

Vitamin D and Vascular Function in Obese Children Clinical Trial Protocol

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Study Title

Vitamin D and Vascular Function in Obese Children

Objectives

The primary objective of this randomized clinical trial is to determine the efficacy of enhanced vitamin D₃ supplementation in improving vascular endothelial function, arterial stiffness, insulin sensitivity, and metabolic syndrome risk status in 10- to 18-year-old vitamin D-deficient (serum 25-hydroxyvitamin D <20 ng/mL) obese or overweight (BMI ≥85th percentile) children; and to assess whether these effects are dose-dependent. As a secondary objective, we will examine the vitamin D supplementation-induced effect on adipokines and inflammatory markers relevant to CVD risk.

Design and Outcomes

In a double-blinded, controlled trial, we propose to randomize 229 eligible children to receive either 600 IU (conventional supplementation, current recommended dietary allowance), or 1000 IU or 2000 IU (enhanced supplementation) of vitamin D₃ daily for six months.

The primary outcome measure will be change in brachial flow-mediated dilation percentage at 6 months (FMD%); FMD% is a measure of vascular endothelial function. The secondary outcome measures will include (1) pulse-wave velocity (PWV) – a measure of arterial stiffness, (2) central and systemic blood pressure, (3) fasting blood glucose concentration, (4) 1/fasting insulin concentration as a surrogate for insulin resistivity, (5) lipid profile indices, (6) adipocytokines (plasma leptin, and adiponectin) and systemic markers of inflammation (plasma high-sensitivity CRP, IL-6, TNF-α).

Eligible participants will be scheduled for enrollment within eight weeks of the initial screening visit. Follow-up visits will occur approximately at three months and six months after enrollment. FMD%, PWV, biochemical measurements, and inflammatory markers will be obtained at each study visit; DXA scan will be done at enrollment and at 6 months.

Interventions and Duration

Enrolled children will receive daily oral supplementation of either 600 IU or 1000 IU or 2000 IU of vitamin D₃ for six months.

Sample Size and Population

We aim to enroll 229 otherwise healthy 10- to 18-year-old, overweight (BMI percentile, ≥85th to <95th) or obese (BMI percentile, ≥95th), vitamin D-deficient (serum 25(OH)D concentration <20 ng/mL) children.

1. STUDY OBJECTIVES

1.1 Primary Objective

To determine whether enhanced vitamin D₃ supplementation in 10- to 18-year-old obese and overweight children with serum 25(OH)D <20 ng/mL results in: (a) improved vascular health (arterial endothelial function and arterial stiffness) and (b) improved insulin sensitivity and metabolic syndrome markers (blood pressure, fasting blood glucose, lipid profile). We hypothesize that vitamin D₃ supplementation with 2000 IU or 1000 IU daily will be more effective than 600 IU in improving vascular health and insulin sensitivity and metabolic syndrome markers.

1.2 Secondary Objectives

To examine whether downregulation of systemic inflammatory markers and adipokines underlie the effects of vitamin D on vasculature and metabolism. We hypothesize that vitamin D effects on vascular health and metabolism may be driven by the attenuation of obesity-associated inflammation.

2. BACKGROUND AND RATIONALE

2.1 Background on Condition, Disease, or Other Primary Study Focus

Childhood obesity is a major public health problem in the United States (US). In 2007-2008, 32% of 2- to 19-year-old US children were either obese (BMI ≥95th percentile) or overweight (BMI 85th - 94th percentile).¹ Obese and overweight children exhibit cardiovascular disease (CVD) risk factors² and have a high risk of metabolic syndrome³ (i.e. large waist circumference, hypertension, fasting hyperglycemia, hypertriglyceridemia, and low levels of high-density lipoprotein (HDL) cholesterol). Obesity and its atherogenic risks track from childhood to adulthood, increasing the risk of adult CVD and pointing to the need for developing primary prevention strategies in affected children.

2.2 Study Rationale

Vitamin D deficiency is emerging as an independent risk factor for CVD and metabolic syndrome in children⁴⁻⁷ and adults.^{8,9} Our preliminary work shows that obese children have an increased risk of vitamin D deficiency; hypovitaminosis D is also associated with other CVD risk factors, such as low HDL cholesterol and lower insulin sensitivity in youth.^{10,11} Accordingly, optimization of vitamin D status in obese children might improve their CVD risk profile and reduce their risk of later CVD. As vitamin D is a potent immune modulator, its cardiovascular and metabolic benefits might be mediated, in part, through downregulation of inflammatory mediators, including adipokines.

In the proposed study, we will test the central hypothesis that enhancement of vitamin D status in otherwise healthy obese and overweight vitamin D-deficient children will improve their vascular health and their CVD and metabolic syndrome risk profile.

3. STUDY DESIGN

We propose a double-masked, randomized, controlled clinical trial of enhanced vitamin D₃ supplementation (600 IU vs 1000 IU vs 2000 IU) for six months in 10- to 18-year-old, overweight (BMI percentile, ≥85th to <95th) or obese (BMI percentile, ≥95th), vitamin D-deficient (serum 25(OH)D concentration <20 ng/mL) children. Children who seek care at the Primary Care Centers of CHP (Oakland, Mercy Hospital, and Turtle Creek) or at affiliated PittNet offices (Practice Based Research Network, PBRN) will be eligible to participate. Our primary aim is to determine whether enhanced supplementation with vitamin D₃ (1000 IU or 2000 IU) compared to the standard of care vitamin D₃ supplementation (600 IU), taken orally once daily, is effective in improving brachial FMD% by 25% or more at the end of six months. Secondary outcomes will determine effects of vitamin D supplementation in reducing arterial stiffness, improving insulin sensitivity, and reducing metabolic syndrome risks. We will also characterize the mechanistic effects of vitamin D on systemic inflammatory markers and adipokines relevant to CVD risk.

