Effect of VSL#3 on Bone Mineral Density in Postmenopausal Women: a Pilot Randomized, Placebo-Controlled Trial

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1. INTRODUCTION AND BACKGROUND

Osteoporosis has a devastating impact on quality of life of postmenopausal women, and is a significant cause of disability and morbidity. Numerous drugs are currently FDA-approved for the prevention and treatment of postmenopausal osteoporosis, the most frequent type of osteoporotic syndromes. However, most cases in the US remain untreated or ineffectively treated because of cost and/or adverse events of currently available drugs, leading to inadequate prescriptions of and poor compliance with anti-osteoporosis medications [1,2]. For example, despite clear efficacy of bisphosphonate drugs in the secondary prevention of hip fracture, a recent large study in patients with hip fracture found that use of bisphosphonates decreased from an already dismal 15% in 2004 to an abysmal 3% in 2013 [3]. These data underscore the critical need to identify inexpensive, safe and effective interventions for both the prevention and treatment of osteoporosis. Animal studies [4-13] and a report in humans [14] suggest that nutritional supplementation with probiotics may represent an effective, safe and inexpensive modality to prevent and treat osteoporosis. Studies from laboratories in the US and Europe have clearly shown that probiotics prevent the bone loss induced by ovariectomy (ovx) [4-6], Adding to this evidence, we recently demonstrated that the probiotics *Lactobacillus rhamnosus GG* (*LGG*) and VSL#3 (containing 8 strains of live bacteria, namely *Bifidobacterium breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. paracasei, L. bulgaricus and Streptococcus thermophilus)* prevent ovariectomy (ovx)-induced bone loss by decreasing cytokine-driven bone resorption[8]. Furthermore, we showed that both *LGG* and VSL#3 induce bone anabolism in estrogen-replete mice.

2. OBJECTIVES AND STUDY PURPOSE

Based on the strong preclinical evidence from our laboratory and others, ***our primary hypothesis is that oral administration of VSL#3 is a feasible, well-tolerated and effective strategy to prevent bone loss in postmenopausal women***. *We further hypothesize that this simple approach will favorably impact biomarkers relevant to bone turnover, including inflammatory and osteoclastogenic cytokine production.*

Specific Aim 1. To conduct a placebo-controlled, double blind, prospective clinical trial in which the commercially available oral probiotic VSL#3 will be administered to otherwise healthy, postmenopausal women for 12 months. The primary objective will be to demonstrate that VSL#3 administration safely maintains or improves bone density in the lumbar (L1-L4) spine, femoral neck, and total hip area in the study subjects at 1 year.

3. CRITERIA FOR PATIENT SELECTION

3.1 Inclusion criteria: 1) Willing and able to give written informed consent for participation in the study, 2) Age range 50-65 years, 3) Menopausal status (defined by >1 yr since last menstrual period or FSH level in the postmenopausal range), 4) Ambulatory, 5) Body mass Index (BMI) must be ≥ 18.0 and ≤ 32 kg/m^2 at screening, 6) Bone mineral density (BMD), expressed as T-scores, must be > - 2.5 in the lumbar spine (L1-L4), the femoral neck, and the total hip, as measured by dual energy X-ray absorptiometry (DXA), 7) Commitment not to use any products that may influence the study outcome (see below), and 8) Ability to understand and comply with the requirements of the study.

3.2 Exclusion criteria: 1) Premenopausal status, 2) History of ≥1 previous atraumatic bone fractures after age 50; 3) Presence of established osteoporosis (T-score ≤ - 2.5, in the lumbar spine, femoral neck or total hip as measured by screening DXA), 4) History of immunological or bone-related disorders including: HIV infection, Type I diabetes mellitus, bone marrow or organ transplantation; Inflammatory bowel disease (ulcerative colitis, Crohn's disease); multiple myeloma; osteomalacia; osteosarcoma; Paget's disease; rheumatoid arthritis; systemic lupus erythematous; parathyroid disorders, 5) uncontrolled type II diabetes mellitus (HgbA1c ≥ 7% within the last 12 months), 6) History of bariatric surgery or other forms of malabsorption (including documented celiac disease, or chronic diarrhea), 7) Alcohol abuse, 8) Clinically significant chronic kidney disease (stage ≥ 2, with total serum creatinine level > 2.5 mg/dL and calculated glomerular filtration rate (GFR) < 60 mL/min by the Modification of Diet in Renal Disease (MDRD equation), 9) Clinically significant cardiovascular disease (myocardial infarction, cerebral vascular accident or acute congestive heart failure within the previous 12 months, 10) Any malignancies, other than localized skin squamous cell carcinoma, diagnosed within the previous 5 years, or any history of metastatic cancer, 11) History of use of oral supplement products containing probiotic bacteria (more than once per week) within four weeks prior to

baseline, 12) Current use (within the past 8 weeks) of any medication with known influences on the immune or skeletal system (e.g. immune modulation therapy, systemic glucocorticoids, systemic steroid hormones, 13) Use of oral or injectable bisphosphonates for more than 1 year within the last 5 years, 14) Current or past use (within 1 year) of Denosumab, Teriparatide, Raloxifene, hormone replacement therapy (HRT), calcitonin, or any other anti-resorptive agent other than bisphosphonates used for the prevention and treatment of osteoporosis, 15) Use of antibiotics during the previous two months or frequent user of antibiotics (>2 courses during the previous 12 months) for any cause, 16) Smoking or use of nicotine-containing products during the last six months, 17) Known hypersensitivity to any of the ingredients in the VSL#3 or the placebo study drug, 18) serum or plasma 25-hydroxyvitamin D [25(OH)D] concentration < 12 ng/mL, 19) uncontrolled thyroid disease (abnormal blood TSH level within the last 12 months and/or changing dose of thyroid replacement therapy within the last 12 months).

3.3. Number of patients to be studied: We propose to study a total of 40 patients. A total of 20 subjects will receive a placebo (control, 2 placebo sachets/day in a single dose), and 20 subjects will receive VSL#3 (2 sachets a day in a single dose).

