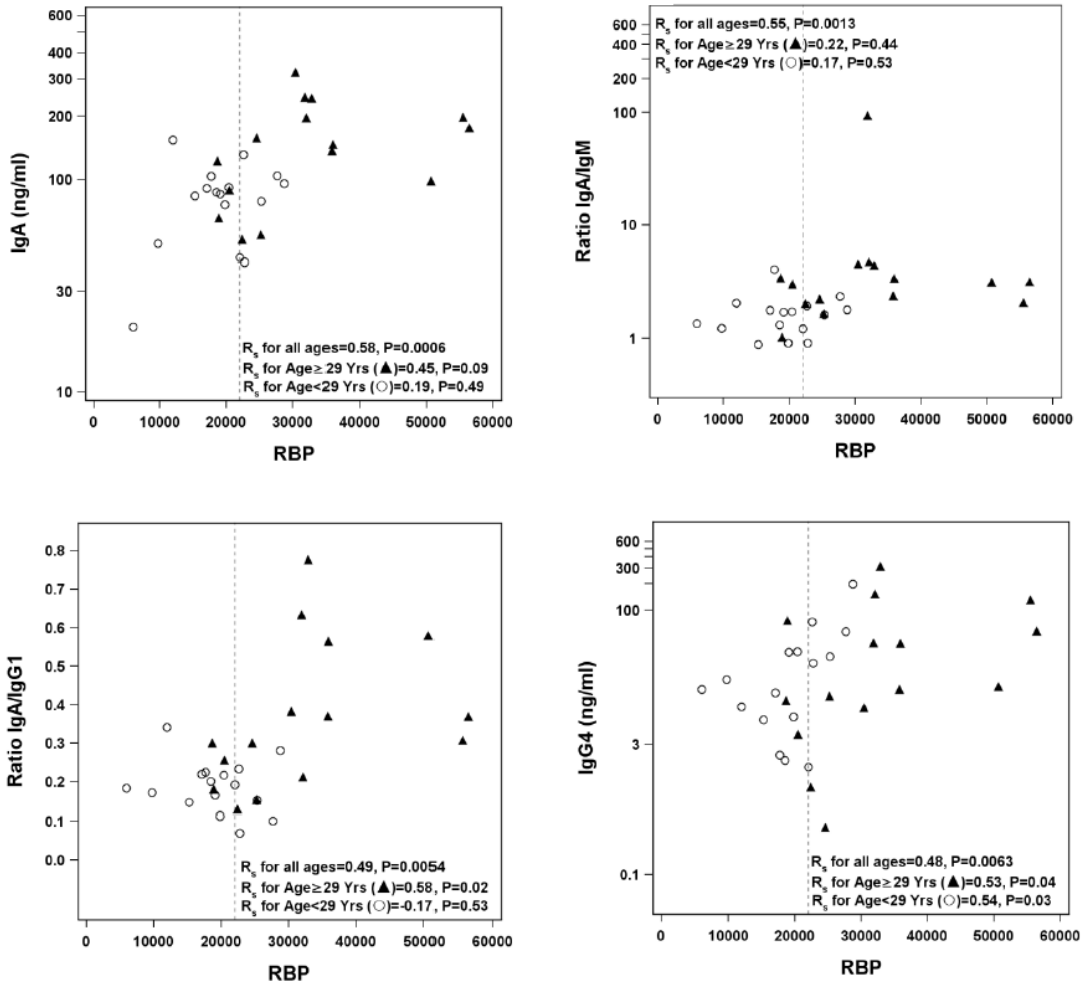


Figure 5. RBP associates with isotypes represented by antibody heavy chain genes located at the 3' side of the immunoglobulin heavy chain locus. Blood samples were examined for RBP levels by ELISA and for total immunoglobulin isotypes using Luminex technology (HGAMMAG-301K Map human isotyping magnetic bead panel, Milliplex, Millipore, St. Charles, MO).

Taken together, our preliminary experiments demonstrate that vitamin A (based on RBP levels) and vitamin D deficiencies are common in Memphis and that vitamin A levels correlate with IgA levels in mice and humans. In the mouse model, the vitamin A and D deficient animals exhibited extremely low IgA levels, which could be corrected when mice received vitamin supplements at the time of vaccination.

Results strongly encourage a test of vitamin supplementation in Memphis residing children who have low vitamin levels and who will receive an influenza virus vaccine. Improvements in influenza virus vaccine efficacy may ultimately protect

children and adults from the morbidity and mortality caused by influenza virus disease. Thus, efforts are underway to examine the efficacy of Vitamin A & D supplementation in conjunction with children receiving the influenza virus vaccine.

Rationale for Two Doses of influenza virus vaccine

Current guidelines are based on the fact that many children younger than 9 years of age have not been infected with influenza viruses previously, and a booster dose is needed for them to produce a protective immune response (36). Previous research among healthy children has also demonstrated the efficacy of two doses of influenza vaccine in preventing laboratory-confirmed cases of influenza illness (37-38). Based on previous evidence and the current guidelines supporting the administration of two doses of influenza vaccine among young children, this study will require two doses of influenza virus vaccine for all participants.

Rationale for Vitamin A and Vitamin D Dose

Consistent with previous literature and our pre-clinical data (as detailed above), high dose vitamin supplementation will be utilized as part of this study (20,000 IU per dose of vitamin A and 2,000 IU per dose of vitamin D). A dose that is 10 times the recommended daily allowance (RDA) at the tolerable upper intake level (UL) will be used for vitamin A. The chosen vitamin A dose is unlikely to cause adverse effects as it is well below that recommended by the WHO for children in developing countries. According to UNICEF's guidelines "the World Health Organization (WHO) has established dosage guidelines; each child between 6 and 11 months is to receive 100,000 international units (IU) of vitamin A. This increases to 200,000 IU every six months from 12 to 59 months of age" (40). Vitamin D levels will be maintained at the UL (41), because although high-dose vitamin D has been successfully studied in humans, and a combination of vitamin A and D supplements support IgA production in research animals deficient in both vitamins, the correlation between vitamin D and IgA is not convincing in our human study. In addition, high-dose vitamin D can yield paradoxical effects, and vitamins A and D can act as antagonists to one another (42-44)