4. SELECTION AND ENROLLMENT OF PARTICIPANTS

Children who seek care at the Primary Care Center of CHP Oakland or at affiliated PittNet offices (Practice Based Research Network, PBRN) will be eligible to participate.

Eligible children will meet the following inclusion and exclusion criteria

4.1 Inclusion Criteria

Potentially eligible children will be

- 10 to 18 years of age
- otherwise healthy
- obese or overweight (BMI ≥85th percentile)
- able to understand all aspects of study procedures
- able to voluntarily comply with all study procedures across the duration of the study
- vitamin D-deficient and normocalcemic during screening (serum 25(OH)D concentration <20 ng/mL; serum calcium concentration: 10-14 years, 8.8-10.8 mg/dL; ≥15 years 8.4–10.2 mg/dL)

4.2 Exclusion Criteria

Children will be excluded from the study if they meet any of the following exclusion criteria:

- Have hepatic or renal disease, metabolic rickets, malabsorptive disorders, primary hyperparathyroidism, hyperthyroidism, previous history of cancer or other chronic disorders that could affect vitamin D or glucose metabolism
- Are receiving treatment with anticonvulsants, systemic glucocorticoids, pharmacologic doses of vitamin D (≥1000 IU of vitamin D₂ or D₃ daily), antihypertensives, vasoactive drugs, antilipidemics, metformin, antipsychotics, other oral insulin regulators, or cancer treatments
- Have cholelithiasis or urolithiasis

- Have type 1 or type 2 diabetes
- Have a condition or underlying abnormality that could compromise the safety of the subject during study procedures (e.g. sickle cell trait/disease)
- Found to be hypercalcemic during screening visit (serum calcium >10.8 mg/dL in 10- to 14-year-old children; >10.2 mg/dL in ≥15-year-old children)
- Significant fasting hyperglycemia (fasting blood glucose concentration ≥125 mg/dL) during the enrollment visit
- Post-menarchial girls with a positive pregnancy test at enrollment will be excluded; however, contraception is not required for post-menarchial girls

4.3 Study Enrollment Procedures

Participants will be recruited from the Primary Care Center of CHP Oakland or at affiliated PittNet offices. In addition to the two recruitment locations, participants may also be recruited through various community outreach programs. The study will also be advertised through the University of Pittsburgh Pitt+Me database to strengthen our recruitment effort.

Potential participants among children attending the CHP Oakland Primary Care Center will be screened for eligibility by reviewing their EPIC electronic medical records. Subjects who are not excluded during the preliminary EPIC chart review will be approached for screening. Reasons for inclusion and exclusion will be electronically documented on a screening form in the study-specific electronic database administered by the University of Pittsburgh Center for Research on Health Care (CRHC).

Written informed consent from a biological or legally-adoptive parent and participants' assent, will be obtained prior to enrollment (both screening and enrollment) of 10- to 17-year-old children. Participants who are 18 years of age can consent for themselves for both screening and enrollment. Legal guardians will have to provide documentation of guardianship from relevant agencies and/or courts.

Participants found to be vitamin D-deficient during screening, and thus eligible for study enrollment, will be randomized to one of three vitamin D supplementation doses (600 IU, 1000 IU, or 2000 IU). The randomization codes will be assigned electronically through the CRHC study database using a block randomization scheme set forth by the study statistician prior to the onset of the study. Both participants and study investigators will be blinded to the assigned supplementation dose throughout the trial.

5. STUDY INTERVENTIONS

5.1 Interventions, Administration, and Duration

Enrolled study participants will be randomized to one of three vitamin D supplementation doses: 600 IU (conventional supplementation), 1000 IU, or 2000 IU (enhanced supplementation). Assigned study vitamin D supplement bottle will be dispensed to the participants at enrollment and 3-month follow-up visit. Supplementation bottles will be given with Medical Event Monitoring System caps (MEMS, manufactured by AARDEX Group Ltd., Sion, Switzerland) for assessing and enhancing adherence. Participants will be instructed to take only one study vitamin D tablet daily for the duration of the study, and to not double their dose if a

previous dose was missed.

5.2 Handling of Study Interventions

Study vitamin D supplements will be manufactured by Douglas Laboratories in Pittsburgh, PA. The procurement of the required tablets will be coordinated in conjunction with the UPMC Investigational Drug Service Research Pharmacist. Study vitamin D supplements will be directly shipped to the research pharmacist who will ensure appropriate masking, labeling, bagging, and coding of the supplement bottles. The study vitamin D supplements will be identical in color, taste, and size. Each dispensed bottle will contain 105 pills.

The coded study vitamin D supplement bottles will be stored in a locked cabinet in the UPMC Osteoporosis Prevention and Treatment Center in the Kauffman Building. Once, the trial is completed, all excess vitamin D supplements will be returned to the UPMC Investigational Drug Service research pharmacist for discarding.

If multiple children of the same household enter the study, the dispensed supplement bottles will be color-coded to ensure identification of their respective medication.

5.3 Concomitant Interventions

Allowed Interventions

For the duration of the study, participants are permitted to take any medications that are not included in the list of prohibited interventions.

Required Interventions

Participants will be required to take one study vitamin D₃ supplement tablet daily for the duration of the study (six months), beginning on the day of their enrollment.

Prohibited Interventions

Participants are expected to not take the following medications during their participation in the study. If these medications are clinically necessary and prescribed by a provider, the participant may be withdrawn from the study based on the PI's discretion.

