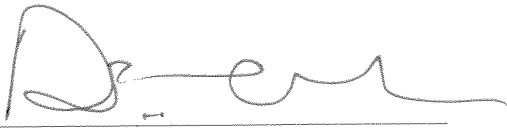
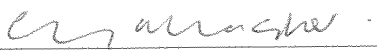



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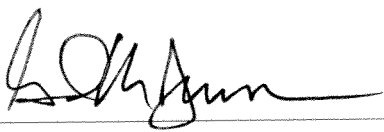
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**ROLE OF VITAMIN D RECEPTOR AND VITAMIN D BINDING PROTEIN
RESTRICTION FRAGMENT LENGTH POLYMORPHISMS IN DETERMINING DOSE
RESPONSE TO VITAMIN D: A RANDOMIZED DOUBLE BLIND PLACEBO
CONTROLLED TRIAL**

By

ADARSH SAI

A (THESIS/DISSERTATION)

Submitted to the faculty of the Graduate School of the Creighton University in Partial Fulfillment
of the Requirements for the degree of Master of Science in the Center for Clinical and
Translational Science

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Abstract

Serum 25-hydroxyvitamin D (25OHD) is considered to be the best biomarker of vitamin D status. Besides the dietary intake of vitamin D and exposure to sunlight, there are other factors that affect serum 25OHD such as weight, race, sex, binding protein and the effect of vitamin D receptor (VDR) and vitamin D binding protein (DBP) single nucleotide polymorphisms (SNPs) on serum 25OHD levels.

The primary objective of this study was to determine the effect of genotypes based on VDR and DBP SNPs on the dose response to vitamin D in terms of serum 25OHD and serum parathyroid hormone (PTH).

We used specimens from an ongoing study (ViDOS - vitamin D supplementation in older subjects) - a double blind randomized placebo-controlled trial that enrolled 160 healthy Caucasian postmenopausal women, with vitamin D insufficiency (mean serum 25OHD 15.6 ng/ml) recruited during winter and followed for one year. The subjects were randomly assigned to placebo, vitamin D3 400, 800, 1600, 2400, 3200, 4000 or 4800 IU/d and calcium to increase total calcium intake to 1200-1400 mg/day based on 7-day food diaries. The *primary outcomes* were serum 25OHD and serum parathyroid hormone (PTH) for this study. Serum 25OHD and PTH were measured by immunoassay. Genotype analysis was performed on DNA isolated from peripheral leucocytes from patients.

There was a significant effect of BMI, baseline serum 25OHD and dose of vitamin D supplementation on final serum 25OHD. However, there was no effect of genotypes based on VDR and DBP SNPs on serum 25OHD at the end of one year after vitamin D supplementation after adjustment for relevant confounders like age, BMI, baseline serum 25OHD, dose, serum creatinine, smoking status, alcohol intake, caffeine intake and calcium intake. This study represents the first longitudinal placebo controlled randomized trial that examined the pharmacogenetics of vitamin D metabolism together with a dose response to vitamin D; these results do not support previous positive association data.

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1. Introduction

Osteoporosis is defined as a progressive systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fractures mostly affecting women (1). The total number of people aged 50 years and older estimated to be at risk for osteoporosis and low bone mass exceeds 44 million (2). The release of first ever Surgeon General's report (3) on the topic of bone health emphasizes the enormity of this public health problem. Development of low-cost and effective strategies is important for prevention of osteoporosis and to reduce osteoporotic fractures. A simple inexpensive strategy to prevent osteoporosis is adequate nutrition with calcium and vitamin D. In addition, there is growing evidence that Vitamin D plays a significant role in many other diseases like cancer (breast (4), prostate (5), skin (6), colon (7)), immune disorders (multiple sclerosis (8), Rheumatoid Arthritis (9), Graves' disease (10), diabetes mellitus type 1 (11), systemic lupus erythematosus (12), inflammatory bowel disease (13) asthma (14,15)), cardiovascular diseases (coronary artery disease (16), hypertension (17)), metabolic diseases (diabetes mellitus type 2, metabolic syndrome) (18,19) and infections particularly respiratory infections (20).

Serum 25OHD is now accepted as the best measure of vitamin D status that includes the nutritional sources of vitamin D from diet and exposure to sun. Serum concentrations of at least 20 ng/ml are needed to reduce serum PTH and reduce bone loss (21). The amount of vitamin D intake required to maintain adequate vitamin D nutrition has not been adequately answered. The current dietary reference intake (DRI) updated recently in 2011 recommends that the RDA (Recommended Dietary Allowance) for vitamin D should be 600 IU/d for adults between 19- 70 years and 800 IU/d for adults >70 years of age. It also defines the EAR (Estimated Average Requirement) as 400 IU/d for adults \geq 19 years (22). The EAR is the daily intake value that is estimated to meet the requirement, as defined by the specified indicator of adequacy, in 50 percent of the individuals in a life stage or gender group. RDA is the average daily dietary intake

level that is sufficient to meet the nutrient requirements of nearly all (97.5 percent) healthy individuals in a specific life stage and gender group. The EAR forms the basis for setting the RDA. In 1997 there was insufficient evidence to calculate an EAR - a reference intake called an AI (Adequate Intake) was used instead of an RDA and this applied to vitamin D in the previous IOM recommendations of 1997 (23). The recent changes in recommendations came as IOM identified new studies on sun exposure and vitamin D content of various foods. However, certain deficiencies were uncovered in determining the dose response effect of vitamin D on serum 25OHD including lack of intervention trials, lack of information about dietary intake and the finding that most studies used calcium and vitamin D together rather than studying each alone.

The relationship between vitamin D intake and serum 25OHD is complex and is dependent on a host of factors including sun exposure (24), body mass index (25,26) and race (26,27). Interpretation of serum 25OHD results is complicated by the assay fluctuations over the years in the radioimmunoassay most commonly used in North America (RIA, Diasorin) for measuring serum 25OHD levels as noted in various NHANES studies (28). The sunlight effect depends on distance from the equator, with the monthly exposure to UVB radiation decreasing as one moves farther away from equator. The time period of effective UVB exposure depends on the angle of the sun (> 35 degree effective), atmospheric pollution and clothing habits.

There is no clear consensus of the amount of vitamin D intake required to maintain optimum serum 25OHD levels. There are no systematic prospective multiple dose response studies of vitamin D in different populations with the aim of establishing a dose of vitamin D that will provide an optimum level of serum 25OHD (20 ng/ml as identified in the recent IOM report). The primary objective of ViDOS (Vitamin D supplementation in older women) was to study the effect of increasing doses of vitamin D₃ in a high risk group of postmenopausal Caucasian and African American women with vitamin D insufficiency (serum 25OHD <20 ng/ml) in winter in presence of sufficient calcium intake, in order to determine the EAR and RDA for vitamin D. WHO

definition of vitamin D insufficiency was used as a starting point for defining optimal vitamin D levels (29).

a. Metabolism of Vitamin D (Figure 1)

Vitamin D is a seco-steroid derived from cyclopentanoperhydrophenanthrene ring structure for steroids (30). It has two major forms – D₂ and D₃. Very few foods like fish, egg yolk, liver and mushrooms contain vitamin D₂ or D₃ (31,32) and strict vegetarians are at an increased risk for vitamin D deficiency especially in winter (33). The major form of Vitamin D₃ synthesis

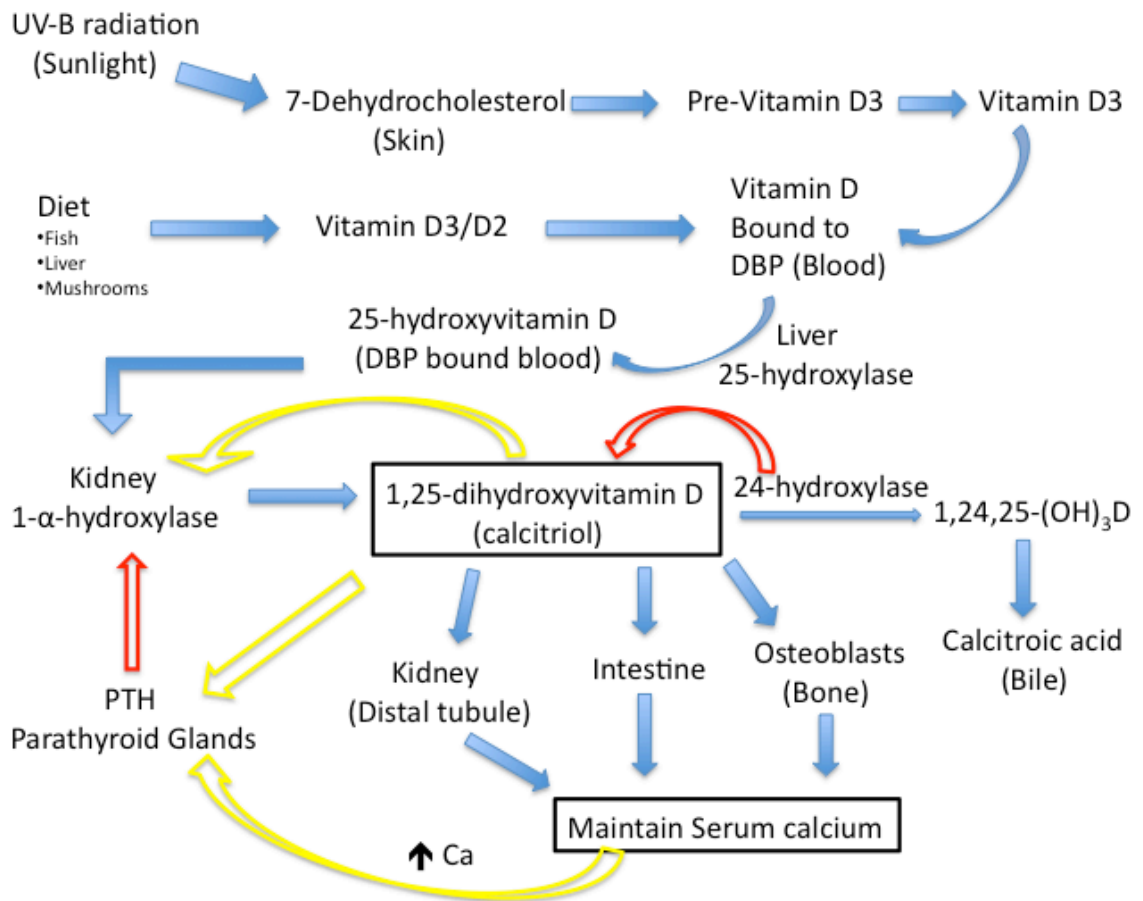


Figure 1 – Metabolism of Vitamin D and calcium homeostasis; red implies stimulation, yellow implies inhibition

is by the action of UV-B radiation in the wavelength of 290-315 nm on a compound, 7-dehydrocholesterol in skin (34). In fact it is estimated that ~ 3000 IU of vitamin D₃ is produced after 5-10 minute exposure of arms and leg to direct sunlight (34). Vitamin D from diet or skin is then carried to liver bound by vitamin D binding protein (DBP) where it is acted upon by the enzyme 25-hydroxylase to form 25-hydroxyvitamin D (25OHD) (35). This 25OHD binds to DBP and is carried to kidney where proximal tubule cells convert 25OHD to 1,25-dihydroxyvitamin D (1,25(OH)₂ D; calcitriol) by the action of enzyme 25-hydroxyvitamin D₃-1 α -hydroxylase (CYP27B1) (35). Calcitriol is the active form of vitamin D that acts on the target cells through vitamin D receptor (VDR). In this classic endocrine system, kidney is the major source of circulating calcitriol (36) although the enzyme CYP27B1 is found in a multitude of cells and tissues including keratinocytes, osteoblasts, brain, mammary epithelial cells, colon, prostate, lung epithelial cells, jejunum, choroid epithelium, testis, macrophages, dendritic cells, placenta and activated T and B lymphocytes (37-44). These organs and cells thus can act as non-classical target organs of vitamin D. The major target organs (classical actions) for calcitriol are bone, intestine and kidney where it interacts with parathyroid hormone (PTH) and calcitonin to maintain serum calcium levels (34). In this endocrine (classical) system, PTH is the main stimulator of enzyme CYP27B1 (45) while substrate levels (25OHD) play a less important role; this effect of PTH declines with age (46). In context of non-classical actions however, it is important to note that PTH does not exert any regulation on CYP27B1, although the enzyme activity is still tightly regulated (47). 1,25(OH)₂ D exerts its action through a nuclear receptor (vitamin D receptor (VDR) which is a member of nuclear receptor superfamily of ligand-activated transcription factors (48).

