Grant Study Title: Trial of vitamin D supplementation and neuromuscular function in older adults
Working Study Title: EVIDENCE - Exploring VItamin D's Effects on NeuromusCular Endpoints Study

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Background, Rationale and Context

The scientific community disagrees on the amount of vitamin D required for optimal health. In 2011, the Institute of Medicine (IOM) concluded that while scientific evidence supports a role of vitamin D for bone health, compelling evidence was lacking for non-bone health outcomes, primarily due to the paucity of randomized trials.[1] The IOM also concluded that 25-hydroxyvitamin D (25[OH]D) concentrations ≥20 ng/mL (50 nmol/L) were sufficient for bone and overall health. However, many vitamin D experts, citing a large volume of observational research, maintain that 25(OH)D concentrations ≥30 ng/mL (75 nmol/L) are beneficial for non-bone health outcomes such as neuromuscular function.[2-4] The resolution of this controversy is vital given that almost 20 million older adults have 25(OH)D concentrations between 20 and 30 ng/mL.[5] If increasing 25(OH)D concentrations above 30 ng/mL is beneficial, it would have an enormous impact on improving the health of approximately one-half of the older U.S. adult population. To resolve these controversies, randomized controlled trials are needed to both establish non-bone health benefits of remediating low 25(OH)D concentrations and determine whether increasing 25(OH)D concentrations from ≥20 to ≥30 ng/mL is beneficial.

Growing evidence indicates that vitamin D's effect on reducing falls is mediated by improvements in neuromuscular function.[6-8] We and others have shown associations between 25(OH)D concentrations and muscle strength and physical performance measures associated with fall risk (e.g., gait, balance) in observational studies.[6,9-13] Yet trials of vitamin D supplementation on changes in muscle strength and physical performance are equivocal,[14] likely due to inadequate trial duration, small sample sizes, insufficient vitamin D dose, and sample heterogeneity. Moreover, vitamin D's effects on the mechanisms underlying neuromuscular function are not well understood. Determining whether increasing 25(OH)D concentrations to ≥30 ng/mL will improve neuromuscular deficits that are risk factors for falls and elucidating the underlying physiological mechanisms linking vitamin D and neuromuscular function could change clinical practice by providing evidence to guide vitamin D supplementation recommendations for neuromuscular-related outcomes in older adults.

Objectives

To conduct a 12-month, double-blind randomized placebo controlled trial in 136 older (65-89 yrs) men and women with initial 25(OH)D concentrations of 18-<30 ng/mL to determine the effect of increasing 25(OH)D concentrations to ≥30 ng/mL through vitamin D_3 supplementation on 1) change in neuromuscular functions that are established risk-factors for falls in older adults; and 2) changes in the underlying physiological mechanisms over 4 months in a subset of 36 randomly selected participants. Participants will be randomized to 2000 IU/d of vitamin D_3 (a dose expected to increase 25(OH)D concentrations by ~14 ng/mL) or placebo.

Hypothesis 1: Compared to participants randomized to placebo, participants randomized to vitamin D_3 will have increased lower extremity muscle power and strength, increased physical performance, decreased postural sway, and reduced rate of falls over 12 months.

Hypothesis 2: Compared to participants randomized to placebo, participants randomized to vitamin D_3 will have increased type II muscle fibers, muscle fiber force, and myofiber regeneration, and decreased muscle fiber denervation over 4 months.

Lower extremity muscle strength and power, physical performance, and postural sway will be assessed at baseline, 4 months and 12 months and falls assessed monthly. Muscle biopsies of the *vastus lateralis* will be taken at baseline and 4 months to assess muscle fiber type, contractility, and denervation, and number and differentiation stage of satellite cells. The primary outcomes are change in lower extremity muscle power (Hypothesis 1) and change in percentage of type II (fast-twitch) muscle fibers (Hypothesis 2). Secondary outcomes include change in lower extremity muscle strength, physical performance, and postural sway (Hypothesis 1) and muscle fiber contractility, muscle fiber denervation, and number and differentiation of satellite cells (Hypothesis 2). Falls will also be assessed as an exploratory outcome (Hypothesis 1).

Methods and Measures

Experimental Overview/Study Design

This study is a 12-month, double-blind randomized placebo controlled trial in 136 older (65-89 yrs) men and women with initial 25(OH)D concentrations of 18-<30 ng/mL to determine the effect of increasing 25(OH)D concentrations to ≥30 ng/mL through vitamin D_3 supplementation on 1) change in neuromuscular functions that are established risk-factors for falls in older adults and 2) changes in the underlying physiological mechanisms over 4 months in a subset of 36 randomly selected participants. Participants will be randomized to vitamin D_3 (2000 IU/d) or matching placebo in a 1:1 ratio stratified by gender and race.

Recruitment of study participants

We plan to recruit and randomize a total of 136 older men and women with baseline 25(OH)D concentrations of 18-<30 ng/mL to 2000 IU/d vitamin D_3 or matching placebo for 12 months in a 1:1 ratio stratified by gender and race. In addition, a subset of participants that are eligible to receive the muscle biopsy, i.e. those not taking anti-coagulants, will be randomly selected in a 1:1 ratio stratified by gender and race to be included in the 4 month muscle biopsy mechanistic substudy. We will stop recruitment into the muscle biopsy substudy when we can ensure that at least 32 participants will complete the pre- and post-muscle biopsy. Our power analyses show that 125 persons total (assuming 90% retention) will yield >80% power to detect between group differences of a 10% relative increase in lower extremity muscle power and 16 persons/group (assuming 32 completers) will yield >80% power to detect a 25% difference in the relative increase in type II muscle fibers between groups in the muscle biopsy substudy. We plan to recruit, screen, and enroll these individuals continuously at the rate of approximately 5-6/mo during years 1-4 of the study. We will target men and women aged 65 to 89 years who live in the surrounding area via media advertisements (newspaper), targeted mass mailings, the VITAL newsletter, and community outreach events. We will also target age-eligible patients being seen at the Downtown Health Plaza (DHP) via posters, flyers, targeted mass mailing, and approaching age-eligible patients following their scheduled DHP care provider visit to see if they would be interested in learning more about the study.

Participant screening and randomization

Telephone screening. All prospective participants will initially be screened via telephone to determine general eligibility. They will be asked about their age, medical history, and current medications and dietary supplements. All participants must meet the inclusion/exclusion criteria in **Table 1**. These selection criteria will eliminate participants with conditions that may affect their ability to safely perform the neuromuscular function tests, consume vitamin D supplements, or undergo a muscle biopsy. The telephone screen will be administered in person to interested DHP patients following their scheduled DHP care provider visit if time allows by the DHP recruitment coordinator; otherwise, it will be administered over the phone.