Additionally, we have conducted vitamin studies in vitamin A deficient mice (VAD) and in mice deficient for both vitamins A and D (VAD+VDD). Caveats to the direct translation of results from these mice to humans are: (i) the vitamin A content of a normal mouse diet is approximately 15X the RDA of humans based

on weight, (ii) vitamin A was completely withheld from our test mice throughout their development prior to vaccination, (iii) the vaccinations in mice were either with influenza virus vaccine, a mouse parainfluenza virus, or a cold-adapted, mouse-adapted influenza virus (PR8), (iv) supplements were administered to VAD mice by either the intranasal or oral route, and (v) mice were anesthetized when vaccinated, a condition conducive to vaccine transmission to the lower airway. We found that a dose of 600 IU retinyl palmitate (high dose) in a ~20 gram mouse (approximately 30,000 IU/kg), corrected an influenza virus vaccine-induced IgA response when administered on Days 0, 3, and 7 relative to vaccination. The same dose corrected responses when administered intranasally on Day 0 to mice that received a parainfluenza virus vaccine. In the latter case, significant, but incomplete improvements were also observed with doses of 60 IU (3,000 IU/kg, medium dose) and 6 IU (300 IU/kg, low dose), the latter being comparatively lower than the vitamin A dose proposed here for the clinical study (45).

More recently, we conducted additional experiments in which mice deficient in both vitamins A and D were given a cold-adapted PR8-based influenza virus vaccine and supplemented orally. When mice received 600 IU retinyl palmitate and 40 IU vitamin D orally on Days 0, 3, and 7 relative to vaccination, the IgA response was rescued. Responses were less well improved when 600 IU retinyl palmitate or 40 IU vitamin D were used alone. In another study, mice received 600 IU retinyl palmitate (~30,000 IU/kg) and 40 IU vitamin D, or 60 IU retinyl palmitate and 40 IU vitamin D, or 6 IU retinyl palmitate and 4 IU vitamin D orally on Day 0 relative to vaccination with the cold-adapted PR8 influenza virus vaccine. The highest dose was most effective at correcting the vaccine-induced IgA response. The medium and lower doses were less effective. To balance safety and efficacy and using the 15X correction factor for human versus mouse diets, we chose a vitamin A dose for young children that converts to ~450 IU in a mouse based on weight, falling between the medium and high doses described above.

Our long-term goal is to identify a logistically-feasible public health strategy to provide all children a vitamin supplement in conjunction with the influenza virus vaccine to enhance their immune protection against influenza virus disease. A dose of 10X the RDA UL given once per month should not pose risk to children who have sufficient levels of vitamins. As stated above, the vitamin A dose by weight is well below that recommended by the WHO for children in developing countries (each child between 6 and 11 months may receive 100,000 international

units (IU) of vitamin A; from 12-59 months of age, children may receive 200,000 IU every six months). If a child of 1 year of age and 10 kg receives a single dose of 200,000 IU vitamin, the dose/kg is 20,000 IU/kg, as opposed to the 1500 IU/kg proposed here. Despite previous indications of vitamin safety, there are also studies that have indicated risk in the context of high vitamin A or D intake. Penniston and Tanumihardjo describe acute toxicities when children ingest >20X the vitamin A RDA over a period of hours or a few days (46). Tolerance to high vitamin intake may be variable among children (47). A dose of 25,000 IU increases the incidence of transient bulging of the fontanelle in infants and a dose of greater than 25,000 IU in pregnant females is discouraged at certain periods of gestation due to a risk of teratogenicity (48). These and other studies were likely considered by the IOM when defining UL RDA guidelines. Vitamins A and D bind nuclear receptors (e.g. RAR-RXR, VDR-RXR, PPAR-RXR, LXR-RXR, and TR-RXR) that in turn bind DNA, impacting transcription at a plethora of loci and influencing a wide variety of cell functions. As stated above, the vitamins and receptors can be agonists or antagonists of other receptors and of one another (42-44). The vitamin A dose proposed here (20,000 IU) is below doses previously associated with toxicities.

The proposed pilot study led by the Department of Infectious Diseases will be a double-blind, randomized, placebo-controlled trial in healthy volunteers. The primary objective will be to assess the vaccine-induced and total antibody (including IgG and IgA) response and IgA/IgG plus IgA/IgM ratios in sera. The secondary objective will be to assess the functional antibody response toward Influenza virus vaccine influenza vaccine in the sera. Participants will be randomly assigned to receive either two doses of an influenza virus vaccine plus Vitamin A + D (intervention group), or two doses of an influenza virus vaccine plus the placebo (control group). The targeted accrual total is 100 with a targeted evaluable 80 participants recruited over 2 influenza seasons (approximately 2 years).

3.0 RESEARCH PARTICIPANT ELIGIBILITY CRITERIA AND STUDY ENROLLMENT

According to institutional and NIH policy, the study will access research participants regardless of gender and ethnic background. Institutional experience confirms broad representation in this regard.

3.1. Inclusion Criteria

Male or female child, age 2 to 8 years (inclusive) at the time of enrollment.

Parent or legal guardian willing and able to give informed consent and comply with study requirements.

3.2. Exclusion Criteria

Current use of investigational or immunosuppressive drugs (e.g., steroids) at the time of enrollment

Currently taking a daily (routine) vitamin A, D, or multivitamin. Note: participants who report occasional or sporadic vitamin use will be allowed to enroll.

History of lung disease, asthma, immunodeficiency, sickle cell disease, or any other serious underlying condition or disease in the opinion of the principal investigator.

Evidence of developmental delay or evolving neurological disorders at screening.

Current use of antibiotics or antivirals at enrollment.

