Vitamin D Deficiency, Insulin Resistance and Cardiovascular Disease

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Background

Type 2 diabetes is the leading cause of chronic renal failure, coronary artery disease (CAD), heart failure and stroke in the USA. The cost for treating diabetes and its complications is estimated to be well over $100 billion annually in the U.S. (1-3). Approximately $69 billion is directed towards treatment of diabetes complications (e.g., cardiovascular disease) in diabetic patients (2). Thus, in perspective, 1/5th of our country's healthcare expenditure is directed towards diabetes management (2). Tight blood pressure control among patients with diabetes is essential to decrease the accelerated rate of cardiovascular disease (4). However, multiple studies have shown that despite the antihypertensive therapies available more than half of the patients with diabetes do not achieve desirable blood pressure values (5,6). Therefore, it is imperative to determine new links between hypertension, diabetes and cardiovascular disease that may lead to novel and affordable therapies to decrease the prevalence of these diseases.

Vitamin D is a nutrient and a pro-hormone largely regulated by environmental factors. It is absorbed from the diet, but its main source depends on a photosynthetic mechanism in the skin by the action of ultraviolet light beta exposure (UVB). Skin derived 7-Dehydrocholesterol is converted to previtamin D_3 by UVB exposure, which then is transformed to vitamin D_3 by thermal induced isomerization. Vitamin D_3 undergoes hydroxylation in the liver to 25(OH) vitamin D_3 [$25(OH)D_3$] and then in the kidney to its active hormonal form $1\alpha,25(OH)_2D_3$. Macrophages, endothelial and smooth muscle cells as well as other cells are also able to transform $25(OH)D_3$ to its active hormonal form. This increased local production of active vitamin D serves as an autocrine/paracrine factor which is fundamental for cell-specific functions. Because levels of 25(OH)D increase in proportion to vitamin D cutaneous synthesis and dietary intake, plasma 25(OH)D levels are commonly used as an indicator of vitamin D status

Vitamin D and Diabetes. There is increasing evidence that low levels of serum 25(OH)D are a risk factor for the development of impaired glucose metabolism and type 2 diabetes (7-9). Vitamin D concentrations are significantly lower in newly diagnosed patients with diabetes and IGT compared with controls (10,11). Analysis of The National Health Nutrition and Examination Survey (NHANES III) and Workforce Diabetes Study confirmed that patients with sufficient 25(OH)D levels (more than 30 ng/ml) have about one-third of the risk of developing Type 2 DM or impaired glucose tolerance when compared with those with insufficient 25(OH)D levels ($\leq$ 24 ng/dl) (12,13). Vitamin D deficiency induces insulin resistance. In vitamin D-deficient rats, vitamin D repletion improves insulin sensitivity in glucose tolerance tests (14). In humans, insulin sensitivity assessed by hyperinsulinemic-hyperglycemic or euglycemic clamps improves in direct relationship with 25(OH)D levels (15,16); moreover, data from the Nurses' Health Study, where combined daily intake of calcium >1,200 mg and vitamin D >800 IU was given, showed a 33% lower risk of type 2 diabetes with a RR of 0.67 compared with an intake of calcium <600 mg and vitamin D 400 IU (17).

Vitamin D and Hypertension. Interventional studies suggest that vitamin D replacement decreases BP. In an 8 week treatment study consisting of oral calcium and vitamin D_3 replacement in elderly non-diabetic women with vitamin D deficiency, plasma 25(OH)D levels increased to > 25 ng/ml and systolic blood pressure (SBP) decreased significantly by 13 mmHg compared with the calcium treated control group. UVB exposure, by skin tanning sessions, increased plasma 25(OH)D levels to 40 ng/ml and decreased blood pressure in mildly hypertensive normoglycemic patients (18,19). However, oral administration of $1\alpha,25(OH)_2D_3$

has not shown consistent BP effects, possibly due to discrepancies in the population plasma vitamin D status as well as different vitamin D doses and duration of the vitamin D replacement (20). Vitamin D suppresses the renin-angiotensin system. In animal models, vitamin D down regulates renin gene promoter activity independent of calcium metabolism. Mice lacking the VDR exhibit hypertension and cardiac hypertrophy due to increased renin expression and plasma angiotensin II production (21). Oral administration of vitamin D_3 in spontaneously hypertensive rats decreased blood pressure and improved endothelial cell–dependent vasodilatation (22,23). In humans, interventional studies with oral synthetic vitamin D (alphacalcidol) replacement lowered plasma renin in non-diabetic vitamin D-deficient patients (24,25). This evidence supports the conceptual relationship between vitamin D and the renin-angiotensin system in essential hypertension and the beneficial effect of vitamin D supplementation on blood pressure.