3.4. Subject recruitment plan: We will employ several methods for identifying potentially eligible participants:

a) We will post approved flyers throughout Emory-affiliated hospitals and clinics, Emory University and the surrounding Atlanta metropolitan area.

b) We will utilize the study subject recruitment resources of the NIH-funded Atlanta Clinical and Translational Science Institute (ACTSI), including its weekly list-serve, clinical trial websites of Emory HealthCare, and ResearchMatch.org.

c) Subjects will also be recruited among participants in an existing cohort of Emory University and Emory Healthcare employees, the Emory/Georgia Tech Predictive Health Initiative (PHI) Cohort, who have consented to be contacted for potential study participation in future research and who meet initial screening criteria based on the most recent data available (within previous 12 months)[15].

d) Participants may also be recruited by advertising (via flyers or in person) at health fairs and other Atlanta community events.

e) We will approach potentially eligible subjects among patients seen in Emory HealthCare-affiliated Endocrinology, Obstetrics/Gynecology, Rheumatology and General Medicine clinics in Atlanta, GA. This will involve a prescreen of the electronic medical record facilitated by a partial HIPAA waiver for screening purposes. We will also identify potentially eligible subjects among patients who have undergone a recent bone density scan at Emory clinics and/or whose data is entered into the Emory BoneStation electronic record.

f) Through the Emory Data Warehouse, we will receive a list of patients in the Emory system who meet eligibility criteria and who have agreed to be contacted about research studies either by phone or through a letter.

g) Individual participating Emory clinics will generate a list of clinic patients who meet eligibility criteria and will send a letter (directly or through the study team) to potentially eligible patients describing the study and providing study contact information.

h) A combination of the above three (e-g) strategies may be used, but the research team will only approach potential study participants in person (during clinic visits) or by letter unless there is available documentation that a potential participant is agreeable to being contacted by phone.

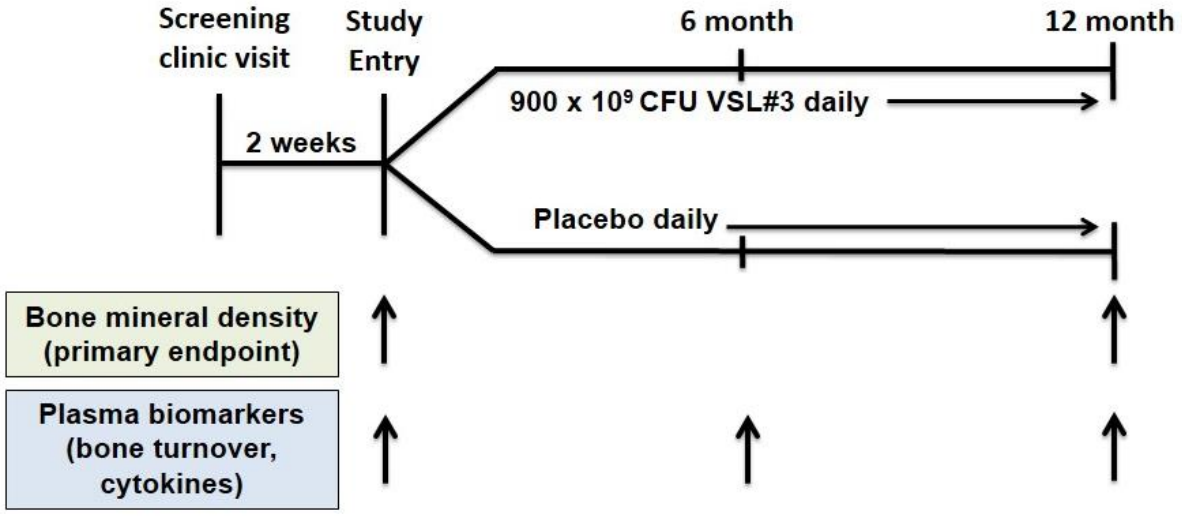
3.5 Study withdrawal: Participants may withdraw from the study at any time. Automatic withdrawal occurs when a participant refuses or is unable to take study drug, or refuses or is unable to complete study procedures, including the return visits to the Clinical Research Center at 6 and 12 months and the twice monthly study-related telephone calls required to ensure tolerance/compliance with the VSL#3 and placebo.

4. STUDY DESIGN AND METHODS

4.1 Overview of the study: This will be a 12-month, double-blind, randomized, placebo-controlled trial of VSL#3 or placebo (**Figure 1).** Participants will be randomized to the placebo group or the VSL#3 group and treated and followed serially for 12 months. Baseline and follow-up assessments will be performed during an outpatient visit in the Emory University Hospital (EUH) Clinical Research Network (CRN) site of the Atlanta Clinical and Translational Science Institute (ACTSI) before initiation of the study drug (entry), and 6, and 12 months following baseline assessments. Subjects will report to the CRN after having fasted for at least 8 hours.

Figure 1: Study schema for protocol "Use of a Commercially Available Combination Probiotic, VSL#3, for Bone Health in Postmenopausal Women"

The study will be conducted in a population of ambulatory, postmenopausal women aged 50-65 (BMI >18.5 and ≤ 30 kg/m^2), with a lumbar spine (L1-L4), femoral neck, and total hip BMD T-score > - 2.5, as measured by DXA (see section 3 above). Women with osteoporosis (T score ≤ -2.5) will not be included in the study because at this stage of knowledge we find it unethical not to offer effective treatment to these individuals. We will recruit subjects of all ethnic groups. Covariates known to influence bone mass, including body mass index (BMI), history of smoking > 6 months prior to entry, alcohol use, baseline BMD of the lumbar spine, femoral neck and total hip area, bone fracture history and co-morbidities will be collected and controlled for in the final analysis. The primary endpoint is the change in bone mineral density (BMD) at the L1-4 lumbar spine over one year of study. Changes in BMD at the femoral neck and total hip area will be secondary endpoints. All BMD data will also be used as a tool for future studies power calculation and design. Additional endpoints will include changes in bone turnover markers and inflammatory/osteoclastogenic cytokines. These indices will provide much needed mechanistic information. To determine additional potential mechanisms of action of probiotics on health, we will conduct serial metabolomics analyses in the plasma, as well as serial assessment of plasma thiol/disulfide redox balance. The data will allow us to establish whether VSL#3 prevents bone loss and/or increases bone mass by regulating bone resorption, formation, or both. Based on our own data in mice following ovx, we hypothesize that treatment with VSL#3 will quickly decrease bone resorption and then stimulate bone formation, resulting in improved net BMD at least one site over the 12-month period of study.



4.2. Screening for eligibility: For study subjects identified among patients seen at the Emory-affiliated Endocrinology, Ob/Gyn, Rheumatology and General Medicine clinics, patients will be pre-screened for eligibility using the Emory Healthcare electronic medical record, and potentially eligible subjects will be approached during their clinic visit by a study coordinator or they will be contacted by phone or letter (as described above) to conduct a full screen. For potential study subjects identified outside of Emory-affiliated Clinics, potential subjects will contact the study team by phone and undergo a pre-screening discussion.