History of having a severe allergy to eggs or to any inactive ingredient in the Influenza virus vaccine

History of a life-threatening reaction to influenza vaccinations

Currently wheezing at the time of enrollment

History of Guillain-Barre Syndrome

History of heart, kidney, or lung conditions

History of diabetes

Use of an anti-influenza medication (including amantadine, rimantadine, oseltamavir, and zanamivir) within 14 days prior to enrollment

Acute febrile [>100.0ºF (37.8ºC) oral] illness or acute respiratory illness (e.g., cough or sore throat) within 3 days prior to enrollment

Previous receipt of current seasonal influenza vaccine

3.3. Research Participant Recruitment and Screening

Children ages 2 to 8 years old will be sought in the Memphis community from a variety of both private and public venues through active recruitment by study staff as well as self-referral in response to advertisements. Types of advertisements that may be used include flyers, postcards, brochures, and newspaper ads. Advertisements may be distributed in many ways including email, mass mailings, and in person by study staff. Verbal permission will be sought at private locations where advertisements will be posted by study staff and a signed letter of authorization will be obtained from any performance sites where recruitment activities take place. All recruitment materials will be approved by the IRB before use. Advertisements will indicate the nature of the study and provide a phone number for further information.

Study investigators or their designees will explain the study to potential research participants, including eligibility and exclusion criteria. Persons who indicate they are interested in participating in the study and potentially eligible will be offered a screening visit.

During the screening visit, study investigators or their designees will assign a participant ID and provide individuals with information about the study and an opportunity to ask questions. Written informed consent for study participation will be obtained from the parent or legal guardian. Participants who fail the screening process due to the time sensitive criteria (e.g., recent febrile illness) will be allowed to rescreen.

3.4. Enrollment on Study at St. Jude

A member of the study team will confirm potential participant eligibility as defined in Section 3.1-3.2, complete and sign the 'Participant Eligibility Checklist'. The study team will enter the eligibility checklist information into the Patient Protocol Manager (PPM) system. Eligibility will be reviewed, and a research participant-specific consent form and assent document (where applicable) will be generated. The complete signed consent/assent form(s) must

be faxed or emailed to the CPDMO at 595-6265 to complete the enrollment process.

The CPDMO is staffed 7:30 am-5:00 pm CST, Monday through Friday. A staff member is on call Saturday, Sunday, and holidays from 8:00 am to 5:00 pm. Enrollments may be requested during weekends or holidays by calling the CPDMO "On Call" cell phone (901-413-8591) or referencing the "On Call Schedule" on the intranet).

3.5. Procedures for Identifying and Randomizing Research Participants

Eligibility of research participants will be confirmed by the PI or study team. A study team member will then approach the participant and his or her legal authorized representative regarding the study. If the participant and/or caregiver agree to participate and meet eligibility criteria, the participant will be randomized.

Once informed consent has been obtained and eligibility confirmed, participants will be randomized to receive influenza virus vaccine + Vitamins A & D (n=40) or influenza virus vaccine + placebo (n=40). Randomization will be performed in the SJCRH pharmacy by an unblinded pharmacist, using the randomization program developed by the Department of Biostatistics. In order to ensure similar enrollment numbers within each group, randomization will be restricted with a permuted block randomization design. All eligible participants who are enrolled and receive at least one administration of influenza virus vaccine will be included in the analyses.

4.0 TREATMENT PLAN

4.1. Treatment

This is a single site, pilot, randomized controlled trial in healthy children. The primary and secondary endpoints assessing vaccine-induced and total antibody (IgG and IgA) response and IgA/IgG plus IgA/IgM ratios in the sera as well as the functional antibody response toward influenza virus vaccine in the sera will be measured at Day 28, and 56. Approximately 100 total and 80 evaluable participants will be enrolled equally into two groups over 2 flu seasons, one of which will receive oral vitamins A and D (intervention), while the other group will receive the influenza virus vaccine and placebo gummy (control).

Potential participants will be approached by study staff. Participants who wish to participate in the study will complete the informed consent process prior to conducting the screening evaluations.

Allocation will be stratified by levels of Vitamin A using the surrogate retinol-binding protein (RBP). Vitamin A insufficiency (inclusive of deficiency) will be defined as ≤1.05 μM or <22,000 ng/ml RBP. RBP will be used to stratify participants based on previous mouse and human study demonstrating that vitamin A (for which RBP is a surrogate) is well correlated with IgA. RBP measurements are variable in humans, distributed somewhat equally above and below sufficiency cut-off. In contrast, our clinical work has indicated that study participants in Memphis are largely homogenous for vitamin D measurements in that most of the serum values fall below the sufficiency cut off. After the screening evaluations are obtained, the participant without co-enrolled siblings will be allocated to the intervention or placebo arm , in the pharmacy department, by means of a computerized stratified permuted block method (block size 4: allocation ratio 1:1) provided by the department of biostatistics. Co-enrolled siblings will first be stratified by their screening RBP levels, and then siblings within the same RBP level stratum will be equally assigned to intervention or control to avoid the chance of treatment imbalance, with the use of a computerized stratified permuted block method (block size 2: allocation ratio 1:1).

Participants will be randomized to receive either influenza virus vaccine plus vitamins A and D supplements (n=40) or influenza virus vaccine plus matched placebo. Gummies for the intervention and control arm (placebo) will be provided in the same container by an unblinded pharmacist to maintain blinding. For the purposes of this study, Study Day 0 is defined for each participant as the day that the first influenza vaccine dose is administered. All participants will receive 2 doses of influenza virus vaccine at least 28 days apart.

Children will have the first influenza virus vaccine dose (dose 1) administered on day 0 of the trial, and the second dose (dose 2) administered 28 days later. Vaccines will be administered by a technique consistent with the manufacturer's specification.
Participants will be observed for 30 minutes following vaccination. Participants will be given a food diary card to record their diet between the Day 0 and Day 56 study visits.

Blood serum samples will be collected from participants at Screening and prior to receiving the influenza virus vaccine on Day 28 as well as during their Day 56 follow-up visit.

Chewable Vitamin or PlaceboAdministration

Chewable Vitamin A & D3 or Placebo Gummy
The chewable gummy contains either Vitamin A (20,000 International Units) and Vitamin D3 (2,000 International Units) or matched placebo, which should be fully chewed under supervision of study staff with documention in the participants research record and eMAR (electronic medical administration record). The chewable gummy should be administered prior to receiving influenza virus vaccine vaccination.