- Multivitamins
- Anticonvulsants
- Systemic glucocorticoids
- Antipsychotics
- Antihypertensives
- Vasoactive drugs
- Antilipidemics
- Metformin or other oral insulin regulators
- Chemotherapeutics or other cancer treatments

5.4 Adherence Assessment

Adherence to the vitamin D supplement will be measured using MEMS cap, pill count, and the medication calendar log. Each supplement bottle will be equipped with a MEMS cap, which will be activated when the study supplement bottle is dispensed during enrollment. Study staff will assess the adherence measures (MEMS cap, pill count, and medication calendar) during follow-up. To promote valid MEMS cap data, subjects will be advised to take their study vitamin D supplement once daily directly from the dispensed bottle—preferably around the same time each day. Use of other medication storage devices or dispensers will be discouraged.

6. STUDY PROCEDURES

6.1 Schedule of Evaluations

Assessment	Screening (Visit 0)	Enrollment (Visit 1)	3-month Follow-Up (Visit 2)	6-month Follow-Up (Visit 3)
Inclusion/Exclusion Criteria	x			
Informed Consent/Assent Form	x	x		
Demographics	x			
Medical History	x	x	x	x
Anthropometric Measurements	x	x	x	x
Skin Color Assessment	x			x
Short Vitamin D and calcium intake FFQ & Sunlight Exposure Questionnaire	x			x
Blood Chemistries	x	x	x	x
Urine Pregnancy Screen*		x		x
Brachial FMD		x	x	x
Pulse Wave Velocity/Analysis		x	x	x
Dual-Energy X-ray Absorptiometry (DXA) Scan		x		x
Activity Survey		x		x
Youth and Adolescent FFQ		x		x
Pubertal Self-Assessment (Tanner Stage)		x		
Dispense Study Vitamin D Supplement		x	x	
Medication Adherence			x	x

*Only post menarchial girls require a urine pregnancy screen as a safety requisite for DXA scan

6.2 Description of Evaluations

Potentially eligible patients passing the inclusion and exclusion criteria will be screened. Screened participants who are found to be vitamin D-deficient (serum 25(OH)D concentration <20 ng/mL) will be eligible to enroll in the study. Eligible participants will be scheduled for enrollment visit within eight weeks of their screening evaluation. Study duration is six months. Randomized participants will return for follow-up evaluations twice. The first follow-up visit will

occur approximately three months after their enrollment visit, and their final study visit will be scheduled approximately three months thereafter.

Screening Evaluation

Consenting Procedure

Written informed consent will be obtained separately for screening and for enrollment, at the respective study visits. The parent or medical legal guardian of the child (as defined by state law) will be given a copy of the informed consent and will be provided an opportunity to read the consent in its entirety. For a legal guardian to consent on behalf of a child to participate in research, the guardian must present a copy of the appropriate legal document proving guardianship under state law before signing the consent document. A copy of the guardianship document will be included in the research subject's chart.

The consent form given at the screening visit will describe the procedures that will take place only during the screening visit. The consent form given at the enrollment visit will detail all procedures to be performed at each study visit. Participants must give their assent and only biological parents, or legally-recognized guardians, may provide consent.

Only trained study coordinators and the principal investigator may obtain informed consent from participants. Person obtaining consent will review all aspects of the consent and allow the parent or legal guardian the opportunity to ask questions. The following points will be explained to the parent(s) or legal guardian(s) at the time of consent:

- The study involves research
- The purpose, background and rationale of the study
- Study involves taking vitamin D supplementation (Vitamin D₃ 600 IU vs. 1000 IU vs. 2000 IU once daily for six months) which has been masked and randomly assigned
- The responsibility of the parent and the participant
- All reasonably foreseeable risks or inconveniences associated with all the expected study procedures (i.e. vascular function tests, DXA scan, blood draw, skin color estimation, completion of physical activity, diet and sun exposure surveys)
- Potential risk of hypervitaminosis D (vitamin D poisoning) and its symptoms
- The expected benefits of the study
- The participation in this study is voluntary and that the parent or subject may refuse to participate or withdraw from the study at any time without penalty of loss of benefits to which he/she may be entitled
- The accessibility of the records to the regulatory authority(ies), monitor(s), auditor(s), or other appropriate reviewers
- Maintenance of confidentiality of the patient record

The consent form will include: the parent(s) or legal guardian(s) signature, the printed name of the child, and the date, as to document that all-of-the-above was completed. Participants who are 18 years of age will sign their own consent. The consenting research staff member will also sign the consent form to indicate that he/she has reviewed and explained the consent form with the subject and was present at the time of signing. After obtaining signed

consent, a copy of an unsigned consent form will be given to the parent(s) or legal guardian(s) for their records. The original signed copy of the consent will be kept within the study folder.

Screening

The following procedures are performed to determine a subject's eligibility:

- *Age Calculation:* To enter the study children should have completed their 10th birthday and not have had their 19th birthday. 18-year-old children are eligible to enroll until the day before their 19th birthday.
- *BMI Calculation:* Height will be measured three times in centimeter (cm) to the nearest 0.1-cm and averaged to determine the participant's height in centimeter. Weight will be measured three times in kilogram (kg) to the nearest 0.1-kg and averaged to determine the participant's weight in kilogram. BMI will be calculated using the following formula:

$$[\text{Weight in kilogram}]/[\text{Height in meter}]^2$$

BMI can also be calculated using metric measurements of height (in cm) and weight (in kg) through NHLBI online BMI calculator available at:

<http://nhlbisupport.com/bmi/bminojs.htm>

- *Medical History Form:* Captures anthropometrics, demographic data (age, sex, race, and ethnicity), level of education, smoking status, current medications, and type of medical insurance.
- *Contact Form:* To be completed by parent unless subject is 18 years of age
- *Vitamin D and Sunlight Exposure Questionnaire:* To be completed by parents unless subject is 18 years of age
- *Waist Circumference Measurement:* Waist circumference will be measured three times, using a non-stretch tape measure, at the mid-point between the lowest rib and the iliac crest at minimal respiration while standing. The respective waist circumference measurements will be measured to the nearest 0.1 cm and averaged.
- *Fitzpatrick Sun-reactive Skin Type estimation:* Sun-reactive skin type estimate will be made by the parent unless the subject is 18 years of age
- *DSM II Colormeter Measurement:* Study staff will normalize the dermatophotospectrometer on the calibration plate and obtain three readings from the forehead, back of the hand, and inner upper arm. The measurements obtained at each site will be averaged to estimate the melanin index at the respective sites.
- *Venipuncture, Specimen Collection, and Specimen Processing:*
 - *Venipuncture:* Venipunctures at the Primary Care Center will be done by the clinic nurses or phlebotomy-trained medical assistants. For subjects consenting for screening at the Pediatric PittNet practices, venipuncture will be performed by the pediatric PittNet nurse, the clinic nurse, or the phlebotomy-trained medical assistants

- *Specimen Collection:* At least 5 mL of blood needs to be drawn in one or two red-top tubes and 10 mL of blood in one or two green-top tubes
- *Specimen Processing:* Label the specimens with screening ID, date of birth, and date of visit. Complete lab test order forms (instructions for lab procedures). Enclose the collected tubes in a medical-biohazard seal bag with the lab order/procedure instruction sheets and drop-off the specimens at the appropriate place in the UPMC Central Lab building or in the clinic for transportation of the samples to the Clinical Chemistry Lab at UPMC Presbyterian.

Enrollment

Enrollment

Participants that are deemed vitamin D deficient (serum 25(OH)D concentration <20 ng/mL) and meet all other inclusionary criteria are eligible to enroll in the study. Prior to entering the study (initiation of baseline assessments and randomization), participants will be required to complete a second informed consent, which outlines the assessments that will take place at the three study visits.

Baseline Assessments at enrollment

Patients who have been successfully screened for the study and have enrolled will undergo the following baseline assessments:

- *Medical history form*
 - *Anthropometric measurements*
 - *Venipuncture, specimen collecting, and specimen processing*
 - *Urine pregnancy screen:* Only post-menarcheal girls require urine pregnancy screen. Only subjects with a negative pregnancy screen will be permitted to enroll.
 - *Fasting glucose concentration screen:* Only participants with fasting serum glucose <125 mg/dL will be permitted to enroll
 - *Activity survey:* Participant may complete this survey, with parental help if they are ≤17 years of age.
 - *Nutritional survey:* Parent may complete a survey regarding subject's food frequency habits for participants ≤17 years of age. If subject is 18 years of age or older, he or she will complete this questionnaire on their own.
 - *Pubertal self-assessment:* Subjects will self-identify their pubertal stage by looking at Tanner Stage drawings and selecting the image that corresponds most closely to their stage of development.
 - *Vascular function testing (pulse wave velocity, pulse wave analysis, and flow mediated dilation, and blood pressure measurements)*
- *Blood pressure.* Measurements will be obtained by a CTSC vascular technician prior to vascular health assessments, and after 10 minutes of participant rest and acclimatization while supine, using a model CONTEC08A automated digital oscillometric device (cuff 22-32 cm). Measurements will be taken three times, one minute apart, and averaged.

- Endothelial function. Brachial artery diameter will be measured using a high-resolution ultrasound machine (GE, Vivid 7, GE Health care, Milwaukee, WI) equipped with a 9-L linear transducer preset to a dedicated vascular scanning protocol. We will then occlude brachial arterial flow at the upper forearm using a 5-cm-wide occlusive cuff (Hokanson SC5) inflated to a pressure of 50 mmHg above the systolic blood pressure or to 200 mmHg, whichever is greater, by a rapid release sphygmomanometer (Hokanson DS 400) for 5 minutes. Post-cuff release diameter measurements during the reactive hyperemic phase, obtained at 60, 120, and 180 seconds, will be used to calculate FMD%.
- Arterial Stiffness Indices. Carotid-femoral pulse wave velocity (PWV), Aortic Augmentation Index at heart rate of 75/min (Alx-75), and central systolic and diastolic blood pressure were measured using arterial tonometry (SphygmoCor CVMS V9, CPVH System, Model EM3, AtCor Medical, Sydney, Australia).
- *DXA scan:* Measures total body adiposity and percent of body fat

Participants should enroll within two months of initial screening. If participants do not enroll within this time window, they will be required to rescreen to ensure eligibility. Subjects that have successfully completed the enrollment process and baseline procedures will be randomized to a study intervention dosage, which is blinded to both study team and participant.

Parents will be instructed to record each dose of intervention given to their child in a calendar supplied by the investigators.

3-month Follow-up Visit

The window for the follow-up visit will be three months after the enrollment date (± 15 days). The following procedures will be performed at this visit:

- Medical history form
- Anthropometric measurements
- Venipuncture, specimen collecting, and specimen processing
- Vascular function testing (pulse wave velocity, pulse wave analysis, and brachial flow mediated dilation, and blood pressure measurements)
- Medication adherence assessment
 - Collect medication calendar
 - Read MEMs cap and obtain Patient Adherence Report
- Dispensation of second supply of vitamin D supplements

Final Visit (6-month Follow-up Visit)

The window for the final visit will be three months after the 3-month follow-up date (± 15 days). The following study procedures are performed at this visit:

- Medical history form
- Anthropometric measurements

- Venipuncture, specimen collecting, and specimen processing
- Urine pregnancy screen (only post-menarchial girls require urine pregnancy screen). Subjects with a positive pregnancy screen will not undergo a DXA scan but can complete the remaining study procedures if they are willing.
- Activity survey
- Nutritional survey
- Vitamin D and Sunlight Exposure Questionnaire
- DSM II Colormeter Measurement: Will be measured at similar body sites as the baseline testing
- Vascular function testing (pulse wave velocity, pulse wave analysis, and brachial flow mediated dilation, and blood pressure measurements)
- DXA scan
- Medication adherence assessment
 - Collect medication calendar
 - Read MEMs cap and obtain Patient Adherence Report