Table 1. Inclusion/Exclusion Criteria

Inclusion Criteria	Exclusion Criteria
Age ≥65 to <90 years	Serious or uncontrolled chronic disease including: insulin-dependent diabetes; cancer requiring treatment in past year, except non-melanoma skin cancers; acute coronary event (within the last 6 months), uncontrolled angina, heart failure (stage 3-4), PAD, or stroke (within the last 6 months); uncontrolled hypertension (BP>200/110 mmHg); chronic respiratory disease requiring the use of oxygen; uncontrolled endocrine/metabolic disease; neurological (e.g., Parkinson's disease) or hematological disease; liver or renal dysfunction (eGFR <45 mL/min/1.73m^2); and musculoskeletal impairments severe enough to preclude functional testing
SPPB ≤10 if chair stand score is ≤3 or <10 if chair stand score is a 4	Evidence of impaired cognitive function (MoCA <18)
Initial serum 25(OH)D concentration of 18 to <30 ng/mL	Taking prescription vitamin D_2 or taking >1000 IU/day of vitamin D_3 (from all sources); taking an oral corticosteriod (i.e., prednisone at 7.5mg/d for 3 mos or equivalent); taking hormone replacement therapy
Not dependent on a walker	Inability or contraindications to consume daily vitamin D supplements (e.g., hypercalcemia, sarcoidosis, history of kidney stones in last 5 years)
Willing to provide informed consent and adhere to the protocol	Knee or hip surgery within the last 6 months or planned knee or hip surgery within the next year
Not involved in another behavioral, exercise, or investigational drug intervention study	Unwillingness to undergo a muscle biopsy

Table 1. Inclusion/Exclusion Criteria	
Inclusion Criteria	**Exclusion Criteria**
Self- reported physical performance difficulty	Weight loss of ≥5% or more in the past 3 months
	BMI >40 kg/m^2
	Eye surgery (e.g., cataract surgery) within the past month or planned within the next month
	If the PI feels the participant is unlikely to follow the protocol

Screening, consent and randomization. Individuals who pass the telephone screening will be scheduled for a screening visit (SV) in the Geriatric Research Clinic (GRC) in the Sticht Center on Aging. Before any data collection, all participants will provide written informed consent and complete a HIPAA authorization form in accordance with the Wake Forest School of Medicine Institutional Review Board policies. DHP patients who pass the in-person telephone screen will be given the option of completing a prescreen visit (PSV; informed consent, MoCA, SPPB, and weight and height for BMI; see below) with the DHP recruitment coordinator at the DHP after their scheduled DHP care provider visit if time allows. If they are eligible following the prescreen visit, they will be scheduled for the full screening visit at the GRC.

Individuals who meet all eligibility criteria and provide informed consent will be invited back for the first baseline visit 1 (BV1). At the end of BV1, participants will be randomized to participate in the muscle biopsy substudy (those who are not on anti-coagulants) using a web-based randomization scheme (developed by Dr. Tooze) with blocking by gender and race with a goal of achieving n=32 pre- and post-muscle biopsy samples. At the end of baseline visit 2 (BV2), participants will be randomized to one of the two study interventions (vitamin D_3 or placebo) using a web-based randomization scheme (developed by Dr. Tooze) with blocking by gender, race, and muscle biopsy subgroup. Block size and the randomization sequences will be unknown to the research staff. Muscle biopsy and intervention assignment will be generated using a program that the study coordinator will access from her PC through the study website.

Schedule and organization of assessment visits

All assessments will be conducted during 5-6 visits according to the timeline in **Table 2**: 1 screening visit; 2 baseline visits; 1 or 2 (participants undergoing a muscle biopsy only) 4-month mid-intervention follow-up visits; and 1 12-month post-intervention follow-up visit. Assessments will take place in the Geriatric Clinical Research Unit, the Geriatric Research Clinic, or the Downtown Health Plaza (prescreen visit only).

Table 2. Assessment timeline **Visit code**	**SV**	**BV1**	**BV2**	**On trial**	**4FV1**	**4FV2**	**12FV**
Activity/assessment **Week number**	**-3**	**-1**	**0**		**16**	**17**	**52**
Informed consent, review inclusion/exclusion criteria, demographics, weight*, height*, BP and pulse, fasting screening blood draw (metabolic blood panel and 25(OH)D), short physical performance battery (SPPB)*, cognitive screen (MoCA)*, medical history, medication/supplement review	X						
Lower extremity muscle power, knee extensor and flexor strength, expanded SPPB, timed up and go, 20-m walk test (usual and fast), postural sway, gait velocity, 4 stair climb, grip strength, CHAMPS Physical Activity Questionnaire, arthritis and joint pain questionnaire, review changes to medical history and medications/supplements		X			X		X
Fasting blood draw (25(OH)D, 1,25(OH)D, storage), MAT-sf, sun exposure questionnaire, calcium and vitamin D diet questionnaire (done only at BV2) *BP, height, weight, and a fasting metabolic panel will also be done at 4FV and 12FV* *Fasting 25(OH)D (LabCorp) will also be done at 4FV and 12FV to determine response to vitamin D supplements*			X		X		X
CES-D, DSST. The MoCA will also be repeated at 12FV			X				X
Muscle biopsy (**subsample only**)			X			X	
Randomization			X				
Monthly supplement adherence and falls assessment calendars				X			

SV = screening visit; BV = baseline visit; FV = follow-up visit; * See PSV below.

The screening visit (SV) will be conducted in the morning following an 8-hour fast for measurement of 25(OH)D concentration and a metabolic panel for screening purposes. After vitals (blood pressure, pulse,

height and weight) are collected and the blood draw completed, participants will be given a snack and continue with the remainder of the visit. First, the short physical performance battery (SPPB)[15] will be administered and scored. If still eligible, participants will then undergo a cognitive screen (assessed using the Montreal Cognitive Assessment, MoCA[16]) and a medical history and review of medications and dietary supplements. A prescreen visit (PSV)* may be scheduled to assess eligibility on the SPPB and the MoCA. If this occurs, the participant will sign consent at the PSV and then weight and height will be obtained to calculate BMI and the SPPB and MoCA will be administered. If the participant qualifies, they will then be scheduled for the SV (and those measures would not be repeated again until follow up). The reason for doing a prescreen visit is those two measures will often disqualify someone from the study so it greatly improves our work flow and scheduling abilities to schedule this as a separate visit since the PSV can be done in the afternoon whereas the SV is a fasting visit and has to be done in the morning. Following the screening visit, the study coordinator will check the lab results to determine if the participant is still eligible based on blood work (25(OH)D between 18 and <30 ng/mL, liver and serum calcium within normal limits, and eGFR≥45). If the participant is eligible, they will be notified and scheduled for a baseline visit. If the participant is not eligible, they will be notified and given a copy of their screening blood work (if it was completed).

At the first baseline visit 1 (BV1), lower extremity muscle power (Nottingham Power Rig) and strength (Biodex dynamometer), physical performance (the expanded SPPB, timed up and go (TUG), and 20-m walk speed), grip strength, postural sway (AMTI AccuSway force platform), timed stair climb, and gait velocity (GAITRite mat) will be measured. The CHAMPS Physical Activity Questionnaire for Older Adults,[17] to estimate physical activity in a typical week over the past 4 weeks, and an arthritis and joint pain questionnaire will be administered. At the completion of BV1, the study coordinator will check to see if the participant has been selected to be in the muscle biopsy substudy and tell the participant whether or not he/she was selected to be in the muscle biopsy substudy. Participants selected for the muscle biopsy substudy will be given oral and written instructions to prepare for the muscle biopsy (not to take aspirin, ibuprofen, naproxen, or other medications that may affect bleeding, platelets, or bruising for 5 days before the procedure and to avoid strenuous physical activity for 36 hours before the procedure). Participants will be scheduled for their BV2 visit approximately one week later.