If participants have difficulty chewing the gummy, another option is to cut the chewable vitamin in half along score line for easier administration.

4.2. Contraindications to an influenza virus vaccine

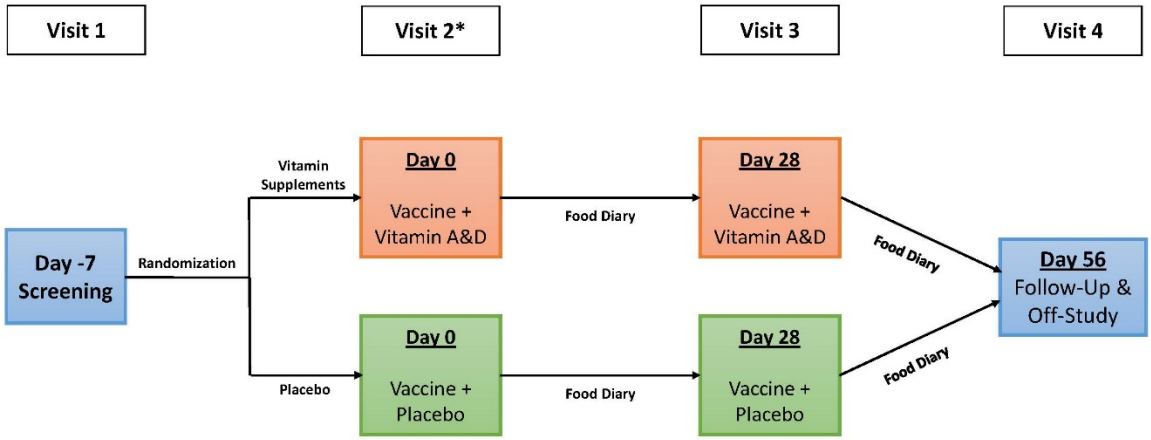
Influenza virus vaccinewill not be administered to anyone with previously known allergic reaction (e.g., anaphylaxis) to any component of the vaccine, or after a previous dose of any influenza vaccine during the same season prior to study participation.

4.3. Concomitant Therapy

Information (including name and indication) regarding medication use by participants will be collected at the time of enrollment. All medications taken from enrollment through Day 56 will also be collected. Concomitant medications reported throughout the course of the study will be recorded including: all prescription medications, over-the-counter preparations, vitamins, nutritional supplements, and herbal preparations.

The use of anti-influenza medications for the prevention of influenza is prohibited during the trial as use of these agents prophylactically may affect culture results. Participants caregivers will be asked to inform study staff should their child require anti-influenza medications while on study. Use of all other concomitant therapies will be allowed.

4.4. Study Visit Flow Diagram



* Day 0 (Visit 2) should occur within 7 days of screening.

5.0 DRUG/DEVICE/BIOLOGIC AGENT INFORMATION

5.1. Influenza Virus Vaccine

The influenza virus vaccine can be of any licensed brand, preferably Fluzone.

5.2. Vitamin A and D3 Nutritional Supplements, Oral

Vitamin A palmitate 20,000 International Units and Vitamin D3 2,000 International Units liquid formulations will be compounded with gelatin base, tangerine oil, polysorbate 20F, citric acid anhydrous USP, and steviol glycosides 95% into a chewable gummy. The gummies will be compounded for participants by Regal PharmaLab (a local compounding pharmacy) following U.S. Pharmacopeial Convention (USP) 795 good compounding practices for pharmaceutical compounding of nonsterile preparations.

The expiration date will be listed on the product label for storage at controlled room temperature. Individual doses will be dispensed with participant name, medical record number, dosing instructions, and date. St. Jude Children's Research Hospital Pharmacy will dispense the gummies and keep accountability records as per randomized assignment.

Certificates of Analysis (CoA) are available for the Vitamin A palmitate liquid, Vitamin D3 liquid, gelatin base, tangerine oil, polysorbate 20 NF, citric acid anhydrous USP, and steviol glycosides 95%. As vitamin supplements, these products are not FDA approved for prevention, diagnosis or treatment of disease.

5.3. Matched Placebo

Placebo gummies will be compounded for participants by Regal PharmaLab (local compounding pharmacy) following U.S. Pharmacopeial Convention (USP) 795 good compounding practices for pharmaceutical compounding of nonsterile preparations. The placebo gummies will be formulated with gelatin base, tangerine oil, polysorbate 20 NF, citric acid anhydrous USP, and steviol glycosides 95% and will match the Vitamin A and D3 gummies in shape, taste, texture, and appearance.

The expiration date will be listed on the product label for storage at controlled room temperature. Individual doses will be dispensed with participant name, medical record number, dosing instructions, and date. St. Jude Children's Research Hospital Pharmacy will dispense the gummies and keep accountability records as per randomized assignment.

Certificates of Analysis (CoA) are available for the gelatin base, tangerine oil, polysorbate 20 NF, citric acid anhydrous USP, and steviol glycosides 95%.

6.0 REQUIRED EVALUATIONS, TESTS, AND OBSERVATIONS

A summary of the evaluations described below and their schedule for completion can be found in Appendix I. Protocol related procedures, evaluations, and labs not performed as scheduled due to malfunctioning equipment, participant non-compliance, participant need or preference (e.g., vacation), participant condition or due to falling on a weekend or holiday will be noted in the participant study record but not be reported as deviations. Adverse events related to commercially available influenza virus vaccine will also not be collected as part of this study.

6.1. Screening Visit

At the screening visit, after informed consent is obtained the following will be completed:

Medical History to include:

- History of a severe allergy to eggs or to any inactive ingredient in the influenza virus vaccine
- History of a life-threatening reaction to influenza vaccinations
- History of Guillain-Barre syndrome
- Currently wheezing or history of wheezing if under 5 years old
- History of immunodeficiency
- History of heart, kidney, or lung problems
- History of diabetes
- Currently taking Tamiflu, Relenza, Amantadine, and/or Rimantadine
- Currently taking daily vitamin or multivitamin

Laboratory Evaluations

- Serum for vitamin studies
- Serum for immunologic studies

Questionnaires

- Socio-demographic questionnaire

Once screening criteria is confirmed and vitamin A retinol protein levels have been resulted, the participant will be randomized.