7. SAFETY ASSESSMENTS

7.1 Specification of Safety Parameter

- Serum calcium levels will be monitored for participant safety. Serum calcium >10.8 mg/dL in 10- to 14-year-old children and >10.2 mg/dL in ≥15-year-old children will prompt further evaluation. An elevated serum calcium level will be repeated and if still abnormal, study medication will be discontinued. Children who discontinued study medication due to hypercalcemia will have serum 25(OH)D measured to rule out vitamin D toxicity (levels ≥100 ng/mL) and will be referred to a pediatric endocrinologist for further evaluation and management. Children with hypercalcemia and normal serum 25(OH)D levels (<100 ng/mL) will be also be referred for further evaluation to their primary care provider and a pediatric endocrinologist to rule out other unusual causes of hypercalcemia (chronic granulomatous disorders, primary hyperparathyroidism, familial hypocalciuric hypercalcemia).
- Significant fasting hyperglycemia (fasting blood glucose >125 mg/dL) during enrollment visit will disqualify subject from enrolling in the study.
- Post-menarchial girls that test positive during pregnancy screen at the enrollment visit will be excluded. Positive screens in the final visit will disqualify subject from getting a DXA scan but will not exclude subject from completing the study.
- At the 3-month follow-up and the final visit, the MEMS cap data will be downloaded to assess adherence.

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

Serum calcium will be measured at the screening visit and only children with normal calcium results for age will be eligible to enter the study. Upon enrollment, serum calcium will be monitored for safety at the 3-month and 6-month follow-up visits, and children found to have elevated serum calcium (>10.8 mg/dL in 10- to 14-year-old children and >10.2 mg/dL in ≥15-year-old children) will be further managed as stated above in section 7.1.

7.3 Adverse Events and Serious Adverse Events

An adverse event is any unintended medical occurrence (symptom, diagnosis, sign, abnormal laboratory finding, or missed study procedure) that occurs during the study that was not present at baseline.

- Any medical condition present at the time of enrollment is considered baseline, and not reported as an adverse event.
- If the severity of a pre-existing medical condition worsens at any time during the study participation, it will be considered an adverse event.
- All adverse events will be assessed by the Principal Investigator to determine the severity of the event (mild, moderate, severe, life-threatening) and whether the event is related to the study product or study intervention
- All AEs will be recorded in the eCRF in the study database. The event description, date of onset and study clinician's assessment of severity and relationship to study product, date of resolution and outcome will be recorded. To elicit any adverse event, the parent will be prompted at each encounter to report any change in their child since the last contact.

A serious adverse event (SAE) is any unintended medical occurrence in which the patient outcome is death or life-threatening hospitalization, disability, or incapacity.

- In the event of an SAE, the SAE eCRF must be completed by the research coordinator and the details of the relevant SAE should be notified to the PI (Dr. Rajakumar) immediately. The event description, date of onset and study clinician's assessment of severity and relationship to study product, and outcome of event will be recorded.
- SAEs will be logged in the electronic database and will be periodically monitored by the study statistician.

7.4 Reporting Procedures

- All AEs will be reported to the Data and Safety Monitoring Board annually.
- Unanticipated protocol-related problems will be notified by the PI (Dr. Rajakumar) to the IRB as and when they occur
- All SAEs must be reported by the research staff to Dr. Rajakumar, the study principal investigator, immediately. Principal investigator will report SAEs to the University of Pittsburgh Institutional Review Board (IRB) according to the local IRB requirements. SAEs related to the research intervention will be notified by the PI (Dr. Rajakumar) to the IRB within 24 hrs. AEs of moderate severity related to the research intervention will be notified by the PI (Dr. Rajakumar) to the IRB within 5 days.

7.5 Follow-up for Adverse Events

- Follow-up of a non-serious adverse event of mild or moderate severity will end when the study reporting period ends [i.e., 6-month follow-up visit (final study visit)]. Study staff will keep apprised of subject's adverse event until the parent/subject provides a date of resolution for the AE; resolution date will be documented in the CRHC database.

- Follow-up for a non-serious adverse event of severe nature will be the following:
 - At the time of the event, the investigator will provide appropriate treatment or follow up, including communicating the event to the child's primary care provider and/or providing a referral to a specialist.
 - The staff will place a follow up phone call to the parent, regardless of the study reporting period, to assure that the child is improving or that the family has connected with the suggested referral. Study staff will keep apprised of subject's adverse event until the parent/subject provides a date of resolution for the AE; resolution date will be documented in the CRHC database.

7.6 Safety Monitoring

- Members of the Data and Safety Monitoring Board will evaluate participants' risks (adverse events) vs. the benefits and other factors that can affect study outcome.
- Real-time reports of SAEs will be sent to the Data and Safety Monitoring Board as determined by the principal investigator.
- We will appoint an Independent Safety Monitor (ISM) who will have expertise in pediatrics and whose primary responsibility will be to provide timely safety monitoring, to ensure good clinical practice and to quickly identify safety concerns. Safety reports by the ISM will be submitted on a regular basis, (e.g., real time, weekly, monthly, quarterly) as determined by the DSMB. To minimize bias, the ISM will evaluate SAEs masked to treatment assignment, unless the DSMB approves partial or complete unmasking. The ISM will participate for the duration of the study.
- The ISM will be able to readily access participant records. The primary focus of the ISM is to independently review SAEs that may be associated with study treatment as well as adverse events of special interest. The ISM will investigate any events considered serious and unexpected. The ISM will be in communication with Dr. Rajakumar and the DSMB chair for any event that needs further evaluation. The ISM will not be directly involved in the trial, will not be under the investigator's supervision, and will have no financial, intellectual, proprietary or professional interest in the outcome of the trial.