Baseline visit 2 (BV2) will be conducted in the morning after an 8-hour fast in which participants will have blood drawn for analyses of serum 25(OH)D and 1,25(OH)D, plasma PTH, bioenergetic profiling (muscle biopsy subsample only), and blood for storage. For participants randomized to the muscle biopsy subsample, a muscle biopsy of the *vastus lateralis* (quadriceps) will also be taken. After vitals are collected and the blood draw (and muscle biopsy for those in the substudy) completed, participants will be given a snack. Participants in the muscle biopsy substudy will be given the option to continue with the BV2 visit following the muscle biopsy or to come back the following week to complete the BV2 questionnaires and be randomized in a separate visit. Then the Mobility Assessment Tool for Disability – short form (MAT-sf) to assess difficulty performing daily tasks, Digit Symbol Substitution Test (DSST) to assess attention and psychomotor speed, the Center for Epidemiologic Studies Depression Scale (CES-D) to measure mood and depression, and a sun exposure questionnaire (a modified version of the NHANES Dermatology Questionnaire) to estimate sun exposure and protection habits will be administered. At the conclusion of BV2, the study coordinator will check to see if the participant is randomized to receive either vitamin D_3 or placebo. The study coordinator will provide the participant with a 4-month supply of vitamin D_3 or placebo pills and monthly fall calendars to complete and provide oral and written instructions for taking the study supplement and completing the monthly fall calendars. Participants in the muscle biopsy substudy who do not elect to return the following week to complete the BV2 visit will receive a phone call 2-4 days following the procedure to check that they are not experiencing any adverse effects that may be related to the procedure (pain/soreness, signs of infection such as fever, excessive bleeding, or limitations in activity). The study physician (Dr. Demons) will be notified of any adverse effects related to the muscle biopsy procedure and any necessary action taken (e.g., follow-up visit scheduled with the study physician).

Monthly phone calls: Participants will be called once a month throughout the study to ensure they are taking and tolerating the supplements and completing the monthly fall calendars. The study coordinator will ask about any falls or adverse events that may have occurred.

The first 4-month follow-up visit (4FV1) will occur during week 16 of the intervention. After an 8-hour overnight fast, participants will have their vitals taken and blood drawn for analyses of serum 25(OH)D and 1,25(OH)D, plasma PTH, metabolic panel, and storage followed by a snack. Lower extremity muscle power and strength, physical performance, grip strength, postural sway, stair climb, and gait velocity will be measured. The CHAMPS Physical Activity Questionnaire, MAT-sf, arthritis and joint pain questionnaire, and sun exposure questionnaire will be administered. Any changes to medical history and current medications and

dietary supplements will be reviewed. The first four months of fall calendars will be collected and reviewed. Participants will return the study supplement bottle with any remaining pills and an 8-month supply of vitamin D_3 or placebo pills will be provided to them to last through the end of the study. Instructions for taking the study supplement and completing the monthly fall calendars will be reviewed. Participants in the muscle biopsy substudy will be scheduled for their follow-up muscle biopsy and given oral and written instructions to prepare for the procedure. Following the 4-month follow-up visit, the study coordinator will check the lab results to determine if the 25(OH)D concentrations are at or above 30 ng/mL in participants randomized to receive vitamin D_3. Participants randomized to vitamin D_3 who have not achieved 25(OH)D concentrations ≥30 ng/mL at the 4-month follow-up visit will be instructed to take 2 pills/d (4000 IU) for the remainder of the study. The study coordinator will also check to ensure that serum calcium levels are ≤10.6 mg/dL. If a participant's serum calcium is >10.6 mg/dL at 4FV1, the protocol to assess whether the participant is hypercalcemic outlined in the Human Subjects Protection section (p. 10) will be followed.

The second 4-month follow-up visit (4FV2) will occur 3-5 days after the 4FV1 visit in the morning for participants in the muscle biopsy substudy who had a viable muscle biopsy sample at BV2 only. After an 8-hour overnight fast, participants will undergo a muscle biopsy of the *vastus lateralis* followed by a snack. Participants will receive a phone call 2-4 days following the procedure to check that they are not experiencing any adverse effects that may be related to the procedure. The study physician (Dr. Demons) will be notified of any adverse effects related to the muscle biopsy procedure and any necessary action taken.

The 12-month follow-up visit (12FV) will occur during week 52 of the intervention. After an 8-hour overnight fast, participants will have their vitals taken and blood drawn for analyses of serum 25(OH)D and 1,25(OH)D, plasma PTH, metabolic panel, and storage followed by a snack. Lower extremity muscle power and strength, physical performance, grip strength, postural sway, stair climb, and gait velocity will be measured. The CHAMPS Physical Activity Questionnaire, MAT-sf, arthritis and joint pain questionnaire, DSST, CES-D, the MoCA, and sun exposure questionnaire will be administered. Medical history and current medications and dietary supplements will be reviewed. Monthly fall calendars will be collected and reviewed. Participants will return the study supplement bottles with any remaining pills. Participants will then be told of their randomization assignment.

Muscle biopsies: Muscle will be obtained in a stratified sample of participants from the *vastus lateralis* using the percutaneous needle biopsy technique.[18] The *vastus lateralis* is a good representative muscle for the *in vitro* study of skeletal muscle in older persons.[19] By conducting an average of 3-4 passes (some may require additional passes depending on body composition) from a single incision, tissue samples of an average of 500mg (more or less depending on body composition) are obtained, which is sufficient for the mechanistic analyses proposed and any remaining tissue will be frozen and stored for future use. Up to 40 participants may be approached for a baseline muscle biopsy to ensure that at least 32 participants complete the 4-month muscle biopsy and allow for biopsy samples that yield insufficient muscle for analyses. Once the specimen is obtained, visible blood and connective tissue is removed, and portions for specific assays are partitioned. A portion for histochemical analysis (≥20 mg) will be oriented such that the fibers run longitudinally, mounted in a histology tray in embedding medium (OCT compound, Miles Laboratory, Naperville, IL), and frozen in isopentane cooled to its freezing point with liquid nitrogen. Another portion to be used for RNA extraction (≥50 mg) will be snap frozen in liquid nitrogen (within 30 seconds of the biopsy) and stored at -80°C until analyses. A portion of muscle tissue will be homogenized for extraction of mitochondria (≥50 mg).