6.2. Day 0 (Vaccine dose #1) Visit

For the purposes of this study, Study Day 0 is defined for each participant as the day that the first vaccine (Influenza virus vaccine) is administered.

Day 0 must be completed within 10 days of the screening assessment. If greater than 10 days passes, the participant must repeat the screening visit to confirm eligibility.

All Screening and Entry visit data must be collected prior to the receipt of vaccination and vitamin (A and D) dose #1. Vitamin or placebo chewable

gummy must be administered prior to influenza virus vaccine dose administration.

The following will be collected as a part of the Entry Visit:

- Chewable gummy (vitamin supplements or placebo depending on randomization) dose administration
- Administration of influenza virus vaccine Dose #1 for intervention and control group and observation in clinic 30 minutes post-vaccine administration
- Dispense food diary to control and intervention participants.
- Serum for vitamin studies
- Serum for immunologic studies

6.3. Day 28 (Vaccine dose #2) Visit

The following will be completed:

- Interim medical history and medication use
- Laboratory evaluations
    - Serum for vitamin studies
    - Serum for immunologic studies
- Collection of food diary from participants
- Administration of influenza virus Vaccine Dose #2 to Intervention and Control group participants
- Administration of chewable gummy dose #2
- Dispense food diary to control and intervention participants.

6.4. Day 56 Follow-Up Visit

The following will be completed:

- Interim Medical History and medication use
- Collect food diary from participants
- Laboratory evaluations:
    - Serum for vitamin studies
    - Serum for immunological studies
- At day 56, participants enrolled after approval of Revision 1.1, will be given the option to complete the Block food frequency questionnaire (FFQ). This questionnaire includes about 90 questions and asks about a child's "usual eating habits in the past 6 months." It takes a caregiver or interviewer approximately 30 minutes to complete. The food list was

developed from NHANES III dietary recall data. The nutrient database was developed from the USDA Nutrient Database for Standard Reference. Individual portion size is asked for beverages but not other foods. Dietary information obtained from this questionnaire is for exploratory purposes only.

6.5. Special Instructions and Definitions of Evaluations

Study Visit Windows

Day 0 (Vaccine dose #1) visit must occur within 10 days of screening.

Day 28 (Vaccine dose #2) visit must be performed within +3 days of the protocol specified date

Day 56 (follow-up)visit must be performed within +/- 7 days of the scheduled protocol visit date.

Vaccination
Influenza virus vaccines will be administered per manufacturer's recommendationsby trained study or clinic staff.
Participant caregivers will be informed to contact their child's PCP should any influenza virus vaccine related reactions occur over the course of the study.

Vitamins A and D3
Chewable vitamin supplements or placebo gummies will be administered orally to participants prior to receiving the influenza virus vaccine on Day 0 and Day 28 by study staff. The gummy will be administered and documented in the participants research record prior to the influenza virus vaccine vaccination..

Laboratory Evaluations
Immune responses (inclusive of IgM, IgG, IgA, and cytokines) will be measured in blood serum collections at Screening, Day 28, and Day 56. ELISAs and functional assays (neutralization or HAI assays) will examine virus-specific and total antibodies. Serum vitamins A and D will be tested by HPLC and/or tests standardized against HPLC, and with a surrogate retinol binding protein assay.

7.0 EVALUATION CRITERIA

7.1. Toxicity Evaluation Criteria

All adverse events will be graded by the Pediatric Toxicity Grading Tables from the Division of Microbiology and Infectious Diseases (DMID). See Appendix III for details.

7.2. Acceptable Percentage of Missed Doses for Research and Standard of Care Drugs

All eligible participants who receive at least one dose of vaccine will be included in the data analyses.

8.0 CRITERIA FOR REMOVAL FROM PROTOCOL THERAPY AND OFF-STUDY CRITERIA

8.1. Off-study criteria

Completion of study (completion of the final follow-up visit)
Development of Influenza infection and/or receipt of anti-influenza medications during the study period
Death
Lost to follow-up
Request of the Participant/Parent
Discretion of the Study PI, such as the following

- The researcher decides that continuing in the study would be harmful
- A treatment is needed that is not allowed on this study
- The participant misses so many appointments that the data cannot be used in the study
- New information is learned that a better treatment is available, or that the study is not in the participant's best interest
- Study evaluations are complete

9.0 SAFETY AND ADVERSE EVENT REPORTING REQUIREMENTS

9.1. Reporting Adverse Experiences and Deaths to St. Jude IRB

Only "unanticipated problems involving risks to participants or others" referred to hereafter as "unanticipated problems" are required to be reported to the St. Jude IRB promptly, but in no event later than 10 working days after the investigator first learns of the unanticipated problem. Regardless of whether the event is internal or external (for example, an IND safety report by the sponsor pursuant to 21 CFR 312.32), only adverse events that constitute unanticipated problems are reportable to the St. Jude IRB. As further described in the definition of unanticipated problem, this includes any event that in the PI's opinion was:

- Unexpected (in terms of nature, severity, or frequency) given (1) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document, as well as other relevant information available about the research; (2) the observed rate of occurrence (compared to a credible baseline for comparison); and (3) the characteristics of the subject population being studied; and
- Related or possibly related to participation 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. An unanticipated problem involving risk to subjects or others may exist even when actual harm does not occur to any participant.

Unrelated, expected deaths do not require reporting to the IRB. Though death is "serious", the event must meet the other two requirements of "related or possibly related" and "unexpected/unanticipated" to be considered reportable.

Deaths meeting reporting requirements are to be reported immediately to the St. Jude IRB, but in no event later than 48 hours after the investigator first learns of the death.