Vitamin D and Vascular Disease. Vitamin D not only alters the hormones involved in the pathophysiology of hypertension and diabetes, but also has a direct effect on the vasculature. The expression of vitamin D receptors and local activation of vitamin D by the 1α hydroxylase enzyme in endothelial (EC) and vascular smooth muscle cells (VSMC) indicate the importance of vitamin D in vascular cell metabolism (26,27). Vitamin D is a potent modulator of the growth of cultured VSMC (28). In animal studies, vitamin D induces the transcription of important genes in vascular remodeling and structure such as vascular endothelial growth factor, matrix metalloproteinases (MMPs), myosin, elastin, and type I collagen (29). In addition to its vascular structural effects, vitamin D rapidly stimulates vascular intracellular calcium mobilization by a non-genomic action activating cellular signaling pathways that are involved in increased vascular contraction and smooth muscle proliferation (cGMP, protein kinase C and MAPK) (30). Recent studies with a national representative sample demonstrate that low plasma 25(OH)D levels have an increased adjusted hazard ratio (1.62) for the incidence of cardiovascular disease compared to those with 25(OH)D levels ≥ 15ng/ml and they were associated with an increased relative risk of developing common cardiovascular risk factors linked to insulin resistance such as: hypertension (OR, 1.30), obesity (OR, 2.29) and hypertriglyceridemia (OR, 1.47) (31,32). In patients with type 2 diabetes, the NHANES III data suggest that low vitamin D levels almost double (OR, 1.70) the relative risk of developing cardiovascular disease compared with diabetic patients with normal vitamin D levels and are strongly associated with increased carotid artery intima-media thickness (CIMT), a reliable marker of atherosclerosis (33,34). However, data from the Women Health Initiative Study showed that supplementation with vitamin D_3 (400 IU) and calcium carbonate (1000 mg) in postmenopausal women for 7 years failed to prevent coronary or vascular disease when compared to those in the placebo group (35). There are several issues in this study that limit its interpretation, such as the low rates of adherence to therapy, higher calcium intake at baseline in study participants, and perhaps the selection of a suboptimal 25(OH)D_3 supplement.

Vitamin D and Vascular Inflammation. Vitamin D has an immunomodulatory effect on the vasculature. Increased vascular inflammation may well account for the high prevalence of hypertension or vascular disease in patients with diabetes. As the cellular and molecular mechanisms of vascular inflammation and hypertension are being more clearly defined, it is becoming apparent that the two processes are interrelated. In humans, elevated circulating inflammatory markers such as C-reactive protein (CRP) are linked to an increased risk of later development of diabetes and hypertension (36,37). In mice, increased hepatic expression of CRP induces hypertension by inhibiting the activation of endothelial NO synthase (38). On the other hand, hypertension promotes vascular inflammation. Flow shear stress stimulates expression of endothelial adhesion markers and production of leukocyte chemo-attractants that induce immune cell adhesion, migration and increased vascular oxidative stress, which are essential factors promoting inflammation, lipid peroxidation and hypertension (39-40).

Although our knowledge of triggering factors for vascular inflammation is limited, oxidized low density lipoprotein (oxLDL), retained within the vascular wall, may represent a direct or indirect initiating agent for leukocyte recruitment. Upregulation of vascular inflammatory mediators and excess LDL driven cholesterol deposits in macrophage cells (foam cells) are the hallmarks of developing hypertension and atherosclerotic lesions. The VDR is present in macrophages and T lymphocytes. Vitamin D promotes monocyte/macrophage differentiation and inhibits its proliferation (41). In freshly isolated monocytes from Type 2 DM patients, vitamin D prevents production of tumor necrosis factor α□□TNFα□ and interleukins (IL)-1, IL-6, and IL-8 after stimulation with gamma interferon. These vitamin D actions confirm its immunomodulatory properties (42). In addition, vitamin D suppresses Th1 cell production of interleukin (IL)-2 and interferon □ and stimulates IL-10 production in lymphoid organs and in target tissues. These events decrease inflammation by altering the capacity of antigen presenting cells to activate Th1-cells and favor the induction of regulatory T cells and Th2 cells (43). Mice lacking the VDR showed accelerated inflammation in models of inflammatory bowel disease, but the effects of VDR deficiency on vascular inflammation have not been determined (44). In humans, there is an inverse relationship between C-reactive protein and vitamin D. Vitamin D supplementation has been found to decrease CRP and MMP9 levels in healthy subjects (45). Therefore, *vitamin D may modulate inflammation associated with vascular disease in humans.*