Vitamin D intervention

Participants will be randomized to either 2000 IU/d of vitamin D_3 or placebo for 12 months. The vitamin D_3 supplements and matching placebo pills will be manufactured by Tishcon Corp (Salisbury, MD). The proposed dose is expected to increase 25(OH)D concentrations by ~14 ng/mL.[20,21] Previous vitamin D supplement dosing studies have observed a plateau in the increase in 25(OH)D concentrations in 6 to 8 weeks.[20,22,23] In a recent dose-response study, a vitamin D_3 dose of 1600 IU/d increased 25(OH)D concentrations from <20 ng/mL to ≥30 ng/mL over 1 year in 97.5% of participants.[24] However, we will measure serum 25(OH)D at the 4-month visit and increase the vitamin D_3 dose to 4,000 IU/d in participants in the intervention group with 25(OH)D concentrations <30 ng/mL (for every participant in the intervention group that has to increase their vitamin D_3 supplement dose to 2 pills/d after the 4-month follow-up visit, a random participant from the placebo group will also be instructed to take 2 placebo pills/d). Supplements will be dispensed at baseline and at the 4-month visit. Participants will be instructed to maintain their regular dietary intake and typical physical activity level and instructed to refrain from starting any other dietary supplements or changing the frequency/dose of any supplements they are currently using during the study. A brown bag review will be done at each visit of all

dietary supplements participants are currently taking; and the brand and type, frequency, and dose of vitamin D and calcium will be recorded on the medication inventory. Supplement adherence will be monitored monthly via phone calls and the remaining supplement pills counted at the 4- and 12-month visits. Since the half-life of 25(OH)D is ~2-3 weeks,[27] occasional missed pills should have a minimal effect on 25(OH)D concentrations. The neuromuscular function assessments, mechanistic studies of the muscle biopsy sample, and assessments of 25(OH)D and $1,25(OH)_2D$ will be conducted by study staff and investigators blinded to the vitamin D intervention assignment.

Outcome measures

All examiners are trained in the standardized conduct of all assessments before data collection. Participants will be instructed to wear appropriate and comfortable clothing, and standardized written instructions will be provided prior to each study visit. Study outcomes (i.e., lower extremity muscle power and strength, expanded short physical performance battery, timed up and go, 20-m walk, stair climbing, grip strength, postural sway, gait velocity, and the mechanistic studies described below for the muscle biopsy substudy) will be assessed by blinded staff.

Lower extremity muscle power will be measured using the Nottingham Power Rig, a safe, convenient method for assessing power output from the lower limb which has been used reliably in older adults.[28] Participants will sit in a chair and unilaterally depress a foot lever attached to a flywheel as hard and as fast as they can. Power output, derived from the acceleration of the flywheel from 5 trials on each leg at maximal effort, will be recorded in Watts. Participants that have had a unilateral hip or knee replacement should not have that side tested. Participants who have had eye surgery (e.g., cataract surgery) within the past month should not be tested. For the primary analyses, the ratio of the overall maximum leg power (right or left leg at baseline) to body weight in kg will be used and the maximum leg power from the same leg used at 4- and 12-month follow-up. For analyses correlating muscle composition and physiology from the muscle biopsy substudy with leg power, the ratio of maximum leg power on the leg biopsied to body weight in kg will be used in analysis.

Lower extremity muscle strength will be measured using an isokinetic dynamometer (Biodex) at one speed (60°/sec) with the participant sitting and the hips and knee flexed at 90°. The dynamometer will be adjusted for each participant and all adjustments will be recorded to duplicate the position for subsequent assessments. Start and stop angles will be set at 90° and 30°. Participants will be asked to extend the knee and push as hard as possible against the resistance pad. Strength is expressed as peak torque in Newton-meters (Nm). Two trials will be done consisting of 4 repetitions each. Participants who have a history of brain aneurysm, cerebral bleeding in the past 6 months, or blood pressure >199/99 mmHg should not be tested. Participants that have had a unilateral knee replacement should not have that side tested. Participants who have had eye surgery (e.g., cataract surgery) within the past month should not be tested. The maximum knee extensor strength of the 4 repetitions from trial 2 for the dominant leg will be will be used in analyses unless unable to test the dominant leg (i.e., knee replacement) in which case the non-dominant leg will be used; the maximum knee extensor strength from the same leg will be used at 4- and 12-month follow-up. For analyses correlating muscle composition and physiology from the muscle biopsy substudy with leg strength, the maximum leg strength on the leg biopsied will be used in analysis.

Physical performance will be assessed using the expanded Short Physical Performance Battery (SPPB). The expanded SPPB consists of 5 repeated chair stands, standing balance (semi- and full-tandem stands and a single leg stand for 30 seconds), a 4-m walk to assess usual gait speed, and a narrow 4-m walk test of balance (walking at usual pace within lines of tape spaced 20 cm apart).[29] Scores for the traditional 0-12 point SPPB can also be obtained from these tests. We will also assess physical performance using the Timed Up and Go (TUG). TUG measures the time a person takes to stand up from a standard chair, walk 3 m, turn, walk back to the chair, and sit down again.[30] A practice trial is given, followed by a timed trial. Usual and fast walking speed over 20 m will be assessed to determine the functional reserve that exists in walking speed and to provide a substantive challenge over the usual pace 6-m walk used in the expanded SPPB. Stair climbing time will be assessed by measuring the time to climb up 4 steps on a 4-step staircase (test will be performed 2 times). The faster of the 2 trials will be used in analysis.

Grip strength will be measured twice in each hand to the nearest 2 kg using an isometric Hydraulic Hand Dynamometer (Jamar, Bolingbrook, IL) and the mean value from the stronger hand at baseline will be used in analysis and the mean value from the same hand used at 4- and 12-month follow-up. Participants will be excluded from performing the test if they report hand-pain or recent hand or wrist surgery.

Postural sway during quiet stance will be assessed from Center-of-Pressure (COP) trajectory data collected at 100 Hz using an Advanced Mechanical Technology Incorporated (AMTI) AccuSway biomechanics force platform. Participants will be barefoot in an upright stance with arms relaxed comfortably at their sides, feet abducted 10 degrees, and heels separated medio-laterally by 6 cm.[31] COP data will be collected in a series of 10 30-sec trials standing on the force plate alone (closed trials) followed by 5 30-sec trials standing on the force plate with a 3 inch Airex foam pad (foam trials) placed on top. Four posturographic parameters (maximum anteroposterior (AP) and mediolateral (ML) displacement, average sway velocity, and 95% confidence ellipse) calculated by the AMTI Balance Clinic software will be used to quantify postural sway and the average of the closed and foam trials used in analysis.[31] Studies have linked indicators of ML stability[32-36] or AP stability[37] to clinical measures of balance performance and/or fall risk.

Gait velocity will be measured using a 4.88 m long instrumented carpet (GAITRite, CIR Systems Inc., Clifton, NJ). The carpet is composed of 18,432 force sensing sensors arranged in a 48 wide x 384 long grid. Data are sampled at either 60, 120, or 240 Hz. The GAITRite is interfaced with a laptop computer that runs software to calculate variables related to gait performance. The GAITRite system is a valid instrument for determining gait velocity and other spatiotemporal parameters. In the testing procedures, participants walk across the carpet, beginning 2 m in front of the carpet and walking 2 m past the end of the carpet to allow for acceleration and deceleration. Participants make multiple passes over the carpet at either the preferred (4 trials) or fast (4 trials) velocity. At the preferred velocity, participants are told to walk at “at a pace that feels comfortable to you (as if you were walking to the mailbox or window shopping at the mall).” At the fast velocity the instructions are to walk “as quickly as you can without running.” The software calculates individual trial and average data on gait velocity and spatiotemporal gait characteristics and these data can be exported for further analysis. Summary data on velocity (m/sec; averaged over the 4 usual and 4 fast trials) and the average of the left and right leg for double support (percent of cycle), single support (percent of cycle), stance (percent of cycle), swing (percent of cycle), stride length (cm), and base width (cm) for both the usual and fast trials will be used in analysis.