The following definitions apply with respect to reporting adverse experiences:

Serious Adverse Event:
Any adverse event temporally associated with the subject's participation in research that meets any of the following criteria:

- results in death;
- is life-threatening (places the subject at immediate risk of death from the event as it occurred);
- requires inpatient hospitalization or prolongation of existing hospitalization;
- results in a persistent or significant disability/incapacity;
- results in a congenital anomaly/birth defect; or
- any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition (examples of such events include: any substantial disruption of the ability to conduct normal life functions, allergic bronchospasm requiring intensive treatment in the 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), a congenital anomaly/birth defect, secondary or concurrent cancer, medication overdose, or is any medical event which requires treatment to prevent any of the medical outcomes previously listed.

Unexpected Adverse Event:

- Any adverse event for which the specificity or severity is not consistent with the protocol-related documents, including the applicable investigator brochure, IRB approved consent form, Investigational New Drug (IND) or Investigational Device Exemption (IDE) application, or other relevant sources of information, such as product labeling and package inserts; or if it does appear in such documents, an event in which the specificity, severity or duration is not consistent with the risk information included therein; or
- The observed rate of occurrence is a clinically significant increase in the expected rate (based on a credible baseline rate for comparison); or
- The occurrence is not consistent with the expected natural progression of any underlying disease, disorder, or condition of the subject(s) experiencing the adverse event and the subject's predisposing risk factor profile for the adverse event.

Internal Events:

Events experienced by a research participant enrolled at a site under the jurisdiction of St. Jude IRB for either multicenter or single-center research projects.

Unanticipated Problem Involving Risks to Subjects or Others:
An unanticipated problem involving risks to subjects or others is an event which was not expected to occur and which increases the degree of risk posed to research participants. Such events, in general, meet all of the following criteria:

- unexpected;
- related or possibly related to participation 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. An unanticipated problem involving risk to subjects or others may exist even when actual harm does not occur to any participant.

Consistent with FDA and OHRP guidance on reporting unanticipated problems and adverse events to IRBs, the St. Jude IRB does not require the submission of external events, for example IND safety reports, nor is a summary of such events/reports required; however, if an event giving rise to an IND safety or other external event report constitutes an "unanticipated problem involving risks to subjects or others" it must be reported in accordance with this policy. In general, to be reportable external events need to have implications for the conduct of the study (for example, requiring a significant and usually safety-related change in the protocol and/or informed consent form).

Although some adverse events will qualify as unanticipated problems involving risks to subjects or others, some will not; and there may be other unanticipated problems that go beyond the definitions of serious and/or unexpected adverse events. Examples of unanticipated problems involving risks to subjects or others include:

- Improperly staging a participant's tumor resulting in the participant being assigned to an incorrect arm of the research study;
- The theft of a research computer containing confidential subject information (breach of confidentiality); and
- The contamination of a study drug. Unanticipated problems generally will warrant consideration of substantive changes in the research protocol or informed consent process/document or other corrective actions in order to protect the safety, welfare, or rights of subjects or others.

9.2. Recording Adverse Events and Serious Adverse Events

Adverse events associated with the influenza virus vaccine will not be recorded for this study. Other adverse events will be graded using the Division of Microbiology and Infectious Diseases (DMID) Pediatric Toxicity Tables (Appendix III). All grades of these events will be recorded.

Adverse events will be collected from enrollment through the participant's last visit. Any AE that has not had a stop date recorded by the participant's last visit will continue to be followed-up until an outcome or resolution has occurred. Study participants will be instructed to notify study personnel at any time during the study if they are hospitalized or have any unusual, alarming or unexpected events.

Adverse events will be assessed by the investigator for severity, relationship to the study product, possible etiologies and whether the event meets criteria as a serious adverse event.

The following guidelines will be used by investigators to assess the relationship of an AE to study product administration:

- Definite: Unquestionable relationship. The event was directly attributable to the study product.
- Probable: Relationship is likely. It is likely that the study product caused the event.
- Possible: Relationship may exist. There is at least a reasonable possibility that the event was caused by the study product, although the likelihood is low.
- Unlikely: Relationship is not likely. Although it is unlikely that the study product caused this event, causality cannot be entirely ruled out.
- Unrelated: No relationship. The study product did not cause the event.

Factors considered in assessing the relationship of an AE to study product include:

- the temporal relationship of the event to the administration of study product;
- whether an alternative etiology has been identified; and
- biological plausibility.

9.3. Un-blinding Procedures

The investigator should follow the trial's randomization procedures and should ensure that the code is broken only in accordance with the protocol. The treating investigator should promptly document and explain to the St. Jude Principal Investigator (PI) any premature un-blinding of the investigational agent (e.g., accidental un-blinding, or un-blinding due to a serious adverse event). The study PI determines the attribution and grading of Adverse Events as well as decides, in sponsor-investigator trials, if breaking of a study blind is necessary. In deciding, the St. Jude PI may consider:

- Are there any specific antidotes for the investigational agent or blinded chemotherapy agent(s),
- Are drug-drug interactions expected that may preclude medical intervention,
- Would discontinuation of IND or blinded agent(s) be likely to have an immediate effect?

- Participant and data management
It is recommended that, when possible and appropriate, the blind be maintained for those persons responsible for analysis and interpretation of results at the study's conclusion.

Any toxicities associated or possibly associated with study treatment should be managed according to standard medical practice.

- Accidental Un-blinding
In the event of accidental un-blinding by study personnel, the following should occur:

- Information on accidental un-blinding (including subject number, person(s) involved, information shared, whether the un-blinded person has patient contact) is sent to the St. Jude site Principal Investigator (PI).
- The St. Jude PI provides a summary of the incident to the Biostatistician and the un-blinded study clinical research monitor.
- The protocol deviation will be recorded in a blinded manner and communicated to the primary blinded monitor.
- Blinded site staff will be re-trained by the blinded MONITOR, and un-blinded site staff will be retrained by the un-blinded MONITOR on procedures for maintaining the blind. A summary of the issue and the re-

training will be documented in both the blinded monitoring visit report and the un-blinded monitoring visit report.