A full screen will consist of any screening medical/surgical and medication use information, blood and urine tests, screening DXA scan, and a physical examination. These may occur in the Emory-affiliated clinics by study personnel and/or in the Emory University Hospital CRN. Informed consent will be obtained prior to any research procedures. A verbal informed consent will be obtained from potential participants being pre-screened by phone prior to collecting information regarding eligibility criteria. In order to reach our goal or 40 randomized participants, up to 100 women may undergo a screening visit.

4.3 Informed consent process: A written and signed informed consent will be obtained in person in a private area. After identification of a person as potentially eligible for the study, approved study personnel will explain the study in detail in the consent form as well as verbally. Potential participants will be informed of the purpose of the study, the study protocol, and the routine and potential risks associated with the study procedures. All risks, costs, and benefits will be discussed. Participants will be assured of their right to withdraw at any time without prejudice to their care. They will be assured of confidentiality in maintaining records and reporting of results. Participants will have the option to provide permission to store biological samples for future studies. Any additional information regarding questions or concerns from potential or confirmed study participants will be provided.

We will request a partial HIPAA waiver to be able to access the medical record for screening.

For subjects recruited in Emory Clinics, the consent process will take place during a patient's clinic visit. Potential study participants will have the option of taking the blank, unsigned consent forms home to consider prior to signing the consent forms, in which case patients who decide to participate will complete a verbal consent by telephone and will complete the full, written consent process during their first study visit (prior to any procedures). For subjects recruited outside of Emory clinics, the consent process will take place during their EUH CRN screening visit. Participants will have ample time to call the study team with questions/concerns and make an informed decision about their participation in the study.

An Institutional Review Board (IRB) approved study designee will provide verbal and written consent. The study is discussed thoroughly with each potential participant, and she will be encouraged to ask questions regarding the study and her participation. If the potential participant decides to enroll, she will sign the written informed consent, as will the study team member conducting informed consent. A copy of the signed consent form will be provided to enrolled study participants. Subjects must be able to read, write and understand the consent documents. Study personnel will subjectively assess the capacity to give informed consent through the use of questions to ensure understanding and comprehension of the study. Only adults will participate.

4.4. Study Initiation and baseline assessments: Following screening and informed consent, baseline assessment and laboratory evaluation will be performed as outlined in **Table 1**. Dietary calcium and vitamin D intake will be reviewed by a registered dietitian. For participants not meeting at least the Recommended Dietary Allowance (RDA) for calcium (1200 mg) and vitamin D (600 IU), a recommendation will be provided for sufficient over-the-counter calcium citrate and/or vitamin D3 to meet the RDA. Participants will be allowed to continue their current regimens; however, they will be asked not to begin any new supplement, medical, or exercise therapies that could influence bone health. For individuals with a screening 25(OH)D < 20, a study physician will communicate with the participant's primary MD (and/or the participant directly) and recommend a standardized dose of vitamin D replacement (daily vitamin D_3 dose of 5,000 IU daily + 50,000 IU weekly for 12 weeks with a recheck of 25(OH)D level in 3-4 months).

Table 1. Study Endpoints by Visit

Assessments	Screen in clinic or CRN unit	Entry visit in CRN unit (max 4 weeks after screen visit)	6 mo visit in CRN unit (+/- 1 month)	12 mo visit in CRN Unit (+/- 1 month)
Medical/Surgical history, medication use, dietary calcium intake, alcohol intake, physical activity	x		x	x
Serum FSH (if indicated)	x			
Physical Exam	x		x	x
Concomitant medications	x	x	x	x
Gastrointestinal Symptom Rating Scale (GSRS)		Determine GCRS every 2 weeks →		
Study drug adherence assessment		Determine adherence every 2 weeks →		
Fasting serum markers of bone turnover (CTX and PINP)		x	x	x
Serum RANKL (free), OPG, TNF, IL-17		x	x	x
DXA scan: (lumbar spine, femoral neck, and total hip, total and regional body fat, total and regional lean body mass, visceral fat)*	x	x		x
Dietary Intake 3-day food record		x	x	x
BioElectrical Impendence Analysis (BIA) Body composition and total body water		x	x	x
Anthropometrics (height, weight, BMI, waist & hip circumference)				
Handgrip strength Hand-held dynamometer		x	x	x
Serum 25(OH)D	x			x
Stool microbiota composition		Xx	Xx	Xx
Plasma metabolomics		x	x	x
Plasma Aminothiols (GSH, GSSG, Cys, CySS)		x	x	x
Serum Intact PTH	x			
Complete blood count (CBC)	x		x	x
Chemistry profile	x		x	x
Urinalysis	x		x	x

*Note: First DXA scan will be done at the screening visit or the entry visit (not both).

4.5 Intervention: A total of 40 matched postmenopausal women meeting all specific inclusion/exclusion criteria will be blindly randomized to one of two study arms. **Arm A** (n=20): VSL#3 two active sachets [containing $450x10^9$ colony-forming units (CFU)/sachet], taken daily in a single administration; **Arm B** (n=20; control): two placebo sachets taken daily in a single administration. Thus, Arm A will receive 900 billion CFU daily and the control group (Arm B) will receive no supplemental probiotics (**Figure 1**).

4.6. Randomization and Stratification: Personnel in the Department of Biostatistics and Bioinformatics at Emory University have developed a centralized electronic randomization system that has been successfully used in several clinical studies [16-19]. Subjects will be blindly assigned in equal proportions, stratified by age (above and below 58 years) using a pseudo-random-number generator with randomly permuted blocks. These assignments will be stored in a backend database table within the iDataFax database management system. This method serves to balance the treatment group assignments over the course of the study to ensure that the desired number of subjects will be allocated to each of the treatment groups at any time point during the randomization period. Permuted block sizes will not be disclosed to the blinded study personnel to minimize the likelihood of their being able to predict the next randomization assignment in the series. Briefly, after obtaining informed consent, all eligibility confirmation data will be entered into the web-based data management system by a research coordinator. If the patient meets all eligibility criteria, computer randomization will be performed and the coordinator will be provided with an audit trial of the process including a verifiable link between the unique study ID and treatment assignment. The application will be password protected with access restricted to designated personnel, including the EUH Investigational Drug Pharmacy staff pharmacist who **will dispense blinded study drug for subjects randomized to Arms A or B.**

4.7. Screening, Entry and Follow-up Evaluations: These will occur as outlined in Table 1. Adverse effects and safety data will be reviewed at least every 6 months by a Data Safety Officer, a gastroenterologist senior Emory faculty member with expertise in clinical trials who is unaffiliated with the study, as described below (Section 7.5).