Falls will be defined as an event whereby an individual unexpectedly comes to rest on the ground or another lower level. Participants will be provided monthly fall calendars, considered the “gold standard”,[38] and asked to mark any falls that occur on the calendar. Completion of monthly fall calendars will be monitored via monthly phone calls and collected at the 4- and 12-month visits. Total number of falls over the 12-month follow-up will be used in analysis.

Single fiber-type composition and fiber size will be examined in muscle biopsies using the ATPase, pH 9.4 and 4.6 technique, combined with laminin immunostaining in 10 μm muscle sections and the absolute and relative number of type I, IIA, IIX fiber subtypes and their cross-sectional area quantified. The relative number of fiber type and their cross-sectional area at baseline and 4-month follow-up will be used in analysis.

Single type I and type II muscle fiber contraction force, shortening velocity, and power will be examined in 10-15 fibers per muscle biopsy sample. For single muscle fiber preparation, physiologic tests, and experimental procedures, we will follow described techniques.[39,40] Briefly, a slack test will be used to determine the unloaded shortening velocity (V_o). The force/velocity relationship will be determined by performing a series of isotonic contractions of the muscle fiber.[41] The muscle fiber will be placed in activating solution (pCa 4.5) and, after it reaches peak force, subjected to three isotonic steps varying from 3-80% of P_o. After the final step, the fiber is rapidly (<1ms) slackened by 20% of its length. This zeroes the force transducer, providing a baseline for force measurement. Shortening velocity and force will be assessed over the final half of each step, and force averaged over the last half of each step. Velocity will be calculated as the slope of the position recorded over the same time period. A total of 5-6 isotonic contraction series will be performed, generating 15-18 pairs of velocity and relative force measurements. Data will be fit to the Hill equation using an iterative, nonlinear curve-fitting procedure to draw the force/power relationship. All forces will be normalized to the fiber’s cross sectional area. At the end of each functional experiment, the single-fiber segment will be used to determine myosin heavy chain (MHC) composition using silver staining.[41,42] Fibers will be excluded from analysis if force declines more than 10% or if fibers break or show partial myofibrillar tearing during the experimental protocol. Absolute fiber force (slk max mN), normalized fiber force to cross-sectional area (slk pk kN/m^2), unloaded shortening velocity (V_o, FL/s), the velocity extrapolated to a force of zero (FV Vmax, FL/s), power (uN*FL/s), and normalized power (W/liter) by fiber type (I or II) at baseline and 4-month follow-up will be used in analysis.

Type I and type II denervated myofibers will be identified using ATPase pH 9 plus NCAM immunostaining analysis. The whole muscle biopsy will be cryosectioned and 10-12 slices will be mounted per slide. Alternate slides will be stained for NCAM and positive fibers and their expression pattern (perimeter or whole cross section) will be reported as a percent of the total number of fibers. Additionally, fiber grouping (the area occupied by at least two contiguous fibers expressing a predominantly similar myosin heavy chain isoform – type I or II, fully surrounded by fibers of the same predominant myosin heavy chain), a classical evidence of a neurogenic process, will be examined. NCAM+ number and density (NCAM+ number per cross sectional area) and fiber grouping (%) by fiber type (I or II) at baseline and 4-month follow-up will be used in analysis.

Regulation of Runx1 and its dependent genes will be assessed by RNA extraction using the TRIzol method (Invitrogen). Runx1 and its dependent genes will be measured by qRT-PCR and normalized to GAPDH expression. We will use the cycle threshold (CT), defined as the number of cycles required for the fluorescent signal to cross the threshold, as the parameter to calculate $2^{-\Delta\Delta CT}$, which is needed to compare the gene expression in two different samples. Expression of Runx1, Chrnγ, Scn5a, Myh3, Myog, NCAM1 at baseline and 4-month follow-up will be used in analysis.

Satellite cell differentiation will be assessed in serial muscle cross sections immunostained for Pax7/NCAM satellite cells (SCs). SC number and density (SC per cross sectional area) by fiber type (I or II) at baseline and 4-month follow-up will be used in analysis.

Vitamin D Receptor (VDR) expression will be measured by Western blots using the C20 antibody raised against a peptide mapping at the C terminus of VDR and normalized to actin used as a loading control. VDR concentration at baseline and 4-month follow-up will be used in analysis.

Exploratory outcomes (Wake Forest Pepper Center Pilot; Anthony Molina, PI)

Respirometric profiling will be used to assess bioenergetic capacity, respiratory control and electron transport chain function. This will be performed by examining the oxygen consumption profile of blood cells and mitochondrial fractions extracted from muscle tissue. Specific outcomes of mitochondria function include respiratory control ratio (RCR), state 3 (maximal oxygen consumption rate), and state 4o (oxygen consumption upon inhibition of ATP synthase).

Respirometry of isolated mitochondria will be performed in order to examine intrinsic mitochondrial function (type 1). Purified mitochondria with intact electron transport chain activity will be obtained from muscle tissue. 5 ug of mitochondria will be loaded into each well of V7 Seahorse plates for analysis using an XF-24-3 system (Seahorse Bioscience, Billerica MA). Specific measures obtained include: baseline respiration (state 2), maximal respiration (state 3), maximal uncoupled respiration (state 3u), and proton leak (state 4).

Mitochondrial mass and biogenesis will be determined by western blot analysis. Changes in mitochondrial mass will be assessed using VDAC/porin and COXIV expression. PGC1a, TFAM, SIRT1 and SIRT3, regulators of mitochondrial biogenesis, will also be measured on the same immunoblot. All primary antibodies will be obtained from Abcam (Cambridge, MA). Quantification of each immunoreactive product will be performed by densitometry using Kodak (Rochester, NY) imaging system.

Blood-based bioenergetic profiling. PBMC's, monocytes, lymphocytes, and platelets will be separated from whole blood and assessed for basal respiration, maximal respiration, ATP linked respiration, spare respiratory capacity, proton leak, and non-mitochondrial respiration as described above.