VDR binds to target DNA sequences as a heterodimer with retinoid X receptor, recruiting a series of coactivators that modify chromatin and approximate the VDR to the basal transcriptional apparatus resulting in the induction of target gene expression (48,49). Excess 25OHD and 1,25(OH)₂ D are metabolized by enzyme 25-hydroxyvitamin D-24-hydroxylase

(CYP24) into 24,25-(OH)₂ D and 1,24,25-(OH)₃ D respectively leading to the formation of calcitriol acid secreted in bile (50).

b. Vitamin D receptor (VDR) (Figure 2):



Figure 2 - Vitamin D receptor protein structure

VDR has been demonstrated in almost all cells of the body. Calcitriol acts through vitamin D receptor (VDR) that is a member of nuclear receptor superfamily of ligand-activated transcription factors. VDR binds to target DNA sequences as a heterodimer with retinoid X receptor, recruiting a series of coactivators that modify chromatin and approximate the VDR to the basal transcriptional apparatus resulting in the induction of target gene expression. VDR protein contains a zinc-finger DNA-binding and transcriptional activation domain and a ligand-binding domain as seen in Figure 1.

The VDR Ligand binding domain is organized into 13 α -helices and 3 β -sheets, which together form a hydrophobic ligand-binding pocket. Helix 12, containing the ligand-dependent AF-2 has a central role in creating a co-activator interaction surface. In response to calcitriol binding, helix 12 folds over top of the globular LBD and caps the ligand-binding cavity. This creates a hydrophobic cleft composed of helices 3, 4, 5, and 12 and the docking surface for many

nuclear receptor coactivators by interacting with a complementary hydrophobic domain in the co-activator containing the consensus LXXLL motif -nuclear receptor box (51,52). The heterodimer of retinoid X receptor (RXR) and vitamin D receptor (VDR) binds to a pair of hexameric sequences separated by three intervening bases (ATG). Upon binding to DNA, the RXR-VDR heterodimer facilitates formation of a transcription initiation complex, which binds to DNA at and near the TATA sequence (53). There are several co-activators involved in this process. Steroid receptor coactivator (SRC)-1 interacts with cAMP response element binding protein (CBP)/p300, a histone acetyltransferase (HAT) that remodels chromatin structure at the promoter. SRCs also possess intrinsic HAT activity. Another large multi-protein complex known as DRIP interacts with VDR. SRCs enter the complex first to remodel the chromatin, followed by DRIP complex entry and subsequent recruitment of RNA polymerase (51).

c. VDR Gene Structure:

The human VDR consists of 11 exons. There have been many polymorphisms described in the human VDR gene (~ 1000), however only a few have been commonly associated with bone metabolism including Fok1, Bsm1, Apa1, Taq1 and Cdx2. These are represented in Figure 3.

VDR polymorphisms

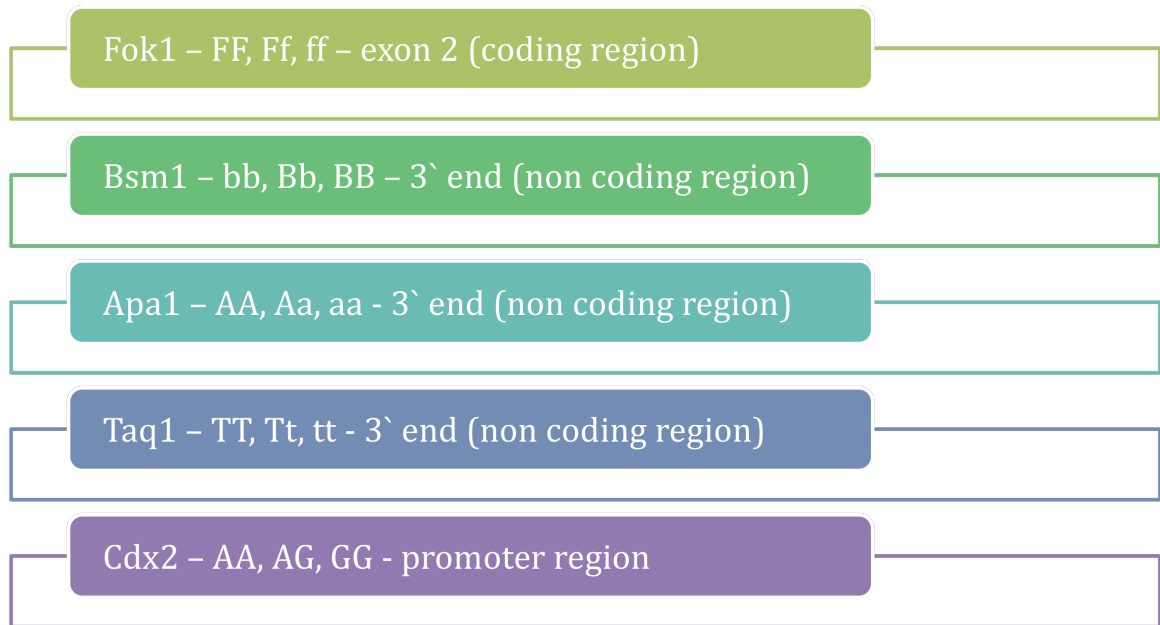


Figure 3 – Polymorphisms in vitamin D receptor gene

Fok1 is the only one that leads to alteration in the VDR protein structure since it is located in exon 2 of coding region (54). Bsm1, Apa1 and Taq1 are located in the 3' untranslated region of the gene.

How these polymorphisms exert their functional effects is still not clear. However, it may be possible that they are in linkage disequilibrium with as yet unidentified functional polymorphisms in VDR (55). It may also affect the binding of the protein product (VDR) with the response elements during target gene activation and transcription.

Several studies have shown an association between polymorphisms of the VDR gene and bone mineral density and also with rates of bone loss (56-60). In previous studies at our center we have shown that VDR gene polymorphisms interacted with environmental factors such as caffeine to influence the rate of bone loss in elderly women (56,61). The subjects with tt genotype showed a significantly higher bone loss at femoral neck as compared to TT genotypes (Change in BMD =

-4.34±3.26 % in tt and 2.31±1.33 % in TT genotype; p = 0.02) when 96 postmenopausal women on placebo were followed for 3 years (56). Some studies have shown that VDR gene polymorphisms can influence the response to vitamin D or calcitriol treatment in terms of increased bone mineral density and serum parathyroid hormone (PTH) suppression (62,63). In one study involving 81 women 70 years or more, the mean increase in bone mineral density at femoral neck was significantly higher in the BB (delta BMD: 4.4%, p = 0.04) and Bb genotype (delta BMD: 4.2%, p = 0.007) as compared to bb genotype (delta BMD: -0.3%, p = 0.61) (62).

Vitamin D binding protein (DBP)

Vitamin D metabolites are transported in the blood bound to DBP (85%) and albumin (15%).

DBP is a glycoprotein synthesized in the liver and binds to 25OHD and 24,25 (OH)₂ D with 30 times greater affinity than 1,25 (OH)₂ and vitamin D. It circulates in blood at about 50 times higher concentrations than Vitamin D metabolites. The binding of DBP to vitamin D metabolites prolongs their half-life by protecting them from hepatic metabolism (64).

DBP protein consists of three domains. Domain 1 is the ligand-binding domain while 2 and 3 are activity domains. Some of the most common studied polymorphisms have been in the exon 11 of DBP gene that encodes domain 3. The two most common polymorphisms studies are rs7041 (HaeIII) at codon 416 and rs4588 (StyI) at codon 420, both located in exon 11. Based on the nucleotide at these codons and amino acid present in protein, there are 3 major haplotypes – Gc1s, Gc2 and Gc1f (65). These haplotypes correspond to GC, TA and TC nucleotides at codons 416 and 420 respectively. These in turn code for glutamate/threonine, aspartate/lysine and aspartate/threonine amino acids respectively.

DBP gene polymorphisms are associated with Rheumatoid Arthritis, Graves' disease and osteoporosis (66-68). It has also been shown that the affinity of 25OHD for the binding protein varies with different DBP haplotypes with highest affinity for Gc1F > Gc1S > Gc2 (69).

In one study conducted in 595 Danish post-menopausal women, the researchers found that serum

25OHD levels were associated with Gc haplotypes (70). They also found that the concentration of Gc protein (DBP) differed in these haplotypes. It could be possible that different Gc haplotypes not only have different affinities but also different serum DBP protein levels and both of these factors might influence the final circulating serum 25OHD levels. These findings suggest that DBP may play an important role in determining serum 25OHD levels in response to vitamin D therapy. Also, little is known about the effect of these RFLPs on the physiological response in terms of serum parathyroid hormone (PTH), serum and urine calcium and calcium absorption. Even so there is a lot of individual variation in the response depending on many factors including genetics. Vitamin D receptor (VDR) and vitamin D binding protein (DBP) gene polymorphisms have been shown to be associated with serum 25OHD (71-82). However, the results of these association studies are conflicting and none have used a systematic multiple dose response approach. Only one study has analyzed effect of DBP polymorphism on treatment response to vitamin D but this was not a randomized placebo controlled trial and only one polymorphism was analyzed (83). Also, since the non-classical actions of vitamin D (cancer, immune and metabolic diseases) might require treatment with very high doses of vitamin D (for e.g. a clinical trial of vitamin D in multiple sclerosis is currently using 35,000 IU/d of vitamin D), these aspects become very important. There are many adverse events associated with high dose vitamin D intake including but not limited to hypercalcemia that may lead to headache, nausea, vomiting, confusion, lethargy and abdominal pain, polyuria, polydipsia, hypercalciuria, weakness, cardiac arrhythmias (QT shortening), soft tissue calcification, bone demineralization with pain and nephrocalcinosis. Although increased risk of myocardial infarction (MI) has not been found with vitamin D, a recent meta-analysis found increased risk of MI with calcium supplements (84). The dose at which these effects occur is not known. Currently, Institute of Medicine has defined the upper tolerable intake of vitamin D as 4,000 IU/d that is much lower than what might be used in clinical trials of vitamin D for non-bone actions (85). Also, some studies have found an increase in total and cancer mortality associated with serum 25OHD levels > 40 ng/ml (86). These levels

are easily attainable especially with high dose vitamin D intake. Thus, there is an unmet need to delineate the pharmacokinetics of vitamin D. Our contribution here is expected to provide a more detailed understanding of vitamin D metabolism with regard to pharmacogenetics. *This contribution could be important clinically since this is the first step in delineating the pharmacokinetics and pharmacogenetics of vitamin D that could affect clinical trials of vitamin D (Phase 2 onwards) in various diseases and the doses used.* Once these aspects of vitamin D are known, future investigators can use these findings to design safe and effective intervention trials of vitamin D in various diseases; in turn this could be expected to lead to important advances in other fields affected by vitamin D such as immunity, cancer and metabolic diseases. Also, since vitamin D is inexpensive it could reduce the overall healthcare costs in a significant manner. It is also expected that what is learned will be equally applicable to the prevention/treatment of diseases in relevant domestic and agricultural animals.