Taken together, these data suggest that vitamin D deficiency promotes hypertension and accelerates atherosclerosis by increasing vascular inflammation and insulin resistance in animal models and subjects with diabetes.

Objective

We would like to understand the mechanisms by which vitamin D affects vascular function on patients with diabetes and hypertension assessed by blood pressure, flow mediated dilatation and systemic inflammatory markers.

Hypothesis

We hypothesize that vitamin D replacement in vitamin D-deficient patients with diabetes will improve systemic inflammatory markers of cardiovascular disease and vascular distensibility measured by blood pressure and flow-mediated dilatation

Inclusion/Exclusion criteria

We will include Type 2 DM patients of both sexes and from all ethnic groups between the ages of 25 and 80 years, with $25(OH)D_3$ levels <25 ng/ml. We will include those diabetes patients with HbA1c between 5.5%-9.5% who are not on insulin therapy and without recent weight loss of more than 5% within 3 months prior to screening. Each patient must have a 24h Ambulatory Blood Pressure (ABPM) with mean systolic BP ≥120 mmHg and / or mean diastolic BP ≥ 80 mmHg without BP lowering medications or after stopping any BP medication for 2 weeks or more (46). We will exclude pregnant women, patients with ABPM showing mean systolic BP (SBP) >160 mmHg or mean diastolic BP (DBP) >100 mmHg, those with heavy alcohol consumption (males >2 drinks per day and females >1 drink per day), patients following extreme diets (Atkins, South Beach diet), those with stage 4 chronic renal failure (calculated by modification of diet in renal disease eGFR equation) and/or patients with >2 + proteinuria on urine dipstick.

Subject Screening

We will screen diabetic hypertensive or pre-hypertensive patients without BP lowering

medications or those who control BP with any anti-hypertensive medication with no known history or clinical manifestations of cardiovascular disease or proteinuria per clinical judgement. If the patient and their physician are agreeable, we will assess the patient's cardiovascular risk and clinical manifestations, and then stop their medication and check their blood pressure supply an automatic blood pressure monitor for them to report blood pressure measurements to the research coordinator 3x/week for 2 weeks (time based on the pharmacokinetics of the most commonly prescribed BP medications). At the end of this wash-out period, we will complete the screening by measuring 24h blood pressure using an ambulatory device to assure that the patient's blood pressure is within our inclusion criteria and will not place the patient at higher risk. If the patient's blood pressure remains below ABPM mean SBP 160 and mean DBP100 mmHg, then we will enroll them in the study with continued close monitoring of their blood pressure.

Protocol

Subjects will be recruited from the Wohl Clinic at Barnes Jewish Hospital, the Diabetes Center, Grace Hill Clinic, Family Medical Center and Volunteers for Health at Washington University. Grace Hill and Family Medical Center covers 40,000 patients. It serves an elderly population with a high prevalence of diabetes and hypertension. Volunteers for Health is a service of Washington University that provides a database with a self-reported medical history from 24,000 individuals. We will also recruit patients at the VA medical center. We will recruit 120 patients between all the sites and no more than 70 total patients from the VA medical center. We will post an ad on Craigslist. This trial will be conducted in compliance with Good Clinical Practice.