- Monitors will ensure appropriate actions are taken (e.g., that the affected study participant is treated according to protocol specifications concerning un-blinding, that a corrective action to prevent unintentional un-blinding is implemented, that the event is recorded in the deviation log in a blinded manner, and that the SMC or other appropriate parties were notified or consulted as applicable)

- Emergency Un-blinding

In cases of patient safety (medical emergency or serious medical condition), the treating physician may request un-blinding a participant randomization code, if knowledge of the treatment assignment is essential for the clinical management or welfare of the participant. Un-blinding of treatment assignment prior to documented disease progression is permitted only for a serious, un-expected adverse event suspected to be study drug-related (as part of the safety reporting process) and if necessary for patient management.

Procedure for Requesting Un-blinding of a Unique Participant Identifier or Randomization Code:

- The study St. Jude PI is the only one who has authority to approve for un-blinding a participant randomization code or identification code.
- In a medical emergency, every effort should be made to consult with the St. Jude PI (insert PI emergency contact phone number) to discuss other options prior to requesting un-blinding of a participant randomization code.
- The treating physician at St. Jude shall make a request to the St. Jude PI.
- The treating physician may hold administration of the investigational agent while waiting for a decision on un-blinding to be made.
- The St. Jude PI may consult with Investigational Pharmacy for un-blinding, if applicable.
- The St. Jude PI will contact the investigational pharmacist to provide the randomization code for the individual participant.
- The un-blinding information for the participant should be limited to as few individuals as possible. The blinded study team and the participant should remain blinded.
- Documentation of the incident will be maintained in the pharmacy binder. All documents associated with the incident should be recorded in a

blinded manner in the EMR and/or other documents, such as, emails, if the participant remains on study and the data will be included in study data analysis.

- If the un-blinding was due to an adverse event, confirm the adverse event was reported and documented in accordance with applicable reporting procedures and timelines.

10.0 DATA COLLECTION, STUDY MONITORING, AND CONFIDENTIALITY

10.1. Data Collection

All study data will be collected by the study staff using designated source documents. Data will be entered and maintained in an institutional relational database with separate backup and monthly off site backup. Standard GCP practices will be followed to ensure accurate, reliable and consistent data collection.

10.2. Study Monitoring

The study team will hold monthly meetings and review case histories or quality summaries on participants.

Source document verification of eligibility and informed consent for 100% of St. Jude participants will be performed by the Eligibility Coordinators within 5 working days of completion of enrollment.

The Clinical Research Monitor will perform monitoring of applicable essential regulatory documentation. Also, reviewing for the timeliness of serious adverse event reporting (type, grade, attribution, duration, timeliness and appropriateness) for selected study participants *semi-annually* and track accrual continuously. The monitor will verify those data points relating to the primary study objective for a certain number of study enrollees as specified in the Moderate Risk monitoring plan checklist for this study. Protocol compliance monitoring will include participant status, safety assessments, eligibility, the informed consent process, participant protocol status, off-study, and off-therapy criteria. The Monitor will generate a formal report which is shared with the Principal Investigator (PI), study team and the Internal Monitoring Committee (IMC).

Monitoring may be conducted more frequently if deemed necessary by the CPDMO or the IMC.

Continuing reviews by the IRB and CT-SRC will occur at least annually. In addition, SAE reports in TRACKS (Total Research and Knowledge System) are reviewed in a timely manner by the IRB/ OHSP.

10.3. Confidentiality

All study procedures will be conducted in private, and every effort will be made to protect participant privacy and confidentiality to the extent possible.

Data collection, process, and administrative forms, laboratory specimens, and other reports will be identified by a coded number only to maintain participant confidentiality. The study database will identify study participants only by the study identification number and will not contain personal health or other identifiers. The list containing the study number and personal identifiers will be maintained in a secured file indefinitely. All study-related information will be stored securely. All participant information will be stored in locked file cabinets in areas with access limited to study staff. All records that contain names or other personal identifiers, such as locator forms and informed consent forms, will be stored separately from study records identified by code number. Databases will be secured with password-protected access systems. Participants' study information will not be released without their written permission, except as necessary for monitoring. Personal identifiers will not be included in any study reports.

All study records will be kept confidential to the extent provided by national and local laws. Authorized representatives of the sponsor(s), and regulatory agencies may examine (and when required by applicable law, to copy) study records for the purposes of quality assurance reviews, audits and evaluation of the study safety and progress.

11.0 STATISTICAL CONSIDERATIONS

11.1. Anticipated Completion Dates

Anticipated Primary Completion Date: 24 months following enrollment of the first participant.
Anticipated Study Completion Date 5 years following approval of the

protocol

11.2. Sample Size

The primary goal of the study is to estimate the effect size of vaccination when incorporating vitamin A/D supplements and standard care. The primary endpoint of interest will be the proportion of 4X increases or conversion from an undetectable to a detectable response in virus-specific antibody after two immunizations (compared to the starting titer) in intervention and control groups. We plan to enroll 40 participants per group in total. Assuming an attrition rate of about 5%, approximately 38 vaccine plus vitamin and 38 vaccine plus placebo participants will provide data for the primary objectives. Because this is a pilot study and there is very limited information about vitamin supplementation with influenza vaccination among children, the sample size was chosen based on past experience with clinical trials of other respiratory virus vaccines among a pediatric population at SJCRH and published literature (39). The sample size proposed will suffice for capturing the response rate difference between groups with a power of over 80% at a significance level of 5%, if the true average response rate overall with the Vitamin A/D supplements is over 62% (EAST 6) while the response rate in the vaccine only group is 30% on average, the majority of the enrolled participants are either vitamin A or D or both deficient as suggested by our experience. If the effect size is smaller, we still believe the estimated effect size with the proposed 80 participants will inform future larger studies.

11.3. Participant Accrual, Follow-up and Retention

Based on previous studies of influenza vaccination among children, the accrual of 100 total and 80 evaluable participants will take approximately 2 years (or 2 flu seasons). Participants lost to follow-up and/or discontinued early from the study will not be replaced. The study team will consider a participant “lost to follow-up” for this trial if he or she has missed 2 consecutive study visits and is unreachable after multiple contact attempts by study staff. However, every effort will be made to complete their regularly scheduled study evaluations. Study staff will target a 95% retention of enrolled participants over the 56 day follow-up period.