My long-term goal is to identify intakes of vitamin D that could be used to achieve specific serum 25OHD levels based upon VDR and DBP Gene restriction fragment length polymorphisms (RFLPs) in patients.

d. Hypothesis and Specific Aims:

Hypothesis: My central hypothesis is that VDR and DBP Gene RFLPs affect the treatment response to Vitamin D in terms of serum 25OHD and serum PTH.

Specific Aims:

- a. To determine the effect of VDR and DBP Gene RFLP on the treatment response to Vitamin D in terms of serum 25OHD

Working Hypothesis: Our working hypothesis is that mean serum 25OHD level at the end of study will be associated with VDR SNPs (ApaI and TaqI) and DBP SNPs (HaeIII and StyI).

- b.** To determine the effect of VDR and DBP Gene RFLP on the treatment response to Vitamin D in terms of serum PTH

Working hypothesis: Our working hypothesis is that mean serum PTH level at the end of study will be associated with VDR SNPs (ApaI and TaqI) and DBP SNPs (HaeIII and StyI).

The results of our study may lead to the development of pharmacogenetics for vitamin D. This is the first longitudinal study of its kind to be carried out and will provide detailed information for clinicians to determine the effect of genotypes on final achieved serum 25OHD levels and other physiological responses to vitamin D supplementation. Future investigators could use the results of our study to develop large clinical trials of vitamin D in various pathological states.

2. MATERIALS AND METHODS

a. Source study

The blood samples for my study were obtained from an ongoing study ViDOS (Vitamin D supplementation in older subjects) being conducted by Dr JC Gallagher in Creighton University Medical Center. This study was a 1 year double blind, randomized prospective clinical trial aimed at establishing the dose of vitamin D₃ required to increase serum 25OHD levels to >30 ng/ml and normalize serum PTH in 97.5% of study subjects in winter in postmenopausal Caucasian and African American women with a starting serum 25OHD level of <20 ng/ml and sufficient intake of calcium.

We here report the results from 160 Caucasian women enrolled in the study. The recruitment of Caucasian women lasted from January 2007 to early May 2008 (pre summer) and the study finished one year later. Due to the slow recruitment, the African American women were not included in the present analysis. All subjects were initially screened for serum 25OHD levels during winter where cutaneous vitamin D₃ synthesis is minimal. Qualified subjects were randomized to 8 treatment arms and followed upto 12 months of supplementation. The subjects were given a calcium supplement to increase their total calcium intake to 1,200-1,400 mg/d based on their dietary intake of calcium. The Creighton University Institutional Review Board approved the study protocol.

b. Study Visits

Volunteers were recruited from local population by advertising the study in local newspapers and church bulletins with a toll free number to call in. Those who had called in were contacted by the recruiter, underwent a telephone screening for the exclusion and inclusion criteria. Any subjects on multivitamins containing vitamin D were told to stop taking them before receiving the study drug. Subjects with significant co-morbidities, those with serum 25OHD <5 ng/ml or > 20 ng/ml, body mass index > 45 Kg/m², serum calcium > 10.6 mg/dl or > 0.3 mg/dl more than upper limit

of normal on 2 baseline tests, 24 hour urine calcium > 290 mg/dl on 2 baseline tests or bone mineral density T-score < -3 on spine or hip were excluded. Participants were also excluded if they were on any drugs interfering with vitamin D metabolism.

The subjects underwent a comprehensive medical history at baseline. Fasting blood samples were collected at all visits – baseline, 3,6,9 and 12 months between 7.00 AM and 10.00 AM and allowed to clot, then centrifuged at 4⁰ C for 15 min at 2056xg to separate serum. All samples were stored frozen at -70⁰ C until analysis. The study subjects were provided information on toxicity of vitamin D₃ supplements in the informed consent form. In addition a separate instruction sheet including this information was given to the subjects while dispensing the study drug. The procedures performed at 12 months included a medical history, recording any adverse events fasting blood sample collection, 24h urine collection and an end of study 7 day food diary was collected.

c. Study groups

One hundred and sixty Caucasian women were randomly assigned to vitamin D₃ 400 IU; 800 IU; 1,600 IU; 2,400 IU; 3,200 IU; 4,000 IU; 4,800 IU/day or placebo. All subjects were given calcium supplements to increase their total calcium intake to 1,200 mg/d based on their dietary intake of calcium.

d. Study medications

Vitamin D₃ capsules and matching placebo capsules were custom- manufactured for the study (Douglas Labs, Pittsburgh, PA). Every participant took 1 vitamin D₃ capsule in the morning. Calcium supplements (Citracal; Mission Pharmacal, Boerne, TX) were given to maintain a total calcium intake of 1,200-1,400 mg/d based on a baseline 7-day food diary, subjects were advised to take calcium tablets in divided doses. The subjects were given tablets every 3 months. At 3, 6

and 9 month follow up periods and at end of study period, the bottles were returned and the pills counted and recorded for compliance purposes.

e. Informed consent and HIPAA

Each subject was required to read and sign a consent form summarizing participation as outlined in the U.S. code of Federal Regulations (Title 21, Part 50). A separate consent form was signed for the DNA study.

All subjects signed the HIPAA form- Health Insurance Portability and Accountability Act regarding confidentiality of subject data. HIPAA provisions were followed during laboratory procedures by numerical coding of DNA samples from patients thus de-identifying them from the patient records for the use of samples in laboratory. All personal information of study subjects remains in the possession of principal investigator and his staff.

f. Laboratory Methods

i. DNA Genotyping:

DNA Isolation

DNA was isolated from peripheral white blood cells by Gentra Puregene blood kit (Qiagen USA) by phenol/chloroform extraction method. DNA was isolated from baseline blood samples. Red Blood Cells (RBCs) were lysed with RBC lysis solution (Triton X-100 in 0.32 M sucrose, 10 mm Tris-HCl and 5 mm MgCl₂ at pH 7.5). 10 ml of blood was added to a 50 ml centrifuge tube containing 30 ml RBC lysis solution, followed by incubation at room temperature for 5 minutes. The sample was then centrifuged at 3,000 rpm for 3 minutes. After discarding the supernatant, the cell lysate was vortexed vigorously to re-suspend cells and 10 ml of cell lysis solution was added. The next step was protein precipitation. The sample was cooled to room temperature by placing on ice for 3 minutes and 3.33 ml of protein precipitation solution was

added. This was then centrifuged at 3,000 rpm for 5 minutes. Precipitated proteins formed a tight brown pellet.

This was followed by DNA precipitation. The supernatant was poured into a new 50 ml centrifuge tube containing 10 ml of 99.5% isopropanol and centrifuged at 3,000 rpm for 3 minutes to reveal small white pellet of DNA. After discarding the supernatant, 10 ml of 70% ethanol was added to the DNA pellet. This was centrifuged at 3,000 rpm for 1 minute followed by carefully pouring off ethanol. The sample was allowed to air dry for 10-15 minutes followed by DNA hydration. This was achieved by adding 1 ml of DNA hydration solution to the sample and incubating at 65°C for 1 hour in heater. The re-hydration was allowed to continue for 24 hours at room temperature. The final step involved removal of RNA by treating samples with RNase A. DNA was re-extracted, precipitated and re-hydrated with the same procedure as described above and stored at -86°C in proper containers.

Polymerase Chain Reaction (PCR)

Conventional polymerase chain reaction amplification of the target DNA sequence was done using GoTaq Master mix and other reagents. A list of reagents used is given in Supplementary Table 1.

The primers for VDR (ApaI and TaqI) and DBP (Hae III and StyI) polymorphisms were generated from NCBI website. The primers used are specified in Table 1.

Table 1 – Primers used for DNA genotyping

RFLP	Primer	T _m (°C)	Product Length	Fragment Size after enzyme digestion	GC (%)
VDR - ApaI/TaqI	Forward Primer 5' GGATCCTAAATGCACGGAG A 3'	54.3	398 bp	ApaI – 188 and 210 bp TaqI – 168 and 230 bp	50
VDR - ApaI/TaqI	Reverse Primer 5' CACTCAGGCTGGAAGGAGA G 3'	57.1			60
DBP – HaeIII/StyI	Forward Primer 5' ACTGGACTTCCAATTCAGCA GCCA 3'	60.7	489 bp	Hae III – 230 and 259 bp StyI – 200 and 289 bp	50
DBP – HaeIII/StyI	Reverse Primer 5' CCAGGAAAAGCCTGTCACA TAATGGCA 3'	61.2			48.1

PCR procedure for VDR RFLPs:

This involved adding 50 µl of GoTaq green master mix to 10 µl of genomic DNA in a PCR tube for each patient. This was followed by addition of 39 µl of nuclease-free water and 1 µl of primer pair. The samples were centrifuged at 1,000 g/min for 5 minutes. PCR conditions consisted of keeping the samples at 94° C for 5 minutes followed by Stage II. Stage II involved keeping the samples at 94° C for 45 seconds, 54.3° C for 45 seconds and 72° C for 45 seconds. This was repeated thirty six times. The final stage consisted of keeping the samples at 72° C for 10 minutes.

Enzyme Digestion Apa

This was done by adding 14.1 µl of nuclease-free water to 12 µl of PCR product for each patient sample. The final step involved adding 0.6 µl of restriction enzyme (ApaI), 3 µl of NE4 buffer and 0.3 µl of BSA (bovine serum albumin – supplied with the enzyme). After centrifuging the samples at 1,000 g/min for 5 minutes, they were incubated at 25° C for 1 hour.

Enzyme Digestion Taq

This was done by adding 13.4 µl of nuclease-free water to 12 µl of PCR product for each patient sample. The final step involved adding 1.3 µl of restriction enzyme (TaqI), 3 µl of NE4 buffer and 0.3 µl of BSA (bovine serum albumin – supplied with the enzyme). After centrifuging the samples at 1000 g/min for 5 minutes, they were incubated at 65° C for 1 hour.

Gel Analysis for VDR RFLPs

The procedure for formation of agarose gel has been described in the appendix (7a). Briefly, the enzyme-digested samples were run on 2 % agarose gel stained with ethidium bromide or Lonza stain and photographed under UV light.

PCR procedure for DBP RFLPs

This involved adding 37.5 µl of GoTaq green master mix to 10 µl of genomic DNA in a PCR tube for each patient. This was followed by addition of 25.6 µl of nuclease-free water and 1.9 µl of primer pair. The samples were centrifuged at 1,000 g/min for 5 minutes. PCR conditions consisted of keeping the samples at 94° C for 5 minutes followed by Stage II. Stage II involved keeping the samples at 94° C for 45 seconds, 55.7° C for 45 seconds and 72° C for 45 seconds. This was repeated thirty six times. The final stage consisted of keeping the samples at 72° C for 10 minutes.