4.8. Procedures to be performed (all for research purposes only):

- **Medical examination:** Medical history and medications will be reviewed by a member of the research team, and a physical exam will be completed by a licensed physician, as required by the clinical research unit. Habitual alcohol intake and dietary calcium intake (based on reported supplements, dairy, and other high-calcium foods) will be assessed.
- **Urine collection:** Urine will be collected for standard urinalysis indexes.
- **Anthropometric measurements:** Height and weight will be measured without shoes. Height will be measured with a manual stadiometer. Weight will be measured with a digital scale. Body mass index (BMI) will be calculated from height and weight. Waist and hip circumference will be measured with a flexible measuring tape.
- **Blood sampling:** Up to approximately 70 mL of fasted blood will be collected at every visit from a peripheral vein. Blood will be drawn for determination of plasma and serum biomarkers, plasma metabolomics, plasma redox markers, and standard clinical blood tests listed in **Table 1** above. Blood will also be stored for use for future studies, with consent from the study participant.
- **Stool collection:** Study subjects will be provided with a kit containing detailed instructions for stool collection and shipping. The kit contains a stool sample shipping tube, an adhesive paper for stool collection, a spatula for placing a small amount of stool into the tube, a biosafety bag and a pre-paid FedEx envelope for shipping the stool sample directly to the study lab within 48 hours.
- **Bone density analysis:** DXA will be used to measure bone density of the spine (L1-L4 segment) and the hip (non-dominant femoral neck and total hip area). Participants will be asked to lie still on a scanning table with their arms at their sides for approximately 20 minutes. All DXA scans will be performed on the same device within the Emory CRN by an experienced technician using a GE Lunar iDXA machine. Areas of interest will include the lumbar spine (L1-4), the femoral neck and the total hip). DEXA will also provide information about total and regional body fat, lean body mass.
- **Three-day food records:** Participants will be asked to complete a food diary for three days (2 weekdays and 1 weekend day) where they will record everything they eat and drink, including supplements. They will be given food records prior to their study visit and asked to return completed food records on the day of their visit. Alternatively, participants may choose to email or mail study investigators the completed food record.
- **Body composition analysis:** In addition to DEXA, body composition may also be assessed using bioelectrical impendence analysis (BIA); for this procedure participants will lie still on a table for <5 minutes with electrodes placed on hands and feet.

- **Handgrip strength:** A small, handheld dynamometer will be used to measure grip strength. Participants will be asked to squeeze the instrument as hard as they can for 3 seconds, which will measure the pounds of force applied.
- **Questionnaires:** Participants will be asked to answer a standardized, validated questionnaire (15 questions) about GI symptoms segregated into 5 symptom domains (see below). They will also complete a questionnaire assessing their habitual physical activity (Modified Baecke Questionnaire)[20].

4.9. Primary Endpoint: The **primary endpoint is** change in BMD of the lumbar spine (L1-L4 segment), as measured by DXA.

4.10 Secondary Endpoints (please see Table 1 above): Serial measures of: 1) bone density of the non-dominant hip (femoral neck and total hip area) as measured by DXA, 2) serum collagen type 1 cross-linked C-telopeptide (CTX) concentrations (a marker of bone resorption), 3) serum procollagen type I N propeptide (PINP) concentrations (a marker of bone formation), 4) serum free receptor activator of nuclear factor kappa-B ligand (RANKL) concentrations, 5) serum osteoprotegrin (OPG) concentrations, 6) serum tumor necrosis factor-α (TNFα), 7) serum interleukin-17 (IL-17) concentrations, 7) total and regional body fat (including visceral fat) and lean body mass as measured by DXA and BIA, 8) plasma thio/disulfide redox biomarkers, 9) compliance with study drug administration10) overall study recruitment and retention, and 11) **) stool microbiota composition**. Measurements of indices of bone turnover and cytokine levels will provide much needed mechanistic information to establish whether VSL#3 may prevent bone loss and/or increases bone mass by regulating bone resorption, formation, or both. Based on animal data, we hypothesize that treatment with VSL#3 will quickly decrease bone resorption and then stimulate bone formation.

Study drug tolerance will be assessed by obtaining serial measures of the Gastrointestinal Symptom Rating Scale (GSRS) every two weeks during the 12-month placebo or probiotic regimen [21]. These will be obtained with in-person interviews at the baseline and month 6 and 12 visits and by telephone contact with all subjects by the study coordinator every 2 weeks. Data will be analyzed within the 5 symptom domains depicting symptoms related to gastric reflux, abdominal pain, indigestion, diarrhea, and constipation. A GSRS total score, the sum of all 5 domains, will also be assessed. To our knowledge there is no published absolute or change in the GSRS score that is demonstrative of intolerance to a study drug or other treatment [21-23]. Therefore we will *a priori* define intolerance to the study drug as a 50% increase in the total GSRS score from baseline at any two consecutive time points, or the new onset of a GSRS classifier score of 2 or 3 in at least three of the 15 gastrointestinal symptoms queried. If either of these signs of intolerance occurs, we will offer the subject the options of: 1) discontinuing from the study; 2) discontinuing the study drug but participating in the serial assessments (e.g. blood draws, DXA) in the research center, or 3) decreasing the dose of blinded study drug to one sachet/daily, with the option of resuming the two sachet/day study regimen upon resolution of symptoms. If intolerance symptoms recur, the same strategy will be followed. All episodes of intolerance to study drug and the twice-monthly GSRS data will be recorded by the study coordinator in the individual subject case report forms (CRFs) (see below).

Compliance with the study placebo or VSL#3 doses will be assessed serially. This will be determined by contacts (twice monthly telephone or at research center visits) of the study coordinator with each subject and responses to three standardized question areas: 1) “Are you having any difficulty, problems or new symptoms with the study medication?” 2) If yes, “What has the problem been?” and 3) “Have you missed any of your study drug doses, and if so how many in the previous 2 week period?” Appropriate notations based on subject responses will be documented in the CRF. Subjects will be instructed at study entry and reminded via serial contacts to return all of their used and unused drug sachets at each CRN visit (6 months +/- 2 weeks and 12 months +/- 2 weeks after entry). Unused and used study drug satchets will be tallied and recorded in the CRF by the study coordinator serially for the entire study. All satchets (used and unused) will be given to the Emory University Investigational Drug Service (IDS) for disposal.