Covariates

Vitamin D status: Fasting blood samples will be collected in the morning by trained and certified phlebotomists at baseline and 4-month and 12-month follow-up. Blood samples will be sent to LabCorp for assessment of serum 25(OH)D for screening purposes and at 4-month follow-up to assess 25(OH)D response to vitamin D supplementation and for assessment of serum calcium at each visit. After processing, approximately 4 ml of the blood from each visit will be aliquotted and stored at -80ºC until it is shipped to the Clinical and Analytical Lab (CACL) at the Human Nutrition Research Center on Aging at Tufts University where the samples will be stored at -80ºC until analysis. Serum total (D_2 and D_3) 25(OH)D will be assessed in duplicate using LC/MS/MS (Waters Acquity UPLC with TQD triple quadrupole mass spectrometer; CV: 6%) along with the 25(OH)D standard reference material (SRM 972) developed by the National Institute of Standards and Technology and the National Institutes of Health Office of Dietary Supplements for assay calibration.[45] Serum $1,25(OH)_2D$ will also be assessed in duplicate using the 5500 QTRAP LC/MS/MS System (AB Sciex LLC) with Electrospray after immunoaffinity extraction, 4-phenyl-1,2,4-triazole-3,5-dione (PTAD)

derivatization, and methylamine adduction (intra- and interassay CVs of 5% and 8.5%, respectively). Repeat analyses will be conducted when duplicate samples differ by 10% or more. Storage: Approximately 10 ml of blood (serum and plasma) from each visit will be stored at -80ºC in the Pepper Tissue Repository (IRB# 1219) for storage. Any person wishing to use this blood will obtain IRB approval first.

Season: The season in which the participant is screened for eligibility will be obtained to examine if the season of enrollment affects the outcomes due to seasonal effects on endogenous vitamin D synthesis and 25(OH)D concentrations.

Supplement adherence: Although we will attempt to enhance adherence by monthly calls to participants, there may be inter-individual variability in adherence to supplementation. At the 4 and 12-month study visits, participants will be asked to return any unused supplements for a pill count to determine participant adherence. Our primary analyses will be an intent-to-treat analysis of the effects of the vitamin D intervention, but secondary analyses will include adherence as a covariate.

Calcium and vitamin D intake: Calcium and vitamin D intake from diet and supplements will be assessed at baseline using a short calcium and vitamin D intake questionnaire.

Cognitive function will be assessed during screening using the Montreal Cognitive Assessment (MoCA), participants must score ≥18 to be eligible. We will also assess psychomotor speed, attention, and working memory using the Digit Symbol Substitution Test (DSST). Participants are given a series of numbered symbols and then asked to draw the appropriate symbols below a list of random numbers. The score is the number of correctly made matches in 2 minutes (120 seconds). The cognitive function tests will be audio recorded by the assessor using a hand held digital recorder. The recordings will be used to properly score the tests as well as be available for quality control testing. The recordings will be deleted once the study is over and the data that was entered has been verified.

Mood will be assessed using the 20-item Center for Epidemiologic Studies Depression Scale (CES-D).

Demographic characteristics including age, gender, race, education level, and socio-economic status will be recorded and used as covariates if necessary.

Body mass index (BMI) will be calculated from measured height and weight.

Physical activity will be measured using the CHAMPS Physical Activity Questionnaire for Older Adults[17] at baseline, 4- and 12-month follow-up. In addition to being a strong correlate of the neuromuscular function measures, more physically active participants will likely have increased sun exposure, and therefore, may have higher endogenous vitamin D production.

Activities of daily living will be assessed using the Mobility Assessment Tool – Short Form (MAT-sf) at baseline, 4- and 12-month follow-up. This is a novel, computerized tool for self-assessment of functional performance designed to reduce bias from factors such as age, gender and body image.

Sun exposure will be assessed at baseline, 4- and 12-month follow-up using a modified version of the NHANES Dermatology Questionnaire that includes questions on sun exposure and sun protection habits.

Medical information regarding co-morbidities will be ascertained at the screening visit and reviewed at the 4- and 12-month follow-up visits. Arthritis and joint pain will be assessed by self-report on a questionnaire. We will record medication and supplement use at screening and 4- and 12-month follow-up. Participants will also be asked to bring in medications and dietary supplements for a "brown bag" review. The medication form will specifically ask about the use of vitamin D-containing supplements, anti-inflammatory agents, protein supplements, and sex steroids or corticosteroids.

Statistical Power

We anticipate that, based on the vitamin D_3 supplement dose of 2000 IU/d, 25(OH)D concentrations will increase by an average of 14 ng/mL in the intervention group at 12-month follow-up. In preliminary data from 17 older individuals with vitamin D concentrations between 20 and 40 ng/mL, we found that a 1 ng/mL difference in 25(OH)D concentration was associated with a 0.0233 watts/kg of body weight difference in lower extremity muscle power for men and women combined. If this cross-sectional relationship holds for the intervention study, we would expect approximately a 0.33 watts/kg difference between the two groups at follow-up (0.0233 x 14 = 0.33). However, due to the uncertainty of extending cross-sectional data to longitudinal follow-up and the potential for non-adherence, we have based the power on a difference in means

of a 10% relative change between the two groups; a magnitude that resulted in a clinically meaningful increase on the SPPB in a previous intervention trial.[47] The mean (SD) of lower extremity muscle power was 1.74 (0.382) watts/kg in our preliminary data; a 10% difference (0.174 watts/kg) corresponds to an effect size of 0.45. A sample size of 125 participants randomized in a 1:1 ratio stratified by gender and race will have >80% power to detect this difference using analysis of covariance (ANCOVA), adjusting for the baseline measure, based on a baseline mean of 1.7 watts/kg (SD=0.444) and a correlation between baseline and 12-month follow-up of 0.708 with a 0.05 two-sided significance level. Allowing for a 10% dropout rate, we will inflate the sample size to 136. We will also have sufficient power to detect a difference of 0.45 SD or greater in our secondary outcomes (**Table 3**).

Table 3. Differences detected with 80% power for secondary outcomes

Outcome measure	SD*	Detectable difference
Short Physical Performance Battery score[30]	2.05	0.92
Timed Up and Go, sec[82]	5.40	2.43
20-m Gait Speed, m/sec[31]	0.15	0.07
Lower extremity muscle strength, Nm[31]	21.0	9.5
Postural Sway, mm[122]		
Mediolateral (ML) displacement	2.7	1.2
Anteroposterior (AP) displacement	3.9	1.8

*SD calculated assuming a correlation of 0.43 between baseline and follow-up measures.

For the muscle biopsy subsample, we have powered on a difference between the two groups of a relative increase in type II fibers of 25% or more. In our preliminary data in older adults with 25(OH)D concentrations <25 ng/mL treated with 2000 IU/d vitamin D_3 plus 1200 mg/d calcium (n=4) or calcium only (n=2), a 1 ng/mL increase in 25(OH)D concentration was associated with a 2.35% relative increase in type II fibers over 4 months. Assuming that 25(OH)D concentrations will increase by an average of 14 ng/mL in the intervention group, we expect to see a relative increase in type II fibers of approximately 33%. To allow for the possibility that there may be some increase in the control group due to "drop in" by starting vitamin D supplements or changes in physical activity, a 25% difference between the two groups in the relative increase in type II fibers was selected. Two previously published vitamin D supplementation studies observed increases in type II fibers of 31-34%[48] and 202%.[49] Thus, the detectable 25% relative increase is in line with independent observations. A sample size of 16 in each group will have 80% power to detect a difference of 25% assuming that the common standard deviation is 23.7 (based on preliminary data of n=17 participants in a behavioral intervention with data on change in type II muscle fibers) using a two group t-test with a 0.05 two-sided significance level. Allowing for 10% drop out, we will inflate the sample size to 18 per group, for a total of 36 participants. However, up to 40 participants may be approached for a baseline muscle biopsy to allow for biopsy samples that yield insufficient muscle for analyses.