Enzyme Digestion Hae III

This was done by adding 14.5 µl of nuclease-free water to 10 µl of PCR product for each patient sample. The final step involved adding 2.5 µl of restriction enzyme (HaeIII) and 3 µl of NE4 buffer. After centrifuging the samples at 1,000 g/min for 5 minutes, they were incubated at 37° C for 1 hour.

Enzyme Digestion StyI

This was done by adding 14.5 µl of nuclease-free water to 10 µl of PCR product for each patient sample. The final step involved adding 2.2 µl of restriction enzyme (StyI), 3 µl of NE3 buffer and 0.3 µl of BSA (bovine serum albumin – supplied with the enzyme). After centrifuging the samples at 1,000 g/min for 5 minutes, they were incubated at 37° C for 1 hour.

Gel Analysis for DBP SNPs

The procedure for formation of agarose gel has been described in the appendix (7a). Briefly, the enzyme-digested samples were run on 2 % agarose gel stained with ethidium bromide or Lonza stain and photographed under UV light.

ii. Biochemical Analysis

Blood and spot urine were collected between 7.00 AM and 10.00 AM to exclude the contribution of diurnal variation in the measurement of bone markers. Blood specimens were allowed to clot and then centrifuged at 4⁰ C for 15 min at 2056xg to separate serum. All samples were stored frozen at –70 ⁰C until analysis.

iii. Serum Calcitropic Hormones

All assays in this category were performed in our research laboratory as previously described (87,88). Serum 25-hydroxyvitamin D (calcidiol, 25OHD) was measured by radioimmunoassay (RIA) using a kit manufactured by Diasorin, Inc. (Stillwater, MN). The minimum detection range reported from Diasorin and in our laboratory is 5 ng/ml. The inter-assay variation is 9.8% and intra-assay variation is 9.2% in our laboratory. Serum intact PTH was measured by Diasorin immunoradiometric assay (Stillwater, MN). The limit for serum PTH detection range in our laboratory is 1.0 pg/ml. The inter-assay variation was 4.1% and intra-assay variation was 2.9%.

We registered to participate in the vitamin D external quality assessment scheme (DEQAS) (89), which is an international program for monitoring the accuracy and precision of 25(OH)D and 1,25-dihydroxy vitamin D assays; our results were within ± 1 SD of the mean (ALTM).

g. Food Diary

The patients recorded everything they ate or drank in a 7-day period. A trained dietician performed the review of the diet. Plastic food models (NASCO, Fort Atkinson, WI) were used to help participants better estimate the quantities consumed as done in our previous studies. This was analyzed by FOOD PROCESSOR II PLUS nutrition and diet analysis system (Esha Research, Salem, OR) for numerous dietary components: protein, fat, carbohydrate, calcium, phosphorus, vitamin D intake, calories, and caffeine. The 7-day food record was done at the beginning and end of the study.

h. Statistical Methods

The primary endpoints for statistical analysis were serum 25OHD and PTH at the final visit. Analysis was done using intent-to-treat strategy. Subject characteristics at baseline were compared between the dose groups, using analysis of variance for continuous variables and chi-square tests for categorical variables.

The effect of dose of vitamin D3 (using doses of 0, 400, 800, 1600, 2400, 3200, 4000, and 4800 IU/d) on final serum 25OHD was modeled using linear regression, with dose as a continuous variable. Linear and quadratic models were explored. The model fit was evaluated using graphical measures, including scatter-plots and residual plots. We formulated 95% prediction limits for the final mean serum 25OHD levels. The RDA (Recommended Daily Allowance) can be interpreted as the dose at which a particular serum 25OHD is below the lower prediction limit for a new individual, we also included 80% prediction limits for final serum 25OHD in which 90% of new individuals would have a value greater than the lower limit.

Modeling dose as a categorical predictor of PTH using Analysis of Variance model, estimated mean levels at each dose are presented with 95% confidence intervals (CI).

Analysis of covariance (ANCOVA) was conducted to test for differences in serum 25OHD and PTH between the 8 dose groups (treated as a categorical variable), while adjusting for known confounders (baseline serum 25OHD, age, BMI, calcium intake, smoking status, alcohol use, average caffeine intake, serum creatinine, baseline serum PTH). Backwards selected models were also examined. If the overall F-tests are significant then pair wise comparisons were made and p-values for these comparisons will be adjusted for multiple comparisons using Tukey's method.

SAS software (SAS Institute Inc., Cary, NC) was used for the statistical analysis. R version 2.11.0 was used to create graphical displays. P-values less than 0.05 are considered statistically significant. The effect of VDR and DBP genotypes on serum 25OHD and serum PTH at 12 months was tested using full factorial multivariate analysis. PASW version 19.0 was used for this analysis. P-values less than 0.05 are considered statistically significant.

3. Results

The mean age of Caucasian women was 67 years. There were 18 withdrawals (11%) leading to 142 completing the study. However, one subject in the 800 IU/d group and one person in placebo who discontinued the study came in for the study visits including the final visit. These two were included in the intent to treat analysis taking the total number analyzed to 144. There were no significant differences at baseline among different treatment groups except that alcohol intake that was lower in placebo and 400 IU/d groups compared to others (Table 2).

Mean dietary intake of vitamin D and calcium at baseline was 115 IU/d and 685 mg/d respectively; this was not different amongst various groups. Mean compliance was 94% for vitamin D and 91% for calcium.

Table 2 – Baseline characteristics

Characteristics	400 (n=18)	800 (n=21)	1600 (n=20)	2400 (n=21)	3200 (n=20)	4000 (n=19)	4800 (n=20)	Placebo (n=21)
Age (years)	70 (8.1)	68 (8.1)	66 (7.4)	66 (6.3)	69 (7.7)	67 (6.8)	65 (6.1)	66 (6.5)
Weight (kg)	79 (14)	75 (17)	77 (15)	79 (12)	79 (16)	77 (17)	84 (18)	82 (17)
Body mass Index	30.3 (5.7)	28.2 (6.1)	30.0 (5.4)	30.4 (5.4)	30.2 (5.7)	29.9 (6.5)	32.1 (6.2)	31.1 (5.3)
N (%)								
Current smoker	2 (11%)	1 (5%)	4 (20%)	1 (5%)	3 (15%)	0	2 (10%)	4 (19%)
Former smoker	7 (39%)	7 (33%)	8 (40%)	8 (38%)	4 (20%)	10 (53%)	9 (45%)	7 (33%)
Non-smoker	9 (50%)	13 (62%)	8 (40%)	12 (57%)	13 (65%)	9 (47%)	9 (45%)	10 (48%)
Alcohol: N (%)								
No*	13 (72%)	9 (43%)	10 (50%)	10 (48%)	6 (30%)	6 (32%)	4 (20%)	14 (67%)
Yes*	5 (28%)	12 (57%)	10 (50%)	11 (52%)	14 (70%)	13 (68%)	16 (80%)	7 (33%)
Serum Creatinine (mg/dl)	0.8 (0.2)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)	0.8 (0.2)
Serum Protein (g/dl)	6.7 (0.3)	6.7 (0.3)	6.9 (0.4)	6.7 (0.4)	6.7 (0.4)	6.8 (0.3)	6.8 (0.3)	6.8 (0.4)
Serum 25OHD (ng/ml)	15.4 (4.4)	16.4 (3.9)	15.3 (4.2)	15.6 (4.1)	16.3 (3.4)	15.4 (3.7)	15.6 (3.8)	15.2 (3.5)
Serum PTH (pg/ml)	37.8 (17.5)	33.0 (10.3)	37.6 (14.1)	35.7 (9.9)	31.9 (10.9)	37.7 (20.7)	35.2 (13.6)	39.6 (14.3)
Food Diary Calcium (mg)	596 (222)	741 (247)	754 (244)	621 (190)	725 (263)	671 (334)	768 (348)	593 (182)
Food Diary Vitamin D (IU)	101 (61)	135 (70)	125 (71)	98 (55)	109 (62)	109 (85)	137 (86)	105 (61)
Food Diary Fiber (g)	16.0 (4.8)	16.3 (7.3)	15.9 (4.7)	14.2 (4.7)	15.3 (4.9)	15.0 (4.8)	17.8 (7.0)	14.1 (5.0)
Food Diary Caffeine (mg)	303 (390)	125 (112)	278 (222)	173 (143)	311 (348)	221 (178)	271 (311)	232 (179)

Data expressed as mean (SD) unless specified

*p<0.05

a. Serum 25OHD at 12 months

The dose response curve for serum 25OHD at the end of study (12 months) as a function of dose of vitamin D is given in Figure 4. A quadratic model was the best fit to data.

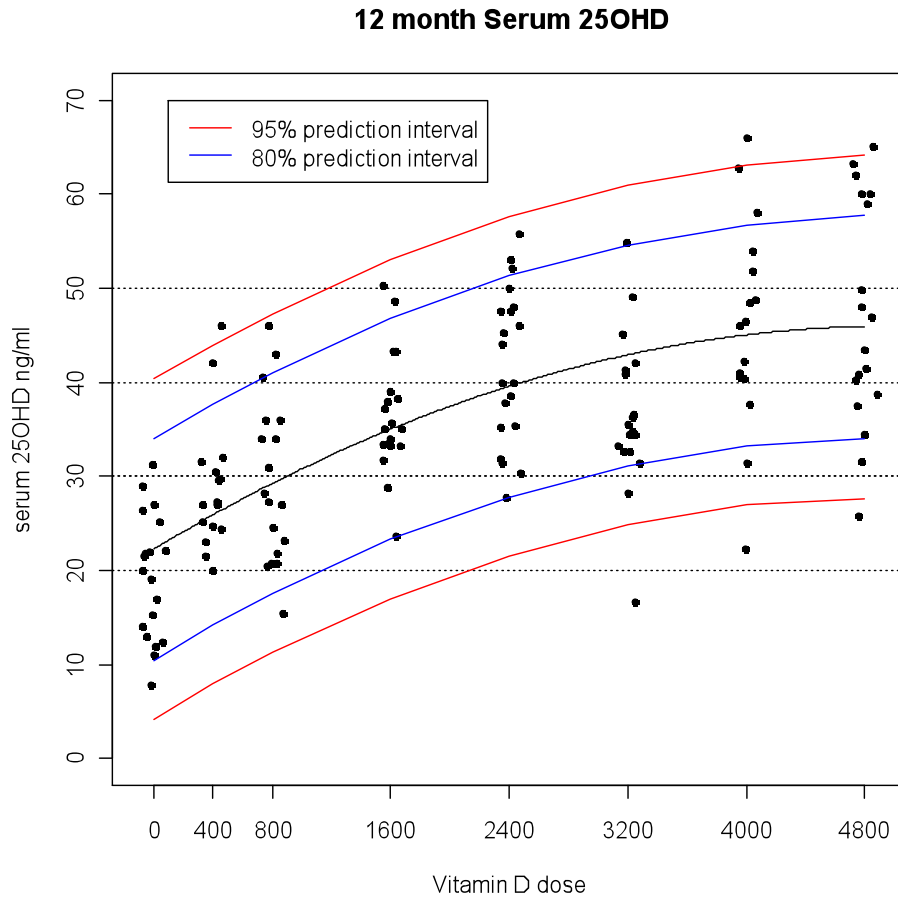


Figure 4 – Dose response of vitamin D

The results of multivariate analysis for predicting serum 25OHD levels at the end of the study (12 months) are presented in Table 3. The only covariates that were statistically significant were BMI and baseline serum 25OHD (Table 3). The overall F-test for final serum 25OHD was significant ($p < 0.0001$), indicating that the final serum 25OHD differed between the dose groups. From the backward selected model including only baseline serum 25OHD, BMI and dose; serum 25OHD at 12 months was significantly lower in the placebo group compared to all vitamin D dose groups individually ($p < 0.05$).