11.4. Summary of Primary and Secondary Objectives

For all analyses, all continuous titer measures will be log transformed unless otherwise specified

Analysis of Primary Objectives

In Section 4.1, we have described a way to equally assign enrolled siblings into two arms. By doing this, ignoring the possible nonindependence in siblings when obtaining group difference should still provide a consistent estimate of the group difference. The precision (i.e., the width of the 95% confidence interval) of the estimate may be inflated, while the degree of inflation of the precision depends on the number of co-enrolled siblings, which is not predictable at this stage. Given the fact that this study is a pilot study, we believe in the value of obtaining an accurate group difference estimate, and allow some leeway in precision. More precise estimates and more power could be achieved through future larger studies.

To assess the vaccine-induced and total antibody (IgG and IgA) response and IgA/IgG plus IgA/IgM ratios in the sera at days 28 and 56.

The serum antibody response described by the proportion of 4X increases or conversion from undetectable to detectable response in virus-specific antibody after two immunizations in intervention and control groups will be estimated and 95% confidence interval will be described for both groups, overall and by stratum. The proportion difference with 95% confidence interval will be reported, overall and by stratum. Due to possible variation in virus strains and the influenza virus vaccine formula in different flu seasons, analyses by season will also be described. Continuous titer values in both groups, overall or by stratum, will also be described and compared with point estimate along with 95% confidence intervals.

Titer ratios, such as IgA/IgG and IgA/IgM will be summarized with descriptive statistics, including mean, standard deviation, median and range, for each intervention group, overall or by season. Two-sample tests (t-test or Wilcoxon rank-sum test) will be applied whenever appropriate.

We will also apply generalized estimating equations (GEE) or mixed models to explore titer levels longitudinally.

As sensitivity analysis, similar analytic procedures will be additionally implemented by treating 4X increases or conversion from undetectable to detectable response in virus-specific antibody after two immunizations as an endpoint.

Analysis of Secondary Objectives

To assess the functional antibody response towared the influenza virus vaccine in the sera

Secondary analyses will examine sero-conversion defined as antibody HAI titers of <1:20 converting to ≥1:20, or a four-fold increase in titer for participants with a starting titer of ≥1:20. Sero-conversion rate will be estimated with proportion and 95% confidence interval for both groups, overall and by stratum. The rate difference will be described with point estimate and 95% confidence interval, overall and by stratum. Due to possible variation in virus strains and influenza virus vaccine compositions in different flu seasons, analyses by season will also be described. Continuous titer values in both groups, overall or by stratum, will also be described and compared with point estimate along with 95% confidence intervals.

We will also apply generalized estimating equations (GEE) or mixed models to explore titer levels longitudinally.

Analysis of Exploratory Objectives

To describe the socio-demographic characteristics and nutritional diet among children participating in the trial.

Socio-demographic and dietary intake will be described with tables and descriptive statistics (frequency, proportion mean, standard deviation, median, range, etc…). The anticipated socio-demographic heterogeneity of participants may preclude further analysis, but if feasible, relationships between socio-demographic characteristics and dietary intake with outcomes will be examined.

To assess the feasibility of the food diary among healthy volunteers as measured by completion of the diary.

To assess the feasibility of the food diary among healthy volunteers, the proportion and 95% confidence interval of participants who complete and return the food diary will be calculated overall and at each visit time point.

To monitor serum Vitamin A and D levels at various time points.

Serum Vitamin A and D levels at screening, days 0, 28 and 56 will be monitored, plotted and described at each time point. Summary statistics, such as mean, standard deviation, median and range, will be provided for all time points. If feasible, paired comparisons and longitudinal modeling will be explored.

12.0 OBTAINING INFORMED CONSENT

12.1. Informed Consent Prior to Research Interventions

This protocol, the informed consent document, and any subsequent modifications will be reviewed and approved by the IRB.

The informed consent will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. The consent discussion will take place in a private room and an opportunity to ask questions will be given. A copy of the signed consent form will be given to the participant. The consent process will be documented in the research record.

All research participants who meet eligibility criteria regardless of gender or minority status are fully eligible to participate in this study.

12.2. Consent at Age of Majority

Age of majority does not apply to this protocol since only children between the ages of 2 to 8 years old will be eligible for study participation. Participation should only last approximately 56 days so participants will not reach the age of 18 before completing the study.

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APPENDIX I: SCHEDULE OF EVALUATIONS

Study Visit	Screening	1st Vaccine Visit[a]	2nd Vaccine Visit	Follow-Up Visit (Off-Study)	Early Discontinuation
Study Day (Window)	**-10[a]**	**0**	**28 (+3 days)**	**56 (+/- 7 days)**	
Clinical Evaluations					
Informed Consent	X				
Randomization[d]		X			
Medical History	X	X	X	X	X
Current Medication Use	X	X	X	X	
Socio-Demographics Questionnaire	X				
Food Frequency Questionnaire				X	
Compensation ($50)	X	X	X	X	X
Laboratory Evaluations					
Blood Sample Collection[c]	X	X	X	X	X
Vaccine and Vitamin Administration					
Vaccine Administration		X[b]	X[b]		
Vitamin A and D or Placebo Administration[d]		X	X		
Participant Diaries					
Food Diary		X	X		X

[a] Day 0 (1st Vaccine Dose) must occur within 10 days of screening.
[b] Participants will be observed for 30 minutes post-vaccination.
[c] Serum for Vitamin and Immunologic Studies
[d] Randomization must occur prior to initiating any Day 0 visit evaluations.

APPENDIX II: CLINICAL AND RESEARCH TESTS

No tests and/or evaluations are performed for good clinical care.

All tests and/or evaluations are research tests.

APPENDIX III: TOXICITY GRADING TABLES

A. Division of Microbiology and Infectious Diseases (DMID) Pediatric Toxicity Tables

https://www.niaid.nih.gov/LabsAndResources/resources/DMIDClinRsrch/Documents/dmidpedtox.pdf