The recruitment process at the VA medical center will consist of obtaining a print out of potentially eligible patients identified during a database search of VISTA for patients meeting inclusion criteria. Screening would occur by a computer search of VISTA data to identify individuals with diabetes (ICD-9 250.x or use of diabetic medications) and 25 OH vitamin D levels less than 20ng/dl. Once this list is generated, then a letter will be sent to these individuals with the details of the study and a contact phone number. If the patient does not call for 2 weeks after the receipt of the letter, then we will call them to give them more study specifics, address any questions or concerns, and assess study suitability. Also patients scheduled to be seen in high potential clinics will be screened using the above criteria. Providers will be given information regarding the study and their permission will be obtained to approach and screen their clinics. The listings will contain the patient's name, SSN and clinic visit information. Individuals will be pre-screened utilizing CPRS for inclusion/exclusion criteria per protocol. If a patient is potentially eligible, a note will be entered in CPRS and sent to their PCP to notify them that their patient is being contacted for possible recruitment into a clinical trial. Also, the study physicians may identify potential patients during their routine clinical duties. If a patient's file is being reviewed or a patient is seen in the clinical area that is possibly eligible, the physician may notify the coordinator for screening. Identified patients will be sent to Washington University for their screening visit if interested. The screening visit or any study procedures will not be conducted until the consent form is signed at Washington University.

At the initial screening visit, anthropometric measurements, ECG, BP and blood samples for CBC, comprehensive metabolic panel (CMP), HbA1c, pregnancy test, urinalysis, urinary albumin-creatinine ratio and 25(OH) D_3 level will be obtained at the Clinical Trial Unit (CTU) or Storz Research Building (Table 1). If the patient is deemed to have low cardiovascular risk and the attending or primary physician agrees, the patient will be advised to stop one anti-

hypertensive agent and proceed with at home blood pressure monitoring 3 times weekly for 2 weeks. Patients whose seated BP in the office is within the range of 120-160/80-100 mmHg (pre-hypertension and Stage 1 hypertension) will have fasting lipid panel, insulin level, and a vitamin D panel (1α,25(OH)$_2$D, 25(OH) D, intact PTH) drawn, and be scheduled for ABPM and tests of endothelial function. Patients must qualify by having a mean SBP and DBP on ABPM in the same range, 120-160/80-100 mmHg and no adverse effects. During the screening visits, adjustments in diabetes medications will be made following ADA treatment guidelines and we will provide information regarding healthy eating and non-pharmacologic treatment of hypertension and diabetes. Patients with a H1AC of ≥9.5% at screening taking maximal doses of appropriate diabetes therapy and showing good adherence to life style and medication intervention will be excluded. Patients will be excluded if they have cardiovascular disease, heart failure, or arrhythmia that prevent them from stopping any oral diuretics, antihypertensive, chest pain medications, heart failure medications. Patient should not be taking multivitamin, calcium or nutritional supplements different than the one provided by the study.

Patients who meet the inclusion and exclusion criteria, and who are otherwise stable without emergent adverse events will have blood drawn for inflammatory markers and will be randomized to 25(OH)D_3, 4,000 IU/day or matching placebo (Tishcon corp, NY). This dose of 25(OH)D_3 was found to be safe in multiple studies (47-52). The pharmacy will dispense the treatment using the Moses-Oakford Algorithm for random assignment. Both treatment groups will receive calcium carbonate 1.2 g/day and compliance will be assessed by pill count at each visit.

Randomized subjects will be followed every 2 weeks for six weeks and then monthly for the remainder of the study for safety checks that include weight, seated BP, BMP, calcium and urine for calcium/creatinine ratio. Medication compliance and adverse events will be recorded. Dietary advice regarding non-pharmacologic treatment for hypertension and diabetes will be provided at monthly intervals. A 3-day food diary and Diabetes Quality of Life Survey will be completed with a dietitian at the baseline, 8 weeks and final visits. Metabolic and hemodynamic testing will be done at baseline, 8 and 16 weeks to include ECG, ABPM, endothelial function tests, a CV panel, vitamin D panel and safety labs. Inflammatory markers will be drawn at baseline and 16 weeks. Blood glucose will be assessed at every visit, A1c every 8 weeks and medication adjustments will be made as needed following ADA treatment guidelines but excluding thiazolidinediones, and insulin. Patients will be contacted 1 year after starting the project for follow-up testing similar to that of last visit to evaluate the persistence of response following discontinuation of the treatment.

Procedures

Ambulatory blood pressure monitoring will be performed with a portable oscillometric recorder (Spacelabs 90207). Ambulatory blood pressure readings will be considered adequate if there are at least 14 readings during the day and seven readings at night as recommended by the British Hypertension Society guidelines for ABP monitoring (53). Sample recording will begin between 8:30 and 9 a.m. The blood pressure will be obtained at 20-minute intervals from 6 a.m. until midnight and at 30-minute intervals from midnight until 6 a.m. The most appropriate size cuff will be selected.