Table 3. Final serum 25OHD at the end of study after one year vitamin D supplementation- Multivariate analysis					
Effect	B Estimate	SE	95% Lower CI	95% Upper CI	P-value^a
Intercept	19.80	10.88	-1.75	41.36	
Baseline serum 25OHD, ng/ml	0.54	0.16	0.23	0.85	0.0009
Age, years	0.06	0.10	-0.14	0.27	0.54
BMI, Kg/m ²	-0.43	0.14	-0.71	-0.15	0.0027
Smoking status, Never	-1.97	2.74	-7.40	3.46	0.77
Smoking status, Former	-1.52	2.71	-6.89	3.85	
Smoking status, Current	Reference	-	-	-	
Alcohol use, Yes	1.81	1.55	-1.26	4.89	0.25
Alcohol use, No	Reference	-	-	-	
Average caffeine intake, mg/d	0.0017	0.0032	-0.0047	0.0080	0.60
Total calcium intake; mg/d	-0.0004	0.0051	-0.0105	0.0098	0.94
Baseline serum creatinine, mg/dl	0.37	6.37	-12.26	13.00	0.95
Baseline serum PTH, pg/ml	-0.0365	0.0581	-0.1517	0.0786	0.53
Dose of Vitamin D, IU/d					
4,800	29.35	2.92	23.57	35.13	<0.0001
4,000	27.44	3.05	21.40	33.48	
3,200	16.46	2.94	10.63	22.28	
2,400	24.28	2.77	18.79	29.77	
1,600	16.98	2.66	11.71	22.24	
800	8.50	2.86	2.83	14.18	
400	10.10	2.97	4.21	15.98	
0	Reference	-	-	-	

b. Serum PTH at 12 months:

The full multivariate model of final serum PTH, showed that dose was not a significant predictor after adjusting for covariates (p=0.098). The only significant predictor was baseline serum PTH (B (SE) = 0.67 (0.06), 95% Confidence Interval (CI) = 0.55-0.78, p < 0.0001) as well as alcohol intake being marginally significant (p=0.053). From a backwards-selected model, final serum PTH was significant after adjusting for baseline serum PTH and alcohol use (p=0.03). However,

after adjusting for multiple comparisons none of the pair-wise comparisons between dose groups were significantly different.

c. Genotype Analysis

The representative photographs for ApaI, TaqI, HaeIII and StyI polymorphisms are depicted in Figure 5 and 6, 7 and 8 respectively.

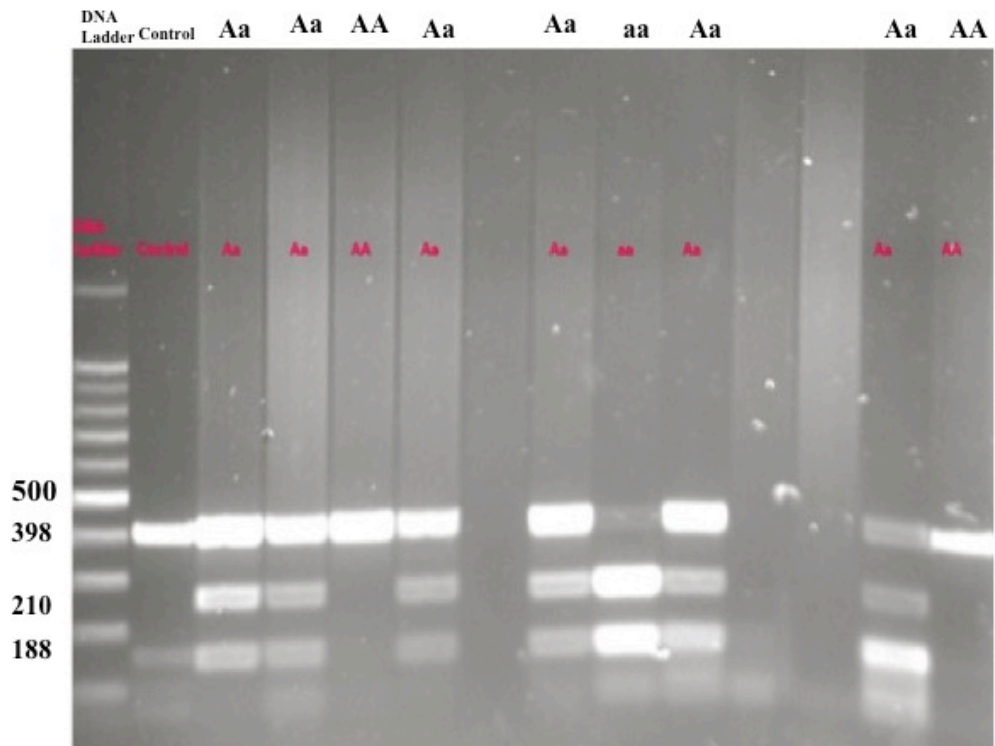


Figure 5 - Representative gel photograph for ApaI polymorphism showing DNA ladder followed by control (undigested PCR product). This is followed by ApaI polymorphism for nine patients

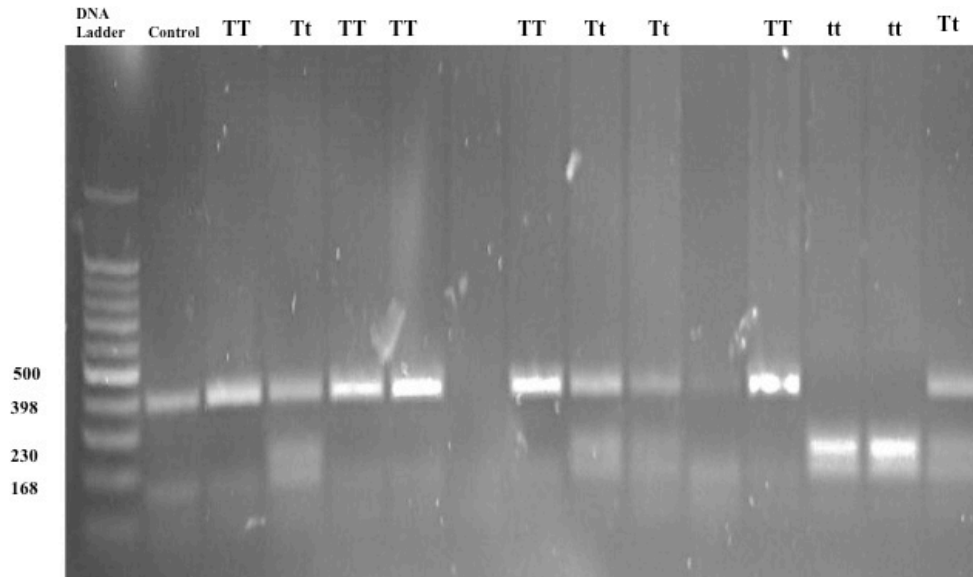


Figure 6 - Representative gel photograph for TaqI polymorphism showing DNA ladder followed by TaqI polymorphism for six patients

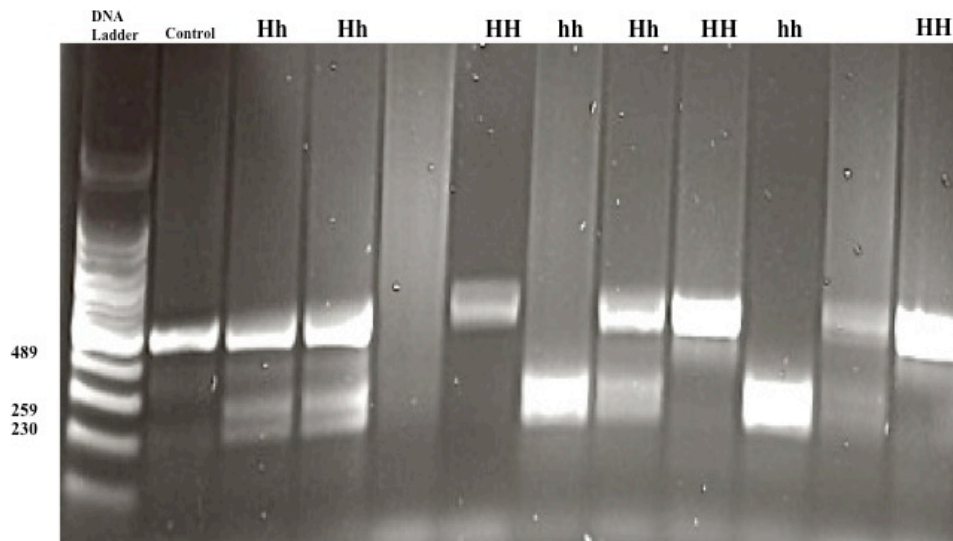


Figure 7 - Representative photographs for HaeIII polymorphism showing DNA ladder followed by control (undigested PCR product). This is followed by HaeIII polymorphism for nine patients

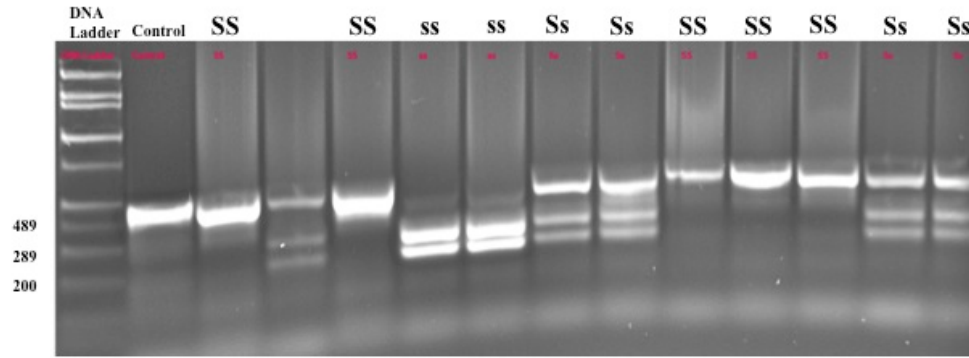


Figure 8 - Representative photographs for StyI polymorphism showing DNA ladder followed by control (undigested PCR product). This is followed by StyI polymorphism for eleven patients

The baseline characteristics of participants according to SNPs HaeIII, StyI, ApaI and TaqI are given in Tables 4,5,6 and 7 respectively. There were no major differences in the baseline characteristics including serum 25OHD and serum PTH in various genotype groups except for fiber intake in HaeIII, caffeine intake and serum protein in StyI and smoking prevalence in ApaI genotypes.