Statistical Analyses

We will examine the distributions of all continuous outcomes of interest, and will transform them to approximate normality if the outcomes show a strong departure from normality as evidenced by examining Q-Q plots and performing the Shapiro-Wilk test. Outliers will be examined on the chosen scale (original or transformed) and may be set to missing if they are 3 or more interquartile ranges above the 75th percentile or below the 25th percentile. Plausible biologic values may be retained even if they are outliers. Implausible biologic values may be set to missing based on a review of health-related events (e.g., AEs related to pain, fractures, surgery) in the interval surrounding the collection of the measurements.

The primary outcome for this trial will be the 12-month measure of muscle power. For the muscle biopsy substudy, the primary outcome will be the change in type II muscle fibers over 4-months. Our primary statistical analyses will proceed on the "intent-to-treat" principle in which data from all randomized participants are used to test the primary hypotheses that compared to placebo, vitamin D_3 supplementation will result in increases in lower extremity muscle power and strength, and improvements in physical performance (expanded SPPB, TUG, and 20-m walk speed) and balance. Even if participants wish to discontinue the supplements, we will still attempt to follow them for outcomes in order to carry out intent-to-treat analysis at 12 months. Characteristics of the participants missing the 12-month follow-up outcomes will be compared to participants without missing data using chi-square tests and t-tests to determine if there are any differences with regard to intervention group, demographic characteristics, and baseline neuromuscular function outcomes. If a particular group exhibits markedly higher drop-out rates, we will attempt to identify baseline covariates that predict attrition. If such covariates can be identified, the analyses of outcome measures may need to be stratified according to these comparisons to decrease bias. We will use a mixed model with an unstructured covariance matrix to model after-treatment neuromuscular function outcomes between the two groups by time, with adjustment for

the baseline neuromuscular function measure, age, gender, and race, using data from baseline, 4, and 12 months, and estimating the baseline to 12 month change using linear contrasts. In secondary analyses, we will fit linear contrasts to compare whether the change between the two groups at 4 months differs from the change at 12 months. These analyses will be adjusted for age, gender, race, and season, as we anticipate there will be an effect of season on the change from baseline and 12 months. If the analyses of the characteristics of those missing the 12-month outcome data indicate that the missing data are informative, we will also consider using the 4-month outcome data to impute the 12-month outcome, using the trajectory models to predict the 12-month outcome.

We will examine factors that may influence the measured responses to the intervention. We anticipate that not all individuals will respond to the vitamin D_3 dose in a similar manner due to genetic differences[50-52] as well as differences in BMI,[53,54] health status, and adherence.[3] First, individuals in the vitamin D intervention group who have low adherence (<80%) will be excluded from the analysis. We will also investigate treatment effects by attained 25(OH)D concentrations including only those in the intervention group whose 12-month 25(OH)D concentrations are ≥30 ng/mL. We will also fit models that include BMI, health status, and other variables to determine if they account for any of the variation in response to the intervention on changes in the outcomes. For our exploratory outcome of falls, we will use a generalized estimating equation (GEE) approach to model repeated measures, assuming a Poisson distribution, to compare the rate of falls in each group with adjustment for age, gender, and race.[55] Differences in, or reasons for, attrition rates between study groups may complicate the analysis of the primary outcomes. Therefore, the attrition rates will also be compared and, if a particular group exhibits markedly higher drop-out rates, we will attempt to identify baseline covariates that predict attrition. If such covariates can be identified, the analyses of outcome measures may need to be stratified according to these comparisons to decrease bias.

For the muscle biopsy subsample (n=16 per group), primary analyses will proceed on the “intent-to-treat” principle in which data from all randomized participants are used in analyses. All participants who complete the 4-month muscle biopsy and on whom the muscle fiber testing can be completed will be included in the analytic sample. Even if participants wish to discontinue the supplements, we will still attempt to follow them for outcomes in order to do the intention-to-treat analysis at 4 months. Characteristics of the participants missing the 4-month follow-up outcomes will be compared to participants without missing data using chi-square tests and t-tests to determine if there are any differences with regard to intervention group, demographic characteristics, baseline physical performance, and baseline muscle fiber testing. Our primary statistical analyses will focus on testing the primary hypotheses that, compared to placebo, vitamin D supplementation will result in changes in skeletal muscle composition and physiology as quantified by: fiber-type composition and size; fiber force, shortening velocity, and power; NCAM+ myofibers; Runx1, Chrnγ, Scn5a, Myh3, Myog, and NCAM1 expression; SC number; VDR expression; and bioenergetic capacity. For our primary outcome of percent type II fibers we will use analysis of covariance (ANCOVA) to model differences in percent of type II fibers between the two groups, with adjustment for the baseline measure, age, gender, and race. Analysis of other continuous measures will also use the ANCOVA model to compare the groups adjusted for age, gender, and race. In secondary analyses, we will examine if the percent of participants who have a relative increase in type II fibers of 25% or more from baseline differs by intervention group using Fisher’s Exact Test, and in logistic regression models adjusted for age, gender, and race. Furthermore, we will explore if the relationship between vitamin D supplementation and neuromuscular function is mediated by change in muscle composition and physiology by including the variables described above as covariates in the ANCOVA model described above. If the relationship between vitamin D supplementation and power decreases by 10% or more, it will be considered evidence of partial mediation.

Human Subjects Protection

Subject Recruitment Methods

We will recruit these individuals using community-based recruitment strategies including newspaper ads and mass mailings. We will also advertise in the VITAL newsletter (BG99-559) and participate in community outreach events.

Informed Consent

Written informed consent will be obtained from each subject. The informed consent process will follow the procedures of the WFSM Institutional Review Board. The study interviewers will explain the purpose, methods and extent of the study to prospective participants. The potential participant is asked to read the informed

consent form and ask questions. The form is written in simple easy to understand language. We require study staff to review all of the key aspects of the study verbally with the potential participants. Staff is provided with a structured checklist for this purpose. Staff is then required to question potential participants to ascertain whether s/he has understood the information. Potential participants who are illiterate or have impaired vision must have the consent read to them, followed by review of the checklist, opportunity for questions, and discussion. This process will take place in a quiet, private room. A copy of the signed and dated consent form will be given to participants, and the original document will be placed in subjects' individual study files, which will be stored in a secure location. In compliance with the Health Insurance Portability and Accountability Act (HIPAA) and the Standards for Privacy of Individually Identifiable Health Information of the Department of Health and Human Services, we will access personal health information only after obtaining informed consent.

Potential Risks

1) Neuromuscular function. There is a small risk of injury during the muscle strength and power testing, stair climb, postural sway and gait velocity testing, and physical performance testing, such as muscle strains or pulls, falls, or joint injury. However, these tests have been performed in large study populations with no significant adverse events reported. Risks will be minimized by having experienced/ trained staff conducting these assessments. A warm-up and range of motion practice will be conducted before maximal strength testing. In addition, if a participant reports pain, dizziness, lightheadedness or other medical problem during the test, the test will be terminated.

2) Blood Draw. Participants may experience temporary pain, bruising, bleeding and a small risk of infection or fainting or dizziness during the blood sample collection process. Only trained staff will be responsible for the collection of blood samples.