Two different periods of ambulatory blood pressure monitoring, will be defined. The daytime period includes all readings obtained from 8 a.m. until 10 p.m., and the nighttime period includes all readings from midnight until 6 a.m. Data from 10 p.m. until midnight and from 6 a.m. until 8 a.m. will not be included in the data for the daytime and nighttime periods, respectively, in

order to minimize overlaps but these data will be included in the analysis of the 24-hour data analysis. The mean values for all the readings of systolic and diastolic pressure during a daytime or nighttime period will be recorded as the systolic and diastolic pressure for that period. A value of 0.9 or lower for the ratio of the mean nighttime systolic pressure to the mean daytime systolic pressure is defined as the normal drop in blood pressure during sleep (54).

Brachial Artery Ultrasound Imaging will be performed at cardiovascular image center in Washington University by a single technician blinded to the study protocol. Measurements of the vessel diameter are performed prior to, during a 5 minute inflation, and immediately after deflation of a pneumatic cuff. Brachial artery blood flow velocity is measured with spectral Doppler. Flow-mediated vasodilation (FMD) was calculated from the diameters as: (reactive hyperemia − baseline)/baseline × 100%. The normal percent increase in diameter of the artery (flow-mediated dilation) and systolic flow after cuff deflation will be used as an index of endothelial-dependent vasodilatation response. All studies will be repeated with a second hyperemic challenge with sublingual nitroglycerin to determine the endothelium-independent response (55).

Patients who are on phosphodiesterase inhibitor medications (i.e. Viagra, Cialis, Levitra) will be asked to not take these medications within 48 hours of their scheduled nitroglycerin challenge. At the time of the scheduled appointment, the patient will be asked by the clinical nurse if this medication has been taken. If the patient states that they have indeed taken the above listed medications, then the nitroglycerin challenge will not be performed. Nitroglycerin in combination with phosphodiesterase inhibitors can cause an unsafe drop in blood pressure.

Measurement of Central Arterial Compliance will assess aortic stiffness by measuring both aortic pulse wave velocity and central systolic aortic pressure augmentation using external Doppler ultrasound against the carotid and the femoral artery by a well-validated commercially available device (56)

Measure of inflammatory markers (hCRP , fibrinogen, TNFα, IL-1, IL-6, VCAM and ICAM) as well as monocyte assessment of cholesterol metabolism, adhesion and migration will be evaluated at baseline ,8 weeks and at the end of the study.

The 3-day food diary will be blinded and analyzed by a registered dietitian using NutritionistPro Software (Stafford, TX).

Statistics

Data analysis will be based on the intention-to-treat principle where all patients will be analyzed as part of their randomization group regardless of subsequent events. We will also perform a secondary analysis on patients that complete at least 8 weeks of treatment. The goal of this aim is to understand the mechanisms by which vitamin D affects vascular function on patients with diabetes and hypertension assessed by blood pressure, flow mediated dilatation and systemic inflammatory markers. Analyses are based on the fact that the primary (SBP and DBP) and secondary (FMD) outcomes are continuous and are measured at multiple time points. Between group comparisons of baseline characteristics will be performed using unpaired t-tests (for continuous measures) or chi-square tests (for categorical measures). To account for the possibility that there will be missing data in some patients, hypotheses regarding the equality of changes over time in the two groups will employ mixed model repeated measures analyses of variance with the primary focus on the significance of the interaction between group and time point. Statistical contrasts will test specific hypotheses regarding between-group differences in changes between select time points. Subsequent analyses of covariance will be performed to evaluate the impact of potential confounding variables that differed between groups at baseline.

Confounds that could influence the response include BMI, body weight, smoking status, season, sun exposure, and dietary intake of sodium, potassium, calcium, and vitamin D. Multiple regression will also be used to determine the influence of changes in these confounds on the outcomes. For all analyses, careful attention will be given to whether the data satisfy the distributional and model-specific assumptions of the procedures used.