Table 4 – Baseline characteristics according to HaeIII SNP

Variables	HH (n = 42)	Hh (n = 52)	Hh (n = 25)
Age (years)	66.2 (6.6)	67.0 (7.1)	66.9 (7.8)
Body mass Index	31.6 (6.7)	30.3 (5.5)	29.4 (6.2)
Weight (kg)	82.9 (18.7)	78.8 (14.4)	76.2 (16.0)
Food Diary nutrient intake/day: Vitamin D (IU)	115.2 (78.2)	121.4 (80.8)	121.9 (83.1)
Food Diary nutrient intake/day: Calcium (mg)	683.5 (257.0)	674.0 (223.6)	736.6(251.4)
Food Diary nutrient intake/day: Caffeine (mg)	182.3 (160.9)	196.3 (236.0)	190.1(168.9)
Food Diary nutrient intake/day: Fiber (g)*	14.8 (5.6)	14.5 (5.1)	17.5 (4.4)
Serum 25OHD at baseline (ng/ml)	15.1 (3.9)	15.3 (4.0)	17.0 (3.8)
Serum parathyroid hormone at baseline (pg/ml)	39.0 (15.4)	37.0 (14.1)	36.8 (12.0)
Serum Protein (g/dl)	6.8 (0.5)	6.8 (0.4)	6.8 (0.2)
Serum Creatinine (mg/dl)	0.7 (0.1)	0.8 (0.1)	0.8 (0.1)
Serum Calcium (mg/dl)	9.5 (0.3)	9.5 (0.3)	9.5 (0.3)
Current Alcohol use: N (%)**	20 (32.8 %)	25 (41 %)	16 (26.2 %)
Current Smokers: N (%)**	4 (33.3 %)	6 (50 %)	2 (16.7%)

Data expressed as mean (SD) unless specified

*p <0.05 unless otherwise specified

**Chi-square test used

Table 5 – Baseline characteristics according to StyI SNP

Variables	SS (n = 48)	Ss (n = 57)	ss (n = 12)
Age (years)	65.4 (6.8)	67.4 (7.8)	67.5 (6.2)
Body mass Index	30.0 (6.3)	30.3 (6.1)	32.6 (5.8)
Weight (kg)	77.8 (16.4)	79.2 (17.0)	85.7 (14.8)
Food Diary nutrient intake/day: Vitamin D (IU)	108.2 (76.0)	133.0 (86.5)	97.1 (58.5)
Food Diary nutrient intake/day: Calcium (mg)	705.1 (238.3)	696.0 (254.8)	674.9 (208.8)
Food Diary nutrient intake/day: Caffeine (mg)*	275.6 (315.7)	160.7 (150.8)	180.7 (159.8)
Food Diary nutrient intake/day: Fiber (g)	15.6 (5.0)	14.9 (5.2)	15.9 (6.6)
Serum 25OHD at baseline (ng/ml)	15.5 (3.9)	15.5 (3.9)	14.6 (4.2)
Serum parathyroid hormone at baseline (pg/ml)	37.5 (13.8)	38.0 (15.3)	36.7 (11.1)
Serum Protein (g/dl)*	6.9 (0.4)	6.7 (0.4)	6.6 (0.5)
Serum Creatinine (mg/dl)	0.8 (0.1)	0.8 (0.1)	0.7 (0.1)
Serum Calcium (mg/dl)	9.6 (0.3)	9.5 (0.3)	9.5 (0.3)
Current Alcohol use: N (%)**	28 (46.7 %)	26 (43.3 %)	6 (10 %)
Current Smokers: N (%)**	8 (57.1 %)	6 (42.9 %)	0

Data expressed as mean (SD) unless specified

*p <0.05 unless otherwise specified

**Chi-square test used

Table 6 – Baseline characteristics according to ApaI SNP

Variables	AA (n = 24)	Aa (n = 75)	Aa (n = 22)
Age (years)	65.8 (7.1)	67.7 (7.2)	65.0 (6.5)
Body mass Index	28.6 (3.9)	30.8 (6.5)	31.1 (6.7)
Weight (kg)	76.7 (11.6)	80.7 (17.6)	78.9 (17.2)
Food Diary nutrient intake/day: Vitamin D (IU)	94.1 (70.7)	123.6 (79.4)	127.0 (85.3)
Food Diary nutrient intake/day: Calcium (mg)	678.0 (229.1)	700.7 (222.4)	700.4 (315.3)
Food Diary nutrient intake/day: Caffeine (mg)	307.1 (343.5)	192.6 (203.1)	175.0 (170.2)
Food Diary nutrient intake/day: Fiber (g)	15.1 (6.1)	15.2 (4.8)	15.2 (6.0)
Serum 25OHD at baseline (ng/ml)	15.1 (4.2)	15.7 (3.9)	15.0 (4.1)
Serum parathyroid hormone at baseline (pg/ml)	35.8 (13.3)	38.2 (13.4)	36.4 (17.9)
Serum Protein (g/dl)	6.8 (0.4)	6.7 (0.4)	6.9 (0.4)
Serum Creatinine (mg/dl)	0.8 (0.1)	0.8 (0.1)	0.7 (0.1)
Serum Calcium (mg/dl)	9.5 (0.3)	9.5 (0.3)	9.5 (0.4)
**Current Alcohol use: N (%)	15 (24.6 %)	36 (59.0 %)	10 (16.4 %)
**Current Smokers: N (%)*	6 (42.9 %)	6 (42.9 %)	2 (14.2 %)

Data expressed as mean (SD) unless specified

*p <0.05 unless otherwise specified

**Chi-square test used; data expressed as mean (SD) unless specified

Table 7 – Baseline characteristics according to TaqI SNP

Variables	TT (n = 57)	Tt (n = 48)	tt (n = 16)
Age (years)	68.1 (7.4)	66.4 (6.8)	63.6 (6.0)
Body mass Index	30.2 (6.0)	30.4 (6.4)	30.9 (6.5)
Weight (kg)	78.5 (15.8)	79.0 (16.6)	83.5 (18.4)
Food Diary nutrient intake/day: Vitamin D (IU)	133.5 (84.0)	105.6 (74.0)	102.0 (71.1)
Food Diary nutrient intake/day: Calcium (mg)	704.4 (255.9)	687.8 (227.3)	715.7 (238.3)
Food Diary nutrient intake/day: Caffeine (mg)	169.2 (153.8)	271.2 (303.2)	173.2 (222.3)
Food Diary nutrient intake/day: Fiber (g)	15.2 (4.9)	15.7 (5.6)	14.9 (6.3)
Serum 25OHD at baseline (ng/ml)	15.9 (3.6)	15.0 (4.0)	14.6 (4.7)
Serum parathyroid hormone at baseline (pg/ml)	36.7 (14.8)	38.7 (14.5)	37.0 (11.3)
Serum Protein (g/dl)	6.8 (0.3)	6.8 (0.4)	6.8 (0.5)
Serum Creatinine (mg/dl)	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)
Serum Calcium (mg/dl)	9.5 (0.3)	9.5 (0.3)	9.5 (0.3)
**Current Alcohol use: N (%)	28 (45.2 %)	23 (37.1 %)	11 (17.7 %)
**Current Smokers: N (%)	4 (28.6 %)	7 (50.0 %)	3 (21.4 %)

Data expressed as mean (SD) unless specified

*p <0.05 unless otherwise specified

**Chi-square test used

Effect of HaeIII SNP on serum 25OHD and serum parathyroid hormone

The results of multivariate analysis are shown in Table 8, supplementary Table 2 (see appendix) and Figures 9 and 10. We found that there was no significant effect of genotypes based on HaeIII SNP on the dose response to vitamin D in terms of serum 25OHD and serum PTH after adjustment for relevant confounders.

Table 8 - Multivariate analysis for HaeIII SNP

Source	Dependent Variable	df	Mean Square	F	Sig.
Corrected Model	Serum 25OHD at 12 months	32	454.258	7.037	.000
	Serum PTH at 12 months	32	209.582	1.580	.051
Intercept	Serum 25OHD at 12 months	1	1113.125	17.244	.000
	Serum PTH at 12 months	1	129.674	.978	.326
Age (years)	Serum 25OHD at 12 months	1	.055	.001	.977
	Serum PTH at 12 months	1	4.418	.033	.856
Serum creatinine	Serum 25OHD at 12 months	1	31.037	.481	.490
	Serum PTH at 12 months	1	141.279	1.065	.305
Dietary vitamin D intake	Serum 25OHD at 12 months	1	72.670	1.126	.292
	Serum PTH at 12 months	1	6.434	.049	.826
Dietary fiber intake	Serum 25OHD at 12 months	1	199.794	3.095	.082
	Serum PTH at 12 months	1	68.875	.519	.473
Dietary caffeine intake	Serum 25OHD at 12 months	1	2.669	.041	.839
	Serum PTH at 12 months	1	409.289	3.085	.083
BMI	Serum 25OHD at 12 months	1	550.994	8.536	.005
	Serum PTH at 12 months	1	631.072	4.757	.032
Baseline serum 25OHD	Serum 25OHD at 12 months	1	980.500	15.190	.000
	Serum PTH at 12 months	1	308.433	2.325	.131
Smokers	Serum 25OHD at 12 months	1	13.110	.203	.653
	Serum PTH at 12 months	1	219.149	1.652	.202
Alcohol use	Serum 25OHD at 12 months	1	.016	.000	.987
	Serum PTH at 12 months	1	249.226	1.879	.174
Genotype_HaeIII	Serum 25OHD at 12 months	2	49.334	.764	.469
	Serum PTH at 12 months	2	340.479	2.567	.083
Dose group	Serum 25OHD at 12 months	7	1293.109	20.032	.000
	Serum PTH at 12 months	7	120.744	.910	.503
Genotype_Hae* Dose group	Serum 25OHD at 12 months	14	74.145	1.149	.331
	Serum PTH at 12 months	14	196.200	1.479	.138
Error	Serum 25OHD at 12 months	81	64.551		
	Serum PTH at 12 months	81	132.654		
Total	Serum 25OHD at 12 months	114			
	Serum PTH at 12 months	114			
Corrected Total	Serum 25OHD at 12 months	113			
	Serum PTH at 12 months	113			

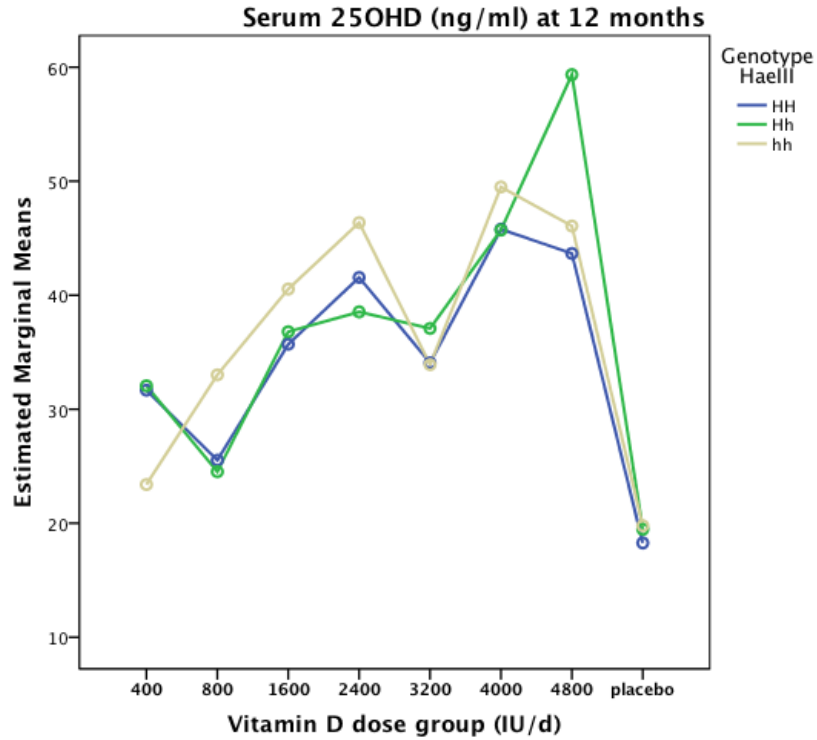


Figure 9 – Serum 25OHD (25-hydroxyvitamin D) at the end of study according to dose group and HaellI genotypes