3) Muscle biopsy. There may be some temporary discomfort and bleeding from the muscle biopsy. However, there is typically no discomfort felt during the procedure due to the use of lidocaine as a local anesthetic injected into the muscle. Temporary numbness of the skin near the sampling site can rarely occur. A small amount of bruising can also occur. There is a slight risk of bleeding into the tissue and infection, but this procedure is done under sterile conditions to protect against this. There is also a risk of a vaso-vagal response during or after this procedure, but there are trained medical personnel performing the procedure and a fully stocked crash cart is available should it be needed. To date, Dr. Demons and Dr. Lyles have performed >600 muscle biopsies for aging-related research studies without a serious adverse event.

4) The risks of the vitamin D supplement intervention are minimal, but may include hypervitaminosis D. Although the proposed vitamin D_3 dose of 2000 IU/d is below the upper limit set by the Institute of Medicine (4000 IU/d), participants could exceed the upper limit if they begin consuming other vitamin D-containing supplements. Participants will be advised not to begin any new dietary supplements during the trial and of the possible side effects of hypervitaminosis D (e.g., non-specific symptoms such as anorexia, weight loss, polyuria, and heart arrhythmias; as well as increased serum calcium which can lead to vascular and tissue calcification including kidney stones). Those with contraindications to vitamin D supplements will be excluded (e.g., kidney impairment (eGFR <45 mL/min/1.73), hypercalcemia, sarcoidosis, and history of kidney stones). Serum calcium will be assessed at screening and at 4- and 12-month follow-up. Participants with hypercalcemia (serum calcium >10.6 mg/dL) at their 4-month visit will have a repeat serum calcium drawn within 2 weeks, and, if confirmed, the study supplement will be discontinued; however, we will continue following participants with confirmed hypercalcemia for outcomes.

Confidentiality and Privacy

All data are obtained for research purposes only. Confidentiality of data is maintained by using research identification numbers which uniquely identify each individual. Data will be used only in aggregate and no identifying characteristics of individuals will be published or presented. The information collected has a low potential for abuse because the data do not address sensitive issues. Nevertheless, appropriate measures are taken to prevent unauthorized use of study information. Research records are kept in a locked room. Data access will be limited to study staff. Information linking IDs to individuals is kept on a secure, password-protected server to which only authorized study personnel will have access. Data and records will be kept locked and secured, with any computer data password protected. A web-based data entry system will be

created for this study which will allow access only to study staff (user id and password required to access). Blinded staff will not have access to blinded study information on the web-based database. Computer files are stored on file servers, which are backed up each night and stored for easy retrieval in case of emergency. Files may not be obtained from the research unit by persons other than the research personnel, who are asked to sign a document agreeing to maintain the confidentiality of the information. No data files distributed for analysis will include personal information. No reference to any individual participant will appear in reports, presentations, or publications that may arise from the study. After the study is completed, the data will be stored with other completed research studies in a locked secure area.

Data and Safety Monitoring

The principal investigator will be responsible for the overall monitoring of the data and safety of study participants. The principal investigator will be assisted by other members of the study staff. A Data Safety Monitoring Board (DSMB) has been established by the NIH, with responsibility to monitor all aspects of the study, including those that require access to any blinded data. The operational plan and all members were approved by the NIH Program Scientist. The NIH-appointed DSMB responsibilities are to:

- review the research protocol, informed consent documents and plans for data safety and monitoring;
- advise the NIA on the readiness of the study staff to initiate recruitment;
- evaluate the progress of the trial, including periodic assessments of data quality and timeliness, recruitment, accrual and retention, participant risk versus benefit, performance of the trial sites, and other factors that can affect study outcome;
- consider factors external to the study when relevant information becomes available, such as scientific or therapeutic developments that may have an impact on the safety of the participants or the ethics of the trial;
- review study performance, make recommendations and assist in the resolution of problems reported by the Principal Investigator;
- protect the safety of the study participants;
- report to NIA on the safety and progress of the trial;
- make recommendations to the NIA, the Principal Investigator, and, if required, to the Food and Drug Administration (FDA) concerning continuation, termination or other modifications of the trial based on the observed beneficial or adverse effects of the treatment under study;
- ensure the confidentiality of the study data and the results of monitoring; and,
- assist the NIA by commenting on any problems with study conduct, enrollment, sample size and/or data collection.

The PI, co-investigators and the biostatistician will meet regularly to review study progress and to examine reports of adverse incidents as well as participant recruitment and follow-up. All *serious* adverse events will be reported to the Institutional Review Board (IRB) and if the event occurred as a direct result of participation in this study, an amendment will be made to the consent form and the PI will request new IRB approval. The PI and the Medical Director will meet quarterly, or as needed, to review all reported events.

Adverse events include any event that occurs during the course of the study that results in a participant suffering physical or mental injury, pain or suffering. Adverse events can be major, such as a subject who suffers cardiac arrest during neuromuscular function testing, or minor such as a subject pulling a muscle during neuromuscular function testing. This includes any events occurring while a subject is enrolled in the study, even if the event did not occur while s/he was actively participating in the activities called for in the research protocol. Deviations from the study's protocol are also considered an adverse, unexpected, or notable event and will be reported to the PI.

Both major and minor events will be reported using the study's Adverse, Unexpected, or Notable Event Reporting Form. Any major event, i.e., any serious injury or life-threatening event, will be reported immediately right after completing any and all actions that are necessary to protect the subject's health and safety. Minor events will be reported within seven days. A description of the event, and the date and location of the event will be recorded on this form, which will be kept in the subject's research file.

In addition, participant safety in all Wake Forest Pepper Center OAIC-supported studies is monitored by a Data and Safety Monitoring Committee (DSMC). It reviews study recruitment, drop-outs, protocol changes,

losses to follow-up, and adverse events every 6 months. The DSMC has authority to recommend changing or stopping the protocol. In the latter case, the PI will immediately act upon the recommendation according to institutional policy and in consultation with the Wake Forest Pepper Center OAIC Executive Committee and the NIH-appointed DSMB committee.

Reporting of Unanticipated Problems, Adverse Events or Protocol Deviations

Any unanticipated problems, serious and unexpected adverse events, deviations or protocol changes will be promptly reported by the principal investigator or designated member of the research team to the IRB and sponsor or appropriate government agency if appropriate.

Use of biological samples by other investigators

Biological samples may be used by investigators other than the investigators of the current study. The use will be limited to non-commercial purposes. The names and other personal identifiers of the study participants will not be sent to any recipients of the blood or muscle samples.

Storage and disposal of biological material

Blood and muscle samples will be stored at Wake Forest Baptist Medical Center for twenty years after the end of the trial at which time the samples will be destroyed. Biological specimens will be stored in locked –70^{o}C alarmed Revco freezers located in a locked room. The Wake Forest Pepper Center Integrative Biology Core lab coordinator (Heather Gregory) and the Core Co-Leader (Dr. Nicklas) have access to the keys of the freezers. All the specimens will have numerical study IDs with no personal identifiers of the participants. These are stored under the Pepper Center Tissue Repository (IRB#1219).

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