Appropriate data transformations or non parametric methods will be used as appropriate. We proposed to recruit 120 patients for this study. Assuming a 20% dropout rate, 90% power calculation was performed to determine the magnitude of between-group differences that can be detected with the sample size of 48 patients per group. The type-I error rate was set at alpha=0.05 with a two-tailed unpaired t-test and conservative projected standard deviations (SDs) based on the investigator's pilot data and Pfeifer, et al. [57] The planned study with 48 patients completing the intervention affords excellent power to detect meaningful differences for the primary outcomes; with minimum detectable differences ranging between 10 to 15 mm Hg and 5 to 10 mm Hg for SBP and DBP, respectively. Although the study is not powered for the secondary endpoint of FMD, the study yields power of more than 0.90 to detect a minimum difference of 2% (assuming an SD=2.5).

Data and Safety Monitoring Plan

We will minimize the patient's risks by careful screening and frequent physician visits (every 2 weeks for the first 6 weeks) to the clinic, where the patient will be educated about the signs and symptoms of hypertension, hyper- and hypocalcemia and kidney stones. We will also monitor the patients' BP, serum calcium, and urinary calcium levels during these visits. To prevent vitamin D toxicity, we will assess a random sample of vitamin D pill content.

All data will be used for research purposes. The risk of loss of confidentiality will be minimized by adherence to HIPAA guidelines established by Washington University. Data analysis will be based on the intention-to-treat principle where all patients will be analyzed as part of their randomization group regardless of subsequent events. We will also perform a secondary analysis on patients that complete at least 8 weeks of treatment. ClinPortal will be used to collect, store and disseminate project-specific clinical and translational research data

The Data Safety and Monitoring plan for this study will include a Data Safety and Monitoring Board (DSMB). The DSMB will consist of Dr. John Turk endocrinologist and diabetes specialist, Dr Babek Razani an adult cardiologist, and Amy Hauch RN, BSN, CDE. All members of the DSMB disclose no conflict of interest related to the proposed trial. The DSMB will have a scheduled meeting twice a year or after an additional 30% of the total anticipated subjects have been enrolled to monitor the study progress and integrity to examine the patient laboratory results, and to assess adverse events (see below) to ensure that patients are not exposed to unnecessary risk during the study.

During screening and baseline evaluation and during the study, serum and urinary calcium levels will be monitored to assess for possible risk of hypercalcemia associated with vitamin D intoxication. Serum calcium >10.5 mg/dl or urinary calcium: creatinine ratio >1.0 at screening or during the course of the study would necessitate that the subject be ineligible for randomization or be withdrawn from the study. This decision will be made by the PI or his designated staff without regard to knowledge of treatment group assignment. In addition, 25(OH) vitamin D levels will be provided to a member of the DSMB to review and alert the PI to any subject who should be withdrawn from the study because of a 25-OHD >200ng/ml during the study (57).

Our *Data Safety Monitoring Plan* includes thorough documentation of any and all Serious Adverse Events (SAE) and reporting to the Human Research Protection Office (HRPO), the Research Subject Advocate and GCRC Advisory Committee. The participant's side effect log will be reviewed at the time of any event, and all participants that note significant concerns about side effects that cannot be easily and safely addressed will be told to stop the study drug. Reporting of adverse events will be conducted with oversight by the Principal Investigator (PI) and the Research Subject Advocate. Summary reports will be provided to the HRPO and the Research Subject Advocate every six months. In case of a SAE, the research coordinator or PI will report the event to the DSMB and to the HRPO according to the current HRPO guidance document. The PI will determine if the SAE is causally related to the study. Other possibly concerning objective findings that indicate potential for harm will be reviewed with the PI, Dr. Carlos Bernal-Mizrachi, and lead to stopping the study for that subject if either the PI or any nurse caring for the patient in the GCRC feels the participant could be harmed by continuing the study. Any clear evidence of potential harm will lead to stopping the study for that patient. The HRPO will get a summary of the situation unless the dropout is secondary to participant's personal reasons and unrelated to the study. An interim efficacy analysis will be performed when 60 of the targeted 120 subjects have been enrolled. This analysis will focus on the primary endpoint and will permit us to make one of the following decisions: a) Stop the study early if the data suggest that efficacy has already been achieved. b) Stop the study early for reasons of futility if the data suggest that it is unlikely that efficacy will ever be achieved. c) Continue the study with an increase in the target sample size if the data indicate smaller effect sizes that were initially projected but that an achievable increase in the sample size will yield adequate statistical power. d) Continue the study as planned if effect sizes appear to be approximately as projected. The interim analysis will be conducted in accordance with guidelines set forth by the O'Brien Fleming stopping rule. [1] We emphasize, however, that this will only be a guideline. Any final decision to modify the sample size or to stop the study early will be based on multiple considerations that also include efficacy with respect to important secondary outcomes and the consistency of results across key subgroups. Our study will be conducted with the ideas of justice, beneficence and autonomy having primary importance.