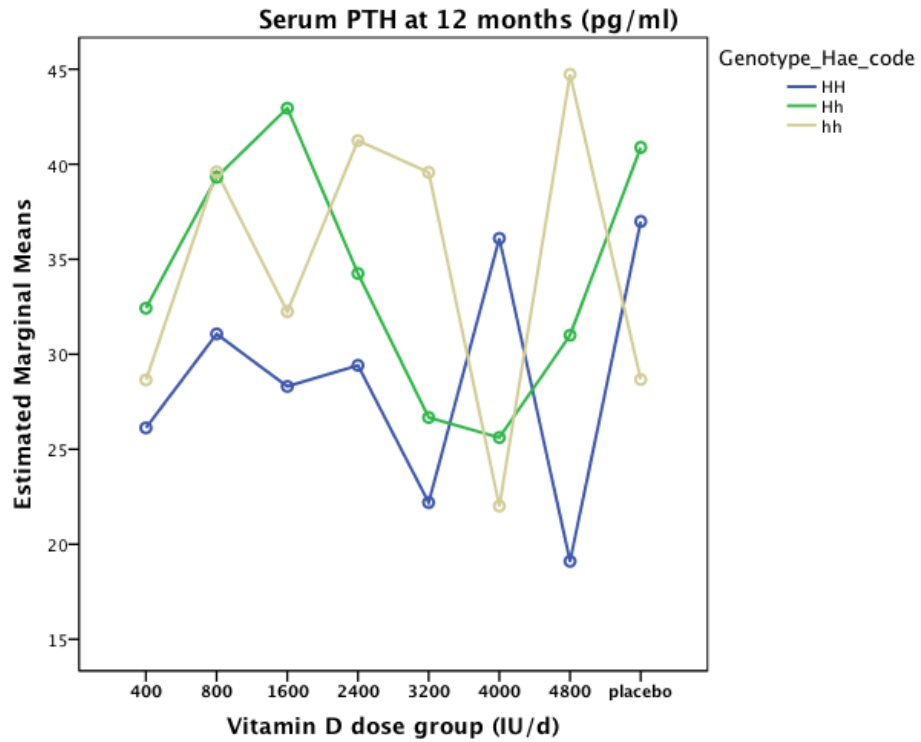


Figure 10 – Serum PTH (parathyroid hormone) at the end of study according to dose group and HaellI genotypes

Effect of StyI SNP on serum 25OHD and serum parathyroid hormone

The results of multivariate analyses are shown in Table 9, supplementary Table 3 (see appendix) and Figures 11 and 12. We found that there was no significant effect of genotypes based on StyI SNP on the dose response to vitamin D in terms of serum 25OHD and serum PTH after adjustment for relevant confounders. However, there was a significant interaction between genotypes based on StyI and dose of vitamin D used ($p = 0.01$, Table 9).

Table 9 - Multivariate analysis for StyI SNP

Source	Dependent Variable	df	Mean Square	F	Sig.
Corrected Model	Serum 25OHD at 12 months	29	491.070	8.718	.000
	Serum PTH at 12 months	29	207.579	1.502	.079
Intercept	Serum 25OHD at 12 months	1	721.766	12.813	.001
	Serum PTH at 12 months	1	167.163	1.209	.275
Age (years)	Serum 25OHD at 12 months	1	4.779	.085	.772
	Serum PTH at 12 months	1	6.485	.047	.829
Serum creatinine	Serum 25OHD at 12 months	1	.058	.001	.975
	Serum PTH at 12 months	1	.084	.001	.980
Dietary vitamin D intake	Serum 25OHD at 12 months	1	20.547	.365	.548
	Serum PTH at 12 months	1	.084	.001	.980
Dietary fiber intake	Serum 25OHD at 12 months	1	233.987	4.154	.045
	Serum PTH at 12 months	1	118.165	.855	.358
Dietary caffeine intake	Serum 25OHD at 12 months	1	.824	.015	.904
	Serum PTH at 12 months	1	390.853	2.828	.096
BMI	Serum 25OHD at 12 months	1	305.703	5.427	.022
	Serum PTH at 12 months	1	303.221	2.194	.142
Baseline serum 25OHD	Serum 25OHD at 12 months	1	772.044	13.706	.000
	Serum PTH at 12 months	1	233.332	1.688	.198
Smokers	Serum 25OHD at 12 months	1	4.679	.083	.774
	Serum PTH at 12 months	1	154.564	1.118	.293
Alcohol use	Serum 25OHD at 12 months	1	56.022	.995	.322
	Serum PTH at 12 months	1	96.206	.696	.407
Genotype StyI	Serum 25OHD at 12 months	2	6.197	.110	.896
	Serum PTH at 12 months	2	8.993	.065	.937
Dose group	Serum 25OHD at 12 months	7	1132.598	20.106	.000
	Serum PTH at 12 months	7	185.603	1.343	.241
Genotype_Sty* Dose group	Serum 25OHD at 12 months	11	138.901	2.466	.010
	Serum PTH at 12 months	11	220.008	1.592	.117
Error	Serum 25OHD at 12 months	82	56.331		
	Serum PTH at 12 months	82	138.231		
Total	Serum 25OHD at 12 months	112			
	Serum PTH at 12 months	112			
Corrected Total	Serum 25OHD at 12 months	111			
	Serum PTH at 12 months	111			

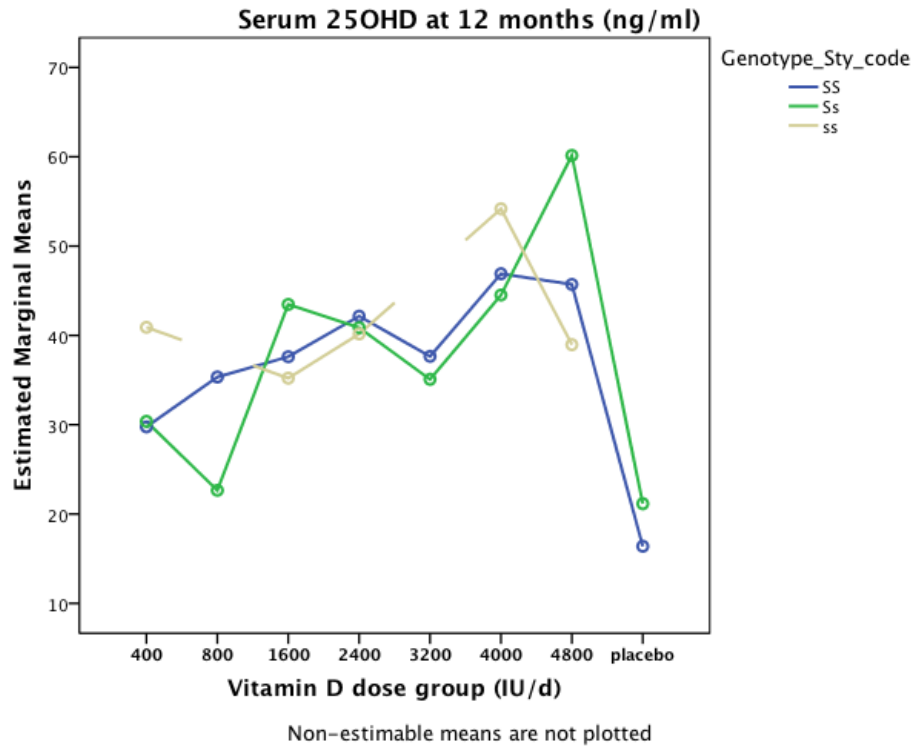


Figure 11 – Serum 25OHD (25-hydroxyvitamin D) at the end of study according to dose group and StyI genotypes

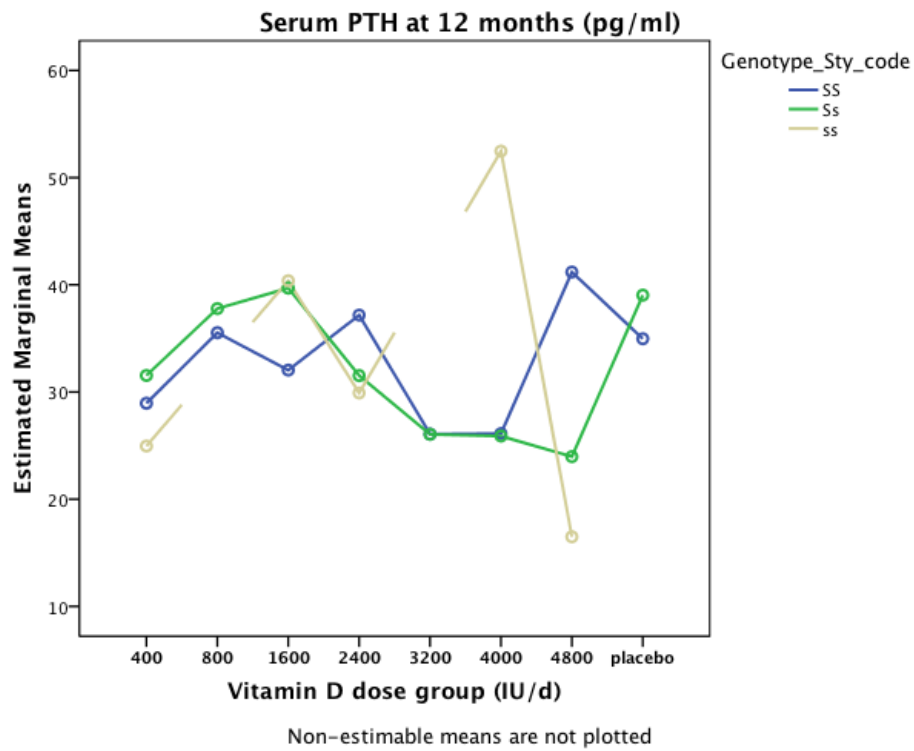


Figure 12 – Serum PTH (parathyroid hormone) at the end of study according to dose group and StyI genotypes

Effect of Apal SNP on serum 25OHD and serum parathyroid hormone

The results multivariate analyses are shown in Table 10, supplementary Table 4 (see appendix) and Figures 13 and 14. We found that there was no significant effect of genotypes based on Apal SNP on the dose response to vitamin D in terms of serum 25OHD and serum PTH after adjustment for relevant confounders.