8. INTERVENTION DISCONTINUATION

To ensure children's safety, we will use a serum calcium cutoff of >10.8 mg/dL in 10- to 14-year-old children and >10.2 mg/dL in ≥15-year-old children to prompt further evaluation. An elevated serum calcium level will be repeated and if it remains abnormal, study medication will be discontinued, and participant will be withdrawn from the study protocol. Children who discontinued study medication due to hypercalcemia will have serum 25(OH)D measured to rule out vitamin D toxicity (levels ≥100 ng/mL) and will be evaluated by a pediatric endocrinologist. Children with hypercalcemia and normal serum 25(OH)D levels (<100 ng/mL) will be referred for further evaluation to their primary care provider and a pediatric endocrinologist to rule out other unusual causes of hypercalcemia (chronic granulomatous disorders, primary hyperparathyroidism, familial hypocalciuric hypercalcemia).

9. STATISTICAL CONSIDERATIONS

9.1 General Design Issues

In this randomized, controlled, double-blinded clinical trial, eligible children will receive in a 1:1:1 ratio either 600 IU or 1000 IU or 2000 IU vitamin D3 supplementation orally once daily for six months. Our primary outcome measure will be brachial FMD at 6-month follow-up. A 25% or more improvement in brachial FMD% in the 1000 IU or the 2000 IU group when compared to the 600 IU group, after six months of vitamin D replacement will be considered significant.

Our secondary outcomes will include: (1) pulse wave velocity (PWV) as a measure of arterial stiffness, (2) 1/fasting insulin concentration as a surrogate for insulin sensitivity, and (3) metabolic syndrome risk factors (blood pressure, waist circumference, HDL cholesterol, triglycerides, and fasting blood glucose) at the 6-month follow-up.

9.2 Sample Size and Randomization

Our sample size estimates are based on showing a relative improvement in baseline brachial artery flow mediated vasodilation (FMD, %) of at least 25% in children receiving each of the higher doses of vitamin D3 (1000 IU and 2000 IU) compared with no improvement in the 600 IU group after six months of treatment. We assumed the baseline FMD was ~6.5% based on previous studies in overweight children similar to our projected population.¹²⁻¹⁴ A sample size of 56 in each treatment group at the end of the trial would have 84% power (two-sided two-sample comparison of means, $\alpha=0.025$) to detect this clinically important difference of 25% relative improvement (absolute improvement= $25\% \times 6.5\% \approx 1.6\%$) assuming a standard deviation of 2.6% (conservative estimates based on published literature).^{12,14} To provide for a projected drop-out rate of 25% by the end of the trial, we propose to recruit 225 children into the trial. We have over 90% power to detect a dose-response effect by testing for linear trend assuming a 0%, 20%, and 30% improvement in vascular health among children receiving 600 IU, 1000 IU, and 2000 IU, respectively.

Our original sample size of $n=252$ was based on 90% power and 20% attrition to allow for subgroup analyses by race. In December of 2015, the PI and funder decided to re-evaluate the targeted sample size due to slower than expected recruitment. Accordingly, with approval from NHLBI, our accrual target was revised from 252 participants to 229 participants based on projected enrollment and power of at least 80% to detect the original effect size with 25%-30% attrition. The lower sample size of $n=225$ would still allow for detection of clinically significant effect of vitamin D supplementation. However, the proportion of African Americans in the recruited sample was much larger than anticipated; the small number of Caucasian subjects in the cohort precludes doing race-stratified analysis. For the secondary outcomes, we have 89% power to detect effect sizes of 0.61 or greater without adjustment for multiplicity.

Treatment Assignment Procedures

In this randomized, controlled, double-blinded clinical trial, eligible children will be randomly allocated, using a permuted block design, in 1:1:1 ratio, to receive either conventional (600 IU)

or enhanced (1000 IU or 2000 IU) vitamin D₃ supplementation orally once daily for six months. Study supplement bottles were masked and labeled by the research pharmacist with appropriate randomization codes. At enrollment, the CRHC database assigned the randomization code based on the permuted block scheme.

Study participants or investigators will not have access to the assigned doses until the end of the clinical trial [timing of planned breaking of randomization code]. Unmasking may occur in the instance of an SAE. The ISM may divulge the masked dosage to the PI or relevant clinicians who are treating the patient.

9.3 Interim analyses and Data and Safety Monitoring Plan

No interim efficacy analysis has been planned. Dr. Moore will work with Dr. Rajakumar to prepare a DSMB report addressing specific concerns that she anticipates the DSMB may have regarding the conduct of the study. This will be distributed to DSMB members along with the Open Session report. Likewise, Dr. Moore's report for the Closed Session will usually contain an assessment of the progress of the trial, including recommendations as to whether it should be terminated or modified. Interim data reports will generally include the following types of information, although only the Closed Session data reports will include comparisons by treatment group:

- Monthly and cumulative accrual compared with targets
- Baseline characteristics, overall and by treatment group
- Completeness and quality of data collection forms
- Status of enrolled participants, overall and by treatment group
- Participants' off-protocol treatments
- Compliance with eligibility criteria and other protocol requirements
- Subject adherence to the treatment regimen, overall and by treatment group
- Individual SAEs by subject ID number and a table of event-specific cumulative rates, overall and by treatment group

Dr. Moore will work to ensure that all the information presented at the DSMB meetings remains confidential. Each report will be marked as such. At the end of each DSMB meeting, all reports will be collected and destroyed. At the end of the meeting, the DSMB will make recommendations regarding the continuation of the trial. Dr. Rajakumar will implement the DSMB recommendations. Dr. Rajakumar will also submit the DSMB recommendations to the IRB.