Recruitment and retention will be documented via conventional CONSORT criteria and documentation of missed study visits, missed telephone communications and compliance with study drug administration. All data will be maintained in the CRFs.

4.13. Sample size and power considerations: Longitudinal data from 380 postmenopausal women form the basis for estimating the sample size and power for lumbar spine BMD as the primary outcome endpoint. BMD at the lumbar spine was available at baseline and 6 and 12 months after baseline (883 measurements across the 3 time points). Mean baseline BMD was 1.108 g/cm^2 (within-subject standard deviation = 0.03 g/cm^2 and the standard deviation on change = 0.02 g/cm^2). Assuming no decline on average in BMD in the VSL#3 treated group and a decline on average of 0.03 g/cm^2 in the control group and an estimated within-subject standard deviation in each group of 0.03 g/cm^2, the proposed sample sizes (n=20 postmenopausal women per group) will provide more than 85% power to detect a difference on change of 0.03 g/cm^2 at the two-sided 5% significance level if the true difference between treatment groups is 0.03 g/cm^2 (two-sided two-sample equal variance t-test).

4.14. Statistical analyses: Demographic data will be summarized with standard descriptive statistics. Repeated-measures analyses for each outcome (i.e., GSRS scale scores, BMD) will be performed with a means model via the SAS MIXED Procedure (version 9.4; SAS Institute, Cary, NC), providing separate estimates of the means by time on study (baseline, 1-year and 2-years) and treatment group. The model will include three predictors (treatment arm, time on study and the statistical interaction between treatment arm and time on study). A compound-symmetric variance-covariance form in repeated measurements will be assumed for each outcome and robust estimates of the standard errors of parameters will be used to perform statistical tests and construct 95% confidence intervals[24]. The model-based means are unbiased with unbalanced and missing data, so long as the missing data are non-informative (missing at random, MAR). A P-value ≤0.05 will be considered statistically significant for the main effects (treatment and time on study) and for the treatment by time on study interaction effect from the repeated measures analysis. The statistical test for interaction between time on study and treatment will be the primary overall hypothesis test to determine whether an outcome in the two study groups changed in significantly different ways during follow-up (i.e., different temporal patterns over time). If a mean outcome in the two groups is consistently different or similar over time (i.e., no statistical interaction) then the main effect test for treatment will be used as the primary test of VSL#3 efficacy. If a significant interaction is detected, then t-tests will be used to compare the differences between the model-based intervention means at each time point and to compare differences over time within each treatment arm. Specific statistical tests will be done within the framework of the mixed effects linear model. Analyses will control for risk factors for osteoporosis, such as age, gender, race, BMI, habitual alcohol consumption, smoking, physical activity, and fracture history, as necessary. All tests will be 2-sided and a P value ≤ 0.05 will be considered significant.

5. DRUG DOSING

5.1. Method for determining doses: Doses were based on the concentration of colony forming units (CFU) within a single, commercially available sachet, and doses used in previous trials in adults with inflammatory bowel diseases (IBD), irritable bowel syndrome, and other gastrointestinal (GI) disorders [25-31].

5.2. Maximum Dosage: The maximum dosage will be 900x10^9 CFU orally daily. This or higher doses have been used in most trials designed to assess the tolerability and efficacy of VSL#3 in IBD and irritable bowel syndrome in adults.

5.3. Drug duration: Patients will take the drug for 1 year.

5.4. Distribution and Storage of Investigational Products: The study drug/placebo will be delivered to the EUH Investigational Drug Service (IDS) directly from the manufacturer. The EUH IDS will store the study drug/placebo under the appropriate storage conditions, as indicated by the manufacturer. The Pharmacy will dispense the study drug/placebo to study subjects (who will remain blinded). Subjects will be provided with sufficient study drug product every two months. IDS will dispense a 2-month supply of the study drug sachets to the study team for each subject serially, who will over-night mail the resupply with a commercial carrier in sealed Styrofoam containers containing cold packs. The IDS will provide subjects with instructions for study drug storage at home. For all products provided, the lot number and expiration date will be recorded in individual subjects case report forms. All study drug products will be labeled with the following, “Caution: New Drug—Limited by Federal (United States) law to investigational use.”

6. OBSERVATIONS AND MEASUREMENTS FOR STUDY OBJECTIVES

Please see Sections 4.8-4.10 and **Table 1** above.

7. PROCEDURES TO MONITOR DRUG EFFECTS AND MINIMIZE RISK

7.1 Potential Risks/Discomforts

The risk of participating in this study is minimal.

a. **VSL#3:** There are few reported side effects of this dietary supplement. Some studies have reported GI effects, such as bloating abdominal pain. Of note, VSL#3 doses of up to $3600x10^9$ CFU orally daily for several weeks or months have been reported to be well-tolerated in adults with irritable bowel syndrome, Crohn's disease, and other GI disorders [32-37].
b. **Venipuncture:** There is some minor discomfort and risk of mild bruising during venipuncture. Standard aseptic techniques will be used during phlebotomy; thus, infection is unlikely. Disposable pre-sterilized needles and syringes will be used for all blood drawing in this study; needles and syringes will not be reused.
c. **DXA:** DXA involves exposure to small amounts of radiation. The radiation dose is equal to or less than the amount of background radiation received in a round-trip flight from New York to Los Angeles or the natural environmental radiation the average person receives in the United States annually. The risk from radiation exposure of this magnitude is considered to be negligible when compared to everyday risks. For screening purposes, if menopausal status is not clear, a urine pregnancy may be performed prior to DXA testing.
d. **Stool collection:** There may be minor inconvenience from the process of collecting a small stool sample, placing a stool sample into the shipping tube and shaking it for a few seconds to mix.
e. **BIA:** The BIA machine passes a weak electrical current through electrodes attached to hands and feet. Participants will not feel anything. There are no known risks associated with the procedure, although participants who have pacemakers or other implantable electronic devices will not undergo the BIA procedure.

f. **Other:** Additional risks will be associated with confidentiality issues surrounding the collection/recording of data, but steps will be taken to minimize these risks, as described below.