The DSMB will be informed of all Serious Adverse Events (SAEs). An SAE will be defined as an event which results in any of the following outcomes: death, life-threatening event, persistent or significant disability, inpatient hospitalization or prolongation of an existing hospitalization, or congenital anomaly. In addition, any symptomatic patients with stroke, intracranial bleeding, heart attack, heart failure, symptomatic hypocalcemia/ hypercalcemia, or increased urinary calcium/creatinine ratio >1.0 that is unresponsive to fluid therapy (at least six to eight glasses of water per day for 5 days) will be considered to be an SAE and withdraw. The PI will determine if the SAE is causally related to the study without regard to knowledge of treatment group assignment. Any patient with an SAE which is determined by the PI to be possibly or probably related to the study intervention will be withdrawn from the study. All SAEs will be reviewed by the DSMB to confirm the PI's assessment of causality and will be reported to the Institutional Review Board (IRB) and CARS Research Subject Advocate. Symptomatic subjects who develop sustained severe symptomatic hypertension during the study defined as SBP >160 mmHg and/or DBP >100 mmHg by office BP measurements (confirmed by ABPM) will be evaluated by Dr. Bernal-Mizrachi and/or Dr. Riek and he/she will determine if patient will be withdrawn by determine the clinical risk to develop hypertensive complications. The physician will also determine if there is a clear triggering factor that is causing the increase in blood pressure and if it is easy to resolve it before patient is dropped from the study. The participant's primary care doctor will be notified and antihypertensive therapy will be started as per their recommendation. If patients do not have a primary care doctor, antihypertensive therapy will be started and they will be referred to a medical provider for further management

that is covered by their insurance. The initial goal is to reduce blood pressure to 160/ 110 mm Hg over several days, we will follow these patients closely until this ambulatory BP is achieved. Patients with increased urinary calcium/creatinine ratio >1.0 that are unresponsive to oral fluid therapy, will be evaluated in the CRU to determine whether IV fluid therapy is required. We will follow this patients until they achieve urinary calcium/creatinine ratio <1.0. Patients with asymptomatic hypocalcemia that are unresponsive to increased PO Calcium intake we will supervise calcium intake by clinic visits and follow until plasma calcium levels return to normal. If the patient becomes symptomatic and requires a hospitalization, the patient will be withdrawn from the study. Patients that have completed at least 8 weeks in the study and are withdrawn for medical reasons, will continue to be evaluated every 4 weeks for BP monitoring, plasma calcium levels and urinary calcium and creatinine ratio until completing 16weeks from the initiation of the trial. At 16 weeks AMBP monitoring and FMD and macrophages will be obtained.

Summary reports of all Adverse Events (non-serious AEs and SAEs) will be provided to the DSMB before each scheduled meeting and at least every 4 months. If the PI or the DSMB determine that there are an unacceptable number of AEs and/or SAEs reported, the PI may request that the DSMB review (or the DSMB may independently decide to review) the events by unmasked treatment group. If treatment-emergent AEs or SAEs are occurring at an unacceptable rate, the DSMB may recommend to the PI that the study be halted or that the protocol be modified.

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Table 1

Study Procedures	Screen 1	Screen 2	Base-line	Week 2	Week 4	Week 8	Week 12	Final
Informed Consent	x							
Study H & P, MD assessment	x		x		x	x	x	x
Anthropometrics (Ht, wt, waist), ECG	x		x			x		x
Seated BP, HR	x	x	x	x	x	x	x	x
25(OH)D level, CMP, A1c	x					x		x
Pregnancy test, if applicable		x		x	x	x	x	x
Dietary Instruction					x	x	x	x
Ambulatory BP		x				x		x
Endothelial function, BART, PWV		x				x		x
Vit D panel*		x				x		x
CV Panel**		x				x		x
BMP, Calcium, Ucalcium/creatinine				x	x	x	x	x
Inflammatory panel***			x					x
Randomize			x					
Dispense study medication			x		x	x	x	
Monitor compliance				x	x	x	x	x
Adverse events			x	x	x	x	x	x