9.4 Outcomes

Primary outcome

Our primary outcome measure will be change in mean FMD% at 6-month follow-up. A 25% or more improvement in FMD% in the 1000 IU or 2000 IU compared to the 600 IU group after six months of vitamin D replacement will be considered significant.

Secondary outcome

Our secondary outcomes will include: (1) pulse wave velocity (PWV) as a measure of arterial stiffness, (2) 1/fasting insulin concentration as a surrogate for insulin sensitivity, and (3) metabolic syndrome risk factors (blood pressure, waist circumference, HDL cholesterol, triglycerides, and fasting blood glucose) at the 6-month follow-up.

9.5 Data Analyses

Our primary analysis will use children's original treatment assignment regardless of adherence (intention to treat). We will begin by calculating descriptive statistics for baseline demographics and clinical characteristics by vitamin D treatment groups (600 IU vs 1000 IU vs 2000 IU). Our primary outcome measure of vascular health is brachial artery FMD expressed as a percentage. We will use mixed-effects models with fixed effects of time (baseline, 3 months, and 6 months), the treatment*time interaction, and random subject effects to test for differential changes in FMD over time and differences in FMD at six months. We are primarily interested in the comparisons of each higher dose (1000 IU and 2000 IU) to the lower dose (600 IU) at six months ($\alpha=0.025$ for each comparisons). The mixed-effects model will allow unbiased estimation of the treatment effects at six months even if some 6-month data are missing because of the incorporated dependence among baseline, 3-month and 6-month assessments (assuming data are missing at random). We will test the main effect of vitamin D supplementation (three groups) at 3 and 6 months and pairwise comparisons of the groups receiving the larger doses of vitamin D with the group receiving 600 IU. If differences are detected across the 3 groups, we will test for a dose response in FMD across doses by using a linear contrast of coefficients test ($\alpha=0.05$). We will use the same methodology to test the effects of higher doses of vitamin D on pulse-wave velocity, our primary measure of arterial stiffness.

The second part of our primary analysis of our trial is to determine whether higher doses of vitamin D will result in improvements in insulin sensitivity and metabolic syndrome markers over time. We will use mixed-effects models with fixed effects of time (baseline, 3 months, and 6 months), and the treatment time interaction with random subject effects to test for differential changes in secondary outcomes over time. We will test the main effect of treatment (three groups) at 3 and 6 months and pairwise comparisons of the groups receiving the larger doses of vitamin D with the group receiving 600 IU. All tests will be two sided with $\alpha=0.05$.

We will explore the mediating effects of inflammatory markers (hs-CRP, IL-6, TNF- α) and adipokines (leptin and adiponectin) on the temporal relationship between vitamin D and vascular health. We hypothesize that vitamin D effects on vascular health may be driven by inflammatory processes and adipokines. An analysis that would complement this hypothesis would be a mediation analysis where we demonstrate that the effect of vitamin D on vascular health is partially mediated by our measures of inflammation and adipokines. We propose to use single-mediator and multiple-mediator methods incorporating product of coefficients to test for mediation.¹⁵ For the simple single-mediator analysis, we first must demonstrate that the high dose vitamin D has a significant effect on each mediator (inflammatory markers and adipokines) at three and six months using a general linear model. We will then test whether 6-month vascular health is correlated with each mediator. Both tests would have to be significant for there to be evidence of mediation. We will estimate the mediated effect by taking the product of the following coefficients: 1) the coefficient for the intervention effect on the mediator, and 2) the coefficient for the mediator in a model testing the intervention effect on

vascular health controlling for the potential mediator. This estimate will provide the reduction in the effect of vitamin D on vascular health when adjusted for the mediator. The confidence interval for the mediated effect will be calculated using bootstrap methods. Since our vitamin D intervention is hypothesized to target multiple mechanisms, we will test for a multiple-mediator model after single mediation is thoroughly investigated.

Missing Data

We do not expect substantial missing item-response data due to the electronic nature of data collection. Based on our pilot work, we anticipate <20% attrition at the 6-month assessment, and we have accounted for this in sample-size analyses. Participants who wish to discontinue the study drug will be asked to continue to participate in all follow-up visits; those who no longer wish to participate in the study will be considered dropouts, and we will monitor these events. We will obtain reasons for dropout in case it appears possible to modify the protocol so as to increase retention. At the end of the study, we will compare baseline characteristics and treatment arms between participants who have received 3- and 6-month assessments and participants who have not, to learn what factors are associated with missing visits.

For participants with missing 6-month outcome data, we will conduct several sensitivity analyses to test the impact on our study conclusions. The methods proposed assume that the data are missing at random (MAR), where the missing data mechanism is related to observed data, not the unobserved values. Although this assumption is difficult to confirm, our sensitivity analyses will allow us to assess the robustness of our findings and threats to the MAR assumption. Given that our outcomes are highly mechanistic and less patient-relevant symptomatic/functional outcomes, missing data at 6 months would likely be related to baseline characteristics or 3-month values. As mentioned previously, we will use linear mixed models for analysis, since these models are more robust to missing data than traditional multivariate models or single-point-in-time ANOVA. From these models, we can estimate the treatment impact at 6 months using the covariance structure of the repeated measurements. This approach will be compared to a complete case analysis (missing complete at random) and a nonignorable missing data method (pattern mixture modeling). These different methods should result in consistent qualitative results if our findings are robust to missing data.

10. DATA COLLECTION AND QUALITY ASSURANCE

10.1 Data Collection Forms

Data based on source documents (eligibility, anthropometrics, demographics, etc.) will be assessed and entered by the research coordinator. Data collected in paper records (skin-typing, sun exposure history, DSM II color-meter readings, pubertal self-assessment, lab results, adverse events, etc.) will be entered into the CRHC study database, and data with electronic outputs (vascular function results and adherence data from MEMs cap) will be merged into the CRHC study database. Tracking reports will be developed to assess recruitment, participant status, upcoming visits, and outstanding assessments. Automated reports for data and safety monitoring will also be developed.