7.2. Assessment of Risk/Benefit

a. **Direct benefit:** Participants will be compensated a stipend for their time and inconvenience. Participants will also receive information on their measured bone density after the 12-month period. Participants will be encouraged to share the results with their healthcare providers. There may be no specific health benefit to patients' participation in the study.
b. I**ndirect benefit:** The long-term goal of this study is to identify a safe, well tolerated and simple intervention strategy, using a commercially available combination probiotic to minimize bone loss associated with menopause. If our hypothesis is correct, VSL#3 would be an inexpensive, easily obtainable commercial probiotic that could be supplemented in this population.
c. The anticipated benefits of the proposed research study outweigh the potential risks to participating subjects. The risks incurred are minimal relative to the potential benefit of finding an effective therapy to reduce bone loss in postmenopausal women.

7.3. Procedures to monitor drug effects

Patients will be called every other week to maintain compliance/adherence with the study drug regimen and to monitor for any adverse effects. Questions will also focus on gastrointestinal symptoms (via the GSRS score), as these are the primary expected side effects of VSL#3. In addition to twice monthly phone calls, subjects will be asked to complete **Subject Diary Cards** to capture adverse events during the course of the trial and up to 1

month following the cessation of the study drug. Subjects will receive a final safety follow-up phone call 1 month following the cessation of the study drug.

Systemic reactions and adverse events (AEs) (e.g. diarrhea, headache, fatigue, rash, and myalgia) will be monitored every other week during study phone calls. The intensity of these events will be assessed on a standard toxicity grading scale for healthy volunteers[38], as shown in **Table 3**. Standard laboratory values will be assessed at baseline, 6 month and 12 month visits and graded as shown in **Table 4**. Decisions to withdraw the study drug from individual subjects, will be at the discretion of both the PI and a Safety Officer (described below), based on a severity grade of 3 or 4 for Graded Adverse Events or Laboratory Values, and based on the likelihood of it being related to study.

Table 3. Graded Adverse Events

Adverse Event	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially Life Threatening (Grade 4)
Nausea/vomiting	No interference with activity or 1 – 2 episodes/24 hours	Some interference with activity or > 2 episodes/24 hours	Prevents daily activity, requires outpatient IV hydration	ER visit or hospitalization for hypotensive shock
Diarrhea	2 – 3 loose stools or < 400 gms/24 hours	4 – 5 stools or 400 – 800 gms/24 hours	6 or more watery stools or > 800gms/24 hours or requires outpatient IV hydration	ER visit or hospitalization
Headache	No interference with activity	Repeated use of non-narcotic pain reliever > 24 hours or some interference with activity	Significant; any use of narcotic pain reliever or prevents daily activity	ER visit or hospitalization
Fatigue	No interference with activity	Some interference with activity	Significant; prevents daily activity	ER visit or hospitalization
Myalgia	No interference with activity	Some interference with activity	Significant; prevents daily activity	ER visit or hospitalization
Rash/Erythrema	2.5 – 5 cm	5.1 – 10 cm	> 10 cm	Necrosis or exfoliative dermatitis
Pruitis/Itching	No interference with activity	Some interference with activity	Significant; prevents daily activity	ER visit or hospitalization
Fever	38.0 – 38.4 °C 100.4 – 101.1°F	38.5 – 38.9°C 101.2 – 102.0°F	39.0 – 40°C 102.1 – 104 °F	> 40°C > 104 °F
Tachycardia - beats per minute	101 – 115	116 – 130	> 130	ER visit or hospitalization for arrhythmia
Bradycardia -	50 – 54	45 – 49	< 45	ER visit or

beats per minute***				hospitalization for arrhythmia
Hypertension (systolic) - mm Hg	141 – 150	151 – 155	> 155	ER visit or hospitalization for malignant hypertension
Hypertension (diastolic) - mm Hg	91 – 95	96 – 100	> 100	ER visit or hospitalization for malignant hypertension
Hypotension (systolic) – mm Hg	85 – 89	80 – 84	< 80	ER visit or hospitalization for hypotensive shock
Respiratory Rate – breaths per minute	17 – 20	21 – 25	> 25	Intubation
Other illness or clinical adverse event (as defined according to applicable regulations)	No interference with activity	Some interference with activity not requiring medical intervention	Prevents daily activity and requires medical intervention	ER visit or hospitalization

Table 4. Laboratory Adverse Events

Blood Parameter	Reference Range*	Mild/Moderate	Severe/Critical
Sodium – Hyponatremia mmol/L	136-144	126-135	≤125
Sodium – Hypernatremia mmol/L	136-144	145 – 155	≥156
Potassium – Hyperkalemia mmol/L	3.6-5.1	5.2-6.0	>6.0
Potassium – Hypokalemia mmol/L	3.6-5.1	3.0-3.5	<3.0
Glucose – Hypoglycemia mg/dL	65 – 110	45-64	<45
Glucose – Hyperglycemia mg/dL	65 – 110	111-450	>450, or insulin requirement, or hyperosmolar coma
Blood Urea Nitrogen, BUN mg/dL	8-25	26-150	> 150 or requires dialysis
Creatinine – mg/dL	0.4-1.0	1.1-5.8	>5.8 or requires dialysis
Calcium – hypocalcemia mg/dL	8.9-10.3	6.6-8.8	≤6.5
Calcium – hypercalcemia mg/dL	8.9-10.3	10.4-12.9	≥13.0
Albumin – Hypoalbuminemia g/dL	3.5-4.8	<3.5	------
Total Protein – Hypoproteinemia g/dL	6.1-7.9	<6.1	------
Alkaline phosphate unit/L	32-91	<91	------
ALT unit/L	≤ 33	>33	------
AST unit/L	15-41	>41	------
Bilirubin mg/dL	0.3-1.2 mg/dL	1.3-20	>20
Cholesterol mg/dL	≤ 199	>199	------
Hemoglobin (Female) - g/dL	11.4-14.4	6.6-11.3	≤ 6.5
WBC low x 10^3/mcL	4-10	2-3.9	< 2.0
WBC high x 10^3/mcL	4-10	11-50	>50
Eosinophils x 10^3/mcL	<0.36	>0.37	Hypereosinophilic
Platelets-high x 10^3/mcL	150-400	401-999	>999
Platelets-low x 10^3/mcL	150-400	51-149	<50

Urine Parameter	Reference Range	Mild/Moderate	Severe/Critical
Protein	none	trace	Hospitalization or dialysis
Glucose	none	trace	Hospitalization for hyperglycemia
Blood (microscopic) – red blood cells per high-power field (RBC/hpf)	none	trace	gross blood or hospitalization or packed red blood cells (PRBC) transfusion

*Specific to EUH Laboratories

7.4. Additional protection against risks

7.4.1 Subject identification and screening for eligibility: Our strict inclusion and exclusion criteria for entry will help to minimize potential risks. All subjects will be monitored closely by frequent clinical assessments and review of safety laboratory tests by experienced clinicians.