All paper records containing identifying information will be kept in locked files accessible only to study staff and unlocked only while a study staff member is working with the files. Information regarding individual subjects will be kept private and shared only with the IRB and appropriate government agencies.

10.2 Data Management

Data management effort for this clinical trial will be coordinated through University of Pittsburgh's Center for Research on Health Care Data Center and will use a paperless data collection systems (eSYSDM), using both the Internet and tablet PCs with backup paper data collection. Dr. Rajakumar will work with Dr. Moore to oversee all aspects of data management. Along with the lead research coordinator, they will develop an operation manual to standardize all procedures and staff training in areas such as participant recruitment; measurements; assessments; and data entry, management, and security.

10.3 Quality Assurance

Training

All key personnel and research staff have completed or will complete the required training and certification for research integrity, protection of human subjects, HIPPA privacy and blood-borne pathogens.

Quality Control Committee

Quality Control Committee will include Dr. Rajakumar, Dr. Moore, and Ms. Kearney.

Metrics

- Monthly and cumulative accrual compared to enrollment/follow-up targets
- Baseline characteristics, overall and by treatment group
- Completeness and quality of data collection forms
- Status of enrolled participants, overall and by treatment group
- Participants' off-protocol treatments
- Compliance with eligibility criteria and other protocol requirements
- Subject adherence to the treatment regimen, overall and by treatment group
- Individual SAEs by subject ID number and a table of event-specific cumulative rates, overall and by treatment group

Protocol Deviations

Any protocol deviations recognized by study staff will be communicated to Dr. Rajakumar (PI). The PI will determine if the IRB needs to be immediately apprised of the deviation or can be informed at the next renewal. This deviation will be documented in the "As Needed" forms in the CRHC database. The protocol deviations and the course of actions taken to address the deviations will be discussed in the next DSMB meeting.

Monitoring

Dr. Rajakumar, Dr. Moore, and Ms. Kearney have extensive experience in all areas of clinical trial oversight, including developing quality assurance benchmarks, enforcing quality control, and monitoring protocol-specific activities through ongoing reporting structures and on-site evaluations. Dr. Moore will be responsible for generating reports on recruitment and follow up; quality control reports, which include the quality of data received; participant follow up adherence data, which include missed visits; and participant characteristics. Ms. Kearney will be responsible for monitoring the study site to ensure that all regulatory procedures are met. This will include ensuring that all study personnel (especially those hired after the trial begins) have completed required training and received certification to conduct human subjects' research.

Within one month of initiating a protocol, we will conduct an audit to review the study procedures and documentation of their use, including standardized procedures for obtaining informed consent, proper recording of data, reporting AEs and SAEs, training of personnel, and protection of patient identification and confidentiality. This process will be repeated every six months to assure that the protocol is being properly implemented, and that data are being collected and entered accurately. Enrollment and retention rates, protocol deviations, all SAEs, and all informed consent documentation will also be reviewed.

The following elements will be reviewed under the Data and Safety Monitoring Plan: study progress, including an assessment of data quality (monitored monthly); outcomes and adverse-events data, including out-of-range laboratory results (ongoing monitoring); any pertinent new information (monitored every six months); study procedures designed to protect the privacy of the subjects and the confidentiality of the data, i.e., that data and charts are stored in locked area and data are recorded to protect the identity of subjects (monitored weekly); interim analysis and final conclusions evaluating benefit-to-risk ratio of study participation. Data and Safety Monitoring Plan reports will be submitted yearly to the Institutional Review Board at the time of renewals.

11. PARTICIPANT RIGHTS AND CONFIDENTIALITY

11.1 Institutional Review Board (IRB) Review

This protocol and the informed consent documents, and any subsequent modifications to these documents, will be reviewed and approved by the IRB committee responsible for oversight of the study.

11.2 Informed Consent Forms

Written informed consent from either a biological or adoptive parent, and participant's assent, are required for enrollment of eligible participants <18 years of age (10- to 17-year-old children). Eligible participants who are 18 years of age can provide legal consent for themselves. Consent forms will include the purpose of the study, why the subject is eligible to participate, the study procedures to be implemented, associated benefits and risks, the voluntary nature of the study, and notices of privacy rights. The original, signed consent form will be retained by the study and a copy of the consent form will be given to the

parent/guardian. If a non-biological legal guardian consents for the subject, a copy of state documentation stating legal guardianship will be retained with the consent form. Consent documents will only be in English; non-English speakers/readers will not be eligible to consent for the study.

11.3 Participant Confidentiality

Subjects will be assigned an anonymizing Participant ID, which will serve as the primary means of identifying subject to non-study related personnel (e.g. clinical labs, etc.). All paper records containing identifying information will be kept in locked files accessible only to study staff and unlocked only while a study staff member is working with the files. All computer and networking systems will comply with the University of Pittsburgh standard for digital security and password-encryption, as necessary. Information regarding individual subjects will be kept private and shared only with the IRB and appropriate government agencies.

11.4 Study Discontinuation

The study may be discontinued at any time by the IRB, the NHLBI, the OHRP, the FDA, or other government agencies as part of their duties to ensure that research participants are protected.

12. COMMITTEES

- Data and Safety Monitoring Board
- Quality Control Committee will include Dr. Rajakumar, Dr. Moore, and Ms. Kearney.

13. PUBLICATION OF RESEARCH FINDINGS

Proposed trial will be registered at ClinicalTrials.gov and the results of the will be submitted to ClinicalTrials.gov as per NIH policy. Finding of the trial will also be published in a peer-reviewed medical journal.

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