7.4.2 Blood sampling: Experienced research nurses will use aseptic techniques for all study blood draws. Participants will be asked to stay hydrated with water the day before the study visit. Universal precautions will be employed in all instances involving human specimens and all blood processed under at least BSL2 conditions. Discomforts associated with venipuncture are rapidly reversible.

7.4.3 DXA: DXA will be performed by experienced technicians who are trained in radiation exposure minimization in patients.

7.4.4 Privacy and confidentiality: The subjects' extracted medical records and data from the study will be initially stored on paper prior to transfer to electronic format. The encrypted electronic record will remain on the principal investigator's password protected computer in his locked office. Confidentiality will be assured by the use of subject codes rather than personal identifiers. The master list connecting the subject codes to identifying information will be secured in the PI's computerized database. All paper subject records will be kept in locked file cabinets in the PI's research office and will be accessible only to the PI and the investigative team. Only institutional review board (IRB)-approved study personnel will have access to individually identifiable information about human subjects. This may include the study PI, co-investigators, research coordinators, and other approved study personnel. All information and materials will be obtained for research purposes only and the data will be kept in strict confidence for use in this proposed research only.

7.4.5 Other: Since the proposed tests are not inherently hazardous, hazard is likely to occur only as the result of impaired participant confidence or sudden unwillingness to complete a test. To avoid this possibility, study personnel will thoroughly explain all tests to potential participants prior to them signing the consent form. Potential participants will have the opportunity to see all test equipment and facilities before giving consent or undergoing testing. Trained personnel will be available at all times to monitor participants' physical and emotional distress. Every effort will be taken to prevent injury or distress that may result from this study. A study physician will be on call for support. A qualified physician will have primary responsibility for outpatient care.

7.5. Data and Safety Monitoring and Reporting: Trained study personnel will be with a participant at all times to monitor patient safety. Adverse events are expected to be uncommon and not pose more than minimal risk to the study participants. The Principal investigator (PI) will review all data collection forms at least semi-annually for completeness and accuracy of the data as well as protocol compliance. The PI will review this protocol on a continuing basis for subject safety and include the results of the review in annual progress reports submitted to the IRB.

7.5.1 Data Safety Officer: A senior faculty Data Safety Officer, Dr. Jennifer Christie, MD, FASGE, Associate Professor of Medicine in the Emory University Division of Digestive Diseases, Clinical Director of Gastroenterology at the Emory Clinic, and Director of Gastrointestinal Motility has been designated for this study. Dr. Christie is an experienced physician-scientist with expertise in randomized controlled clinical trials and translational science. She will review semi-annual reports created by the Principal Investigator (PI) Dr.

Roberto Pacifici and the Data Coordinating Committee, directed by Mr. Kirk Easley, MS, and make recommendations regarding the continuation of study.

7.5.2 Participant Safety Data Examination, Monitoring Procedures/Oversight: Participant monitoring will be performed by the PI, Co-Investigators, and members of the study team. The IRB Reportable Events Guidelines will be followed. The standard Emory IRB reporting guidelines for AE and SAE reporting will also be followed. Any adverse events will be reported to the IRB within 10 days of the event if it is (a) an unanticipated problem involving risk to participants or others, (b) a death that is possibly, probably, or definitely related to the research, (c) an anticipated event occurring with a greater frequency, duration, or severity than what is described in the protocol related documents, or (d) any other information that suggests the research places participants or others at greater risk of harm than was previously known. A reportable events log will be maintained by the PI and reported annually to the IRB (or more frequently if required.)

All adverse events will be graded as to their attribution (unrelated to protocol, or possibly, probably, or definitely related to protocol). Any AE that is reported to either the PI or the study team by a study subject or by medical staff caring for the subject and which meets the criteria will be documented as such. Adverse events will also be further labeled as “expected” AEs (which are predefined and listed as potential risks in the informed consent), “unanticipated” AEs, and serious adverse events. Serious adverse events (SAE) are predefined as: any experience that suggests a significant hazard, such as events which: a) are fatal, b) are life threatening, c) result in permanent disability, d) require inpatient hospitalization, or e) involve cancer, a congenital anomaly, or drug overdose.

7.5.3 Performance Standards: The Data Coordinating Center (DCC) has worked closely with the clinical team during protocol development to specify performance standards that should be met to achieve high quality of trial conduct. These standards will address screening and recruitment plans and rates, retention plans and rates, missing data, definition of dropout, the intention-to-treat principle (both as a design and analysis issue), completeness of data, procedural adherence, quality control plus plans to address problems that may arise among these interrelated issues. The proposed trial affords investigators an opportunity to revise and standardize the recruitment and retention plans that will be helpful for future trials and cohorts.

7.5.4 Recruitment, Retention and an Intent-To-Treat Design: The clinical investigators and the DCC will work together to establish successful strategies for patient recruitment and patient retention. Screening and recruitment reports will be generated monthly that include actual and expected recruitment statistics. Strategies to address potential problems with recruitment include every two-week telephone calls to each subject to address treatment/intervention adherence, medical record review of previous compliance with study visits, and patient education during the informed consent process about the scientific relevance of their data even if they discontinue the intervention, and the deleterious effect that missing data has on study integrity and credibility. Wording to this effect has been successfully added to informed consent forms to enable patients to make more informed decisions about their willingness to join a trial and to participate in continued follow-up without feeling inappropriately pressured to do so [39].

Plans to minimize missed visits/assessments and minimize dropout will be an important focus during study design. Randomization alone is not sufficient to provide unbiased comparisons of interventions, but one sufficient condition to provide an unbiased comparison is to obtain complete data on all randomized participants. Therefore retention of the cohort is key to ensuring an intention-to-treat design and intention-to-treat analysis (full compliance with treatment/intervention, no missing data and complete follow-up of all participants). Only intent-to-treat analyses fully preserve the validity of assumptions between intervention groups established by randomization.

Valid reasons a patient can be off study include withdrawal of consent at any time during the study or the achievement of all required efficacy and safety end point information. Withdrawal of consent will be used only when the patient no longer wishes to participate in the trial and no longer authorizes the investigators to make efforts to continue to obtain their outcome data. Valid reasons for non-adherence to treatment include an inability to tolerate the intervention, toxicity, physician choice or need for other therapies. All patients will continue to be followed with all scheduled outcome evaluations until the end of the study. This strategy will

allow a true all-inclusive, intent-to-treat design and analysis. Patients will not be labeled 'dropouts' but as 'temporarily inactive' and the patient will be welcomed back to the trial when possible. The designation of 'lost-to-follow-up' will be applied at the end of the trial to patients that remained inactive. Monthly reports will summarize recruitment and retention rates, treatment adherence rates, observed and expected data on attendance to routine clinical visits and the degree of completeness of data capture. The DCC will establish performance standards before the trial. These standards will be reviewed and revised as needed during the trial to enhance the quality of trial conduct.

7.5.5 Data Management Considerations: The DCC will establish processes and work-flow to create and maintain a high quality research database for the trial while preserving the confidentiality of all subjects' data. It is difficult to implement a statistical analysis plan unless a data management plan has been followed to assure data quality and procedural adherence. Quality control will be applied to each phase of data handling to ensure that all data are reliable and have been processed correctly.

7.5.5.1 Case Report Forms (CRFs): The clinical investigators and the DCC will collaborate on the design of CRFs for each study. All CRFs will be designed using Adobe InDesign. Forms questions and lay-out will be optimized for ease of forms completion and ease of data entry.

7.5.5.2 Design of the Data Management System: CRF data as defined and dictated by protocol will be collected and managed using iDataFax or web-based tools (DF Web). iDataFax is a commercially available electronic data capture (EDC) system used to enter, review and modify patient data, and to submit it over the internet to a DataFax server maintained at the Rollins School of Public Health at Emory University. iDataFax provides secure 128 bit SSL encryption of all data and document transmissions over the internet. DataFax is fully 21 CFR Part 11 compliant and its use has been fully validated to FDA standards. The DCC has used iDataFax since 1998 for several NIH funded multicenter clinical trials and multicenter cohort studies. The EDC system has extensive data management features including a data query system to help ensure study credibility. Beyond EDC, DataFax allows data worksheets, source documents, and other participant records to be scanned and securely attached to any data record.

Data queries are reduced through the use of DataFax's powerful edit check features. Extensive data checks, both within and across forms, will be built into the computerized data capture system developed for the trial. Data are checked by comparing values of a field to predefined specifications (for example, minimum and maximum values or specific codes) and by programs written in an internal C-like language that allow for intricate comparisons among multiple fields on the same or on different CRFs. System generated queries requesting correction or clarification of values can be responded to on-line or by faxing/ emailing a corrected version of the CRF. The system provides a mechanism for defining the prescribed patient visit schedule so that queries indicating overdue CRFs are automatically generated. DataFax has a module for exporting CRF data by creating Statistical Analysis System (SAS) datasets. Department of Biostatistics personnel have developed S-Plus functions that produce the code for making tables and graphs in LaTeX, a mark-up language commonly used in mathematical fields for document processing. This set of tools are used by the DCC for generating automated DSMB reports.

7.5.5.3 Data Acquisition Plans and Reports: Data entry will be distributed and the study database will be centralized. For the proposed studies, personnel at the clinical center will enter patient data from the screening, baseline, and follow-up visits into eCRFs on the server at the DCC via the internet. The iDataFax system will generate regular reports (overall and by clinical center) that summarize and track routine data collection. These reports will help the investigative team monitor and maintain data completeness during follow-up and achieve high data capture performance standards by minimizing missed clinical visits, preventing missing data and maintaining high cohort retention rates. Monthly reports will be generated for clinical sites on the status of patient screening and enrollment and on the status of data queries issued by the DCC. These reports will be available to appropriate study personnel on a website developed and maintained by the DCC.

7.5.5.4 Study Material Access: The DCC will maintain a web dashboard. The investigators will access study-related documents through this dashboard including CRFs, the Protocol, meeting minutes, monthly data reports, enrollment and recruitment curves, presentations and publications.

7.5.5.5 Quality Control

Quality control (QC) is the responsibility of all study personnel. QC covers all phases of the studies from developing good data collection instruments, to training of clinical center staff, and monitoring adherence to study protocols, procedures and progress of the studies. The quality control process includes data entry plus all data is reviewed for completeness and accuracy.

7.5.5.6 Training: The DCC will train clinical personnel in study procedures for data collection. Training will include providing instruction on completion of CRFs, use of the iDataFax system for data entry, data quality control via QC reports generated by iDataFax, and use of the web dashboard.

7.5.6 Policies and Methods for Ensuring Blinding of Study Results: Only the IDS pharmacy will know which study drug was given to the subject. The study team, parents, subjects, individuals entering the data, statistician, individuals performing laboratory tests and DXA scans, research center nurses and investigators will remain blinded as to which treatment the subjects received. Each subject will be assigned a unique identifier, which will be used in the database. In addition, all lab specimens will only include the patient's unique identifier. Permuted block sizes will not be disclosed to the blinded study personnel to minimize the likelihood of their being able to predict the next randomization assignment in the series. Investigators and all study personnel will remain blinded until the final subject has completed her final visit and primary and secondary endpoints have been analyzed.

8. INCIDENTAL FINDINGS

Subjects will be receiving a DXA scan at screening/entry and again at 12 months for research purposes only. The scans will not be used for clinical or diagnostic purposes. If a potential subject is screened and found to have a T-score ≤ - 2.5, in the lumbar spine, femoral neck or total hip as measured by DXA, the individual will be provided with a referral to an endocrinologist and/or primary care physician and will have an option to have the scan sent directly to the physician of choice for review. The DXA procedure generates a report that includes bone mineral density of the spine and hip. For enrolled study participants, they will be given their 12-month DXA report, and they will be encouraged to share this information with their healthcare providers in making a diagnosis about the state of her bones. For enrolled subjects found to have a 12-month T-score ≤ -2.5, in the lumbar spine, femoral neck or total hip as measured by DXA, the individual will be provided with a referral to an endocrinologist and/or primary care physician and will have an option to have the scan sent directly to the physician of choice for review. Study participants may receive a copy of DEXA results on their 12 month study visit. They will be reminded that the DEXA scan is for research only, and participants will be encouraged to share the results with their healthcare provider.

9. COMPENSATION

Participants will be paid $25 for a screening visit and $50 for each additional study visit, for a total of up to $175 for full completion of the study, in the form of cash. Participants will be paid upon the completion of each visit. Participants will only be paid for visits in which they participated. There will be no compensation or reimbursement for travel, parking or other expenses.

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