Table 10 - Multivariate analysis for Apal SNP

Source	Dependent Variable	df	Mean Square	F	Sig.
Corrected Model	Serum 25OHD at 12 months	32	430.555	6.699	.000
	Serum PTH at 12 months	32	193.727	1.341	.146
Intercept	Serum 25OHD at 12 months	1	756.988	11.777	.001
	Serum PTH at 12 months	1	460.398	3.186	.078
Age (years)	Serum 25OHD at 12 months	1	22.330	.347	.557
	Serum PTH at 12 months	1	90.365	.625	.431
Serum creatinine	Serum 25OHD at 12 months	1	5.916	.092	.762
	Serum PTH at 12 months	1	1.660	.011	.915
Dietary vitamin D intake	Serum 25OHD at 12 months	1	7.754	.121	.729
	Serum PTH at 12 months	1	44.651	.309	.580
Dietary fiber intake	Serum 25OHD at 12 months	1	184.292	2.867	.094
	Serum PTH at 12 months	1	525.609	3.637	.060
Dietary caffeine intake	Serum 25OHD at 12 months	1	21.520	.335	.564
	Serum PTH at 12 months	1	93.574	.648	.423
BMI	Serum 25OHD at 12 months	1	524.425	8.159	.005
	Serum PTH at 12 months	1	262.380	1.816	.182
Baseline serum 25OHD	Serum 25OHD at 12 months	1	839.776	13.065	.001
	Serum PTH at 12 months	1	258.000	1.785	.185
Smokers	Serum 25OHD at 12 months	1	.032	.001	.982
	Serum PTH at 12 months	1	1.247	.009	.926
Alcohol use	Serum 25OHD at 12 months	1	77.788	1.210	.275
	Serum PTH at 12 months	1	55.496	.384	.537
Genotype Apal	Serum 25OHD at 12 months	2	135.262	2.104	.128
	Serum PTH at 12 months	2	57.126	.395	.675
Dose group	Serum 25OHD at 12 months	7	879.155	13.678	.000
	Serum PTH at 12 months	7	346.492	2.398	.028
Genotype Apal* Dose group	Serum 25OHD at 12 months	14	51.070	.795	.672
	Serum PTH at 12 months	14	185.520	1.284	.235
Error	Serum 25OHD at 12 months	82	64.276		
	Serum PTH at 12 months	82	144.503		
Total	Serum 25OHD at 12 months	115			
	Serum PTH at 12 months	115			
Corrected Total	Serum 25OHD at 12 months	114			
	Serum PTH at 12 months	114			

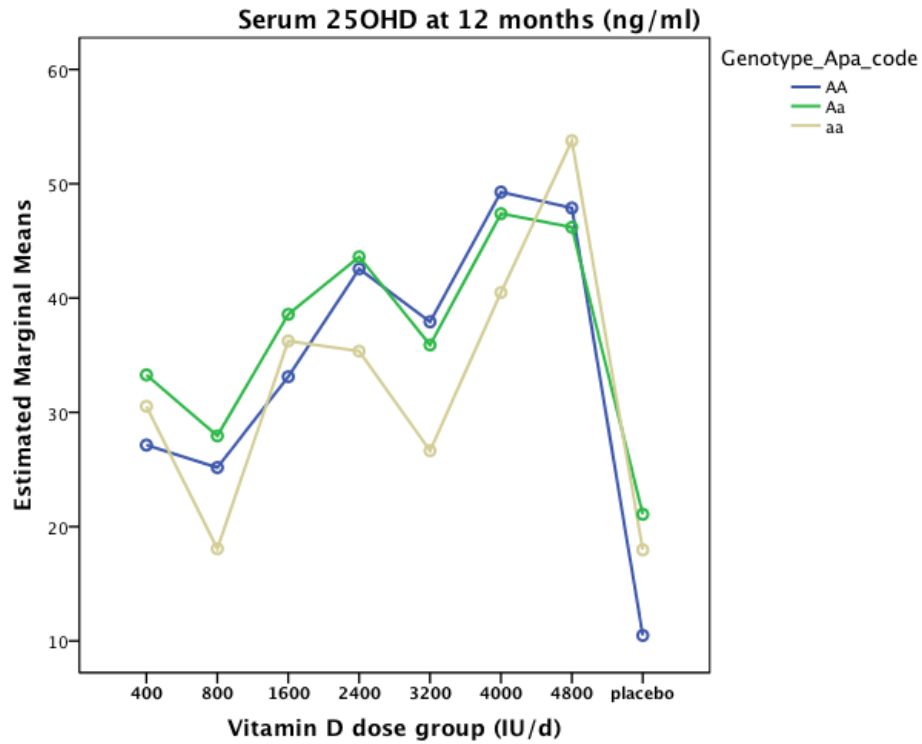


Figure 13 – Serum 25OHD (25-hydroxyvitamin D) at the end of study according to dose group and Apal genotypes

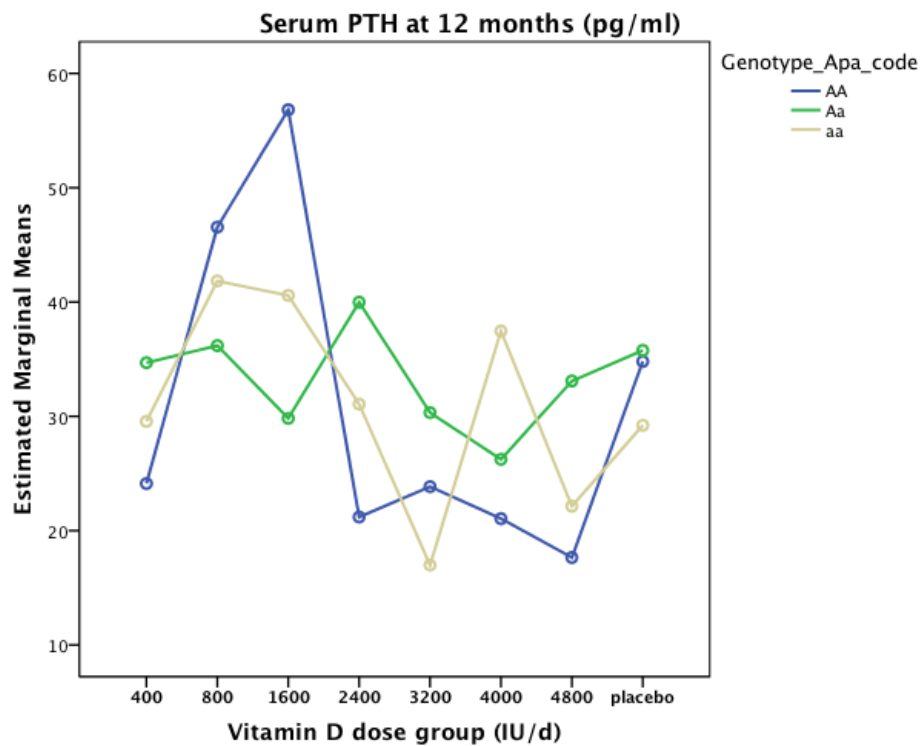


Figure 14 – Serum PTH (parathyroid hormone) at the end of study according to dose group and Apal genotypes

Effect of TaqI SNP on serum 25OHD and serum parathyroid hormone

The results multivariate analyses are shown in Table 11, supplementary Table 5 (see appendix), Figures 15 and 16. We found that there was no significant effect of genotypes based on TaqI SNP on the dose response to vitamin D in terms of serum 25OHD and serum PTH after adjustment for relevant confounders.

Table 11 - Multivariate analysis for TaqI SNP

Source	Dependent Variable	df	Mean Square	F	Sig.
Corrected Model	Serum 25OHD at 12 months	32	424.090	6.353	.000
	Serum PTH at 12 months	32	204.644	1.469	.084
Intercept	Serum 25OHD at 12 months	1	884.054	13.244	.000
	Serum PTH at 12 months	1	145.842	1.047	.309
Age (years)	Serum 25OHD at 12 months	1	25.773	.386	.536
	Serum PTH at 12 months	1	40.116	.288	.593
Serum creatinine	Serum 25OHD at 12 months	1	.776	.012	.914
	Serum PTH at 12 months	1	7.024	.050	.823
Dietary vitamin D intake	Serum 25OHD at 12 months	1	94.595	1.417	.237
	Serum PTH at 12 months	1	2.371	.017	.897
Dietary fiber intake	Serum 25OHD at 12 months	1	152.883	2.290	.134
	Serum PTH at 12 months	1	432.580	3.105	.082
Dietary caffeine intake	Serum 25OHD at 12 months	1	60.329	.904	.345
	Serum PTH at 12 months	1	143.906	1.033	.312
BMI	Serum 25OHD at 12 months	1	676.024	10.127	.002
	Serum PTH at 12 months	1	811.918	5.828	.018
Baseline serum 25OHD	Serum 25OHD at 12 months	1	1036.969	15.535	.000
	Serum PTH at 12 months	1	713.484	5.122	.026
Smokers	Serum 25OHD at 12 months	1	.095	.001	.970
	Serum PTH at 12 months	1	91.080	.654	.421
Alcohol use	Serum 25OHD at 12 months	1	105.182	1.576	.213
	Serum PTH at 12 months	1	220.359	1.582	.212
Genotype TaqI	Serum 25OHD at 12 months	2	92.012	1.378	.258
	Serum PTH at 12 months	2	9.507E-5	.000	1.000
Dose group	Serum 25OHD at 12 months	7	904.094	13.544	.000
	Serum PTH at 12 months	7	250.745	1.800	.098
Genotype Taq* Dose group	Serum 25OHD at 12 months	14	60.073	.900	.562
	Serum PTH at 12 months	14	235.848	1.693	.073
Error	Serum 25OHD at 12 months	82	66.752		
	Serum PTH at 12 months	82	139.307		
Total	Serum 25OHD at 12 months	115			
	Serum PTH at 12 months	115			
Corrected Total	Serum 25OHD at 12 months	114			
	Serum PTH at 12 months	114			

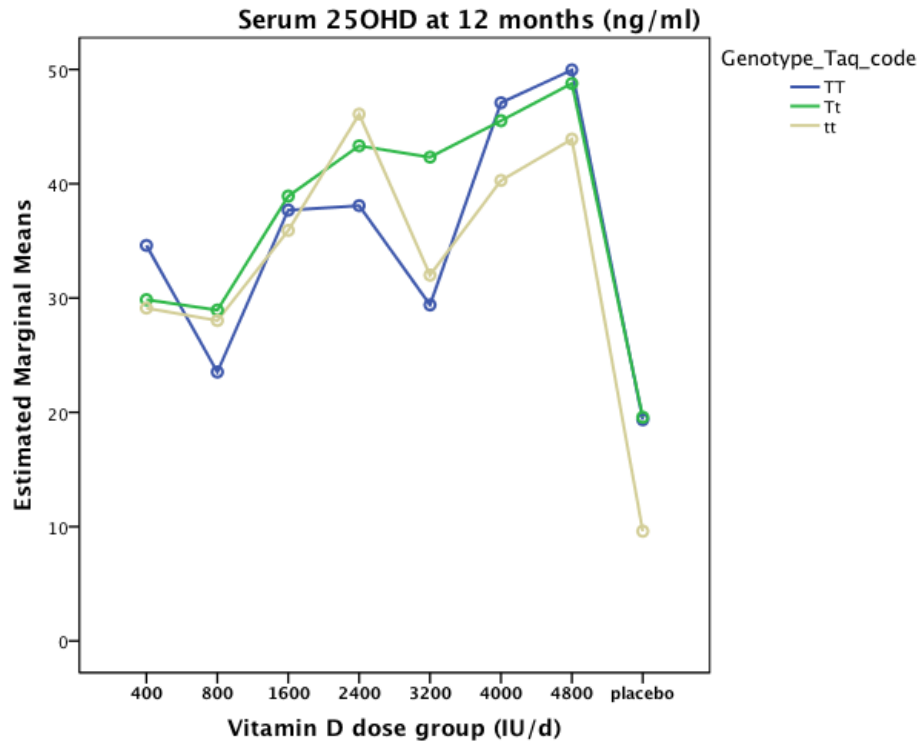


Figure 15 – Serum 25OHD (25-hydroxyvitamin D) at the end of study according to dose group and TaqI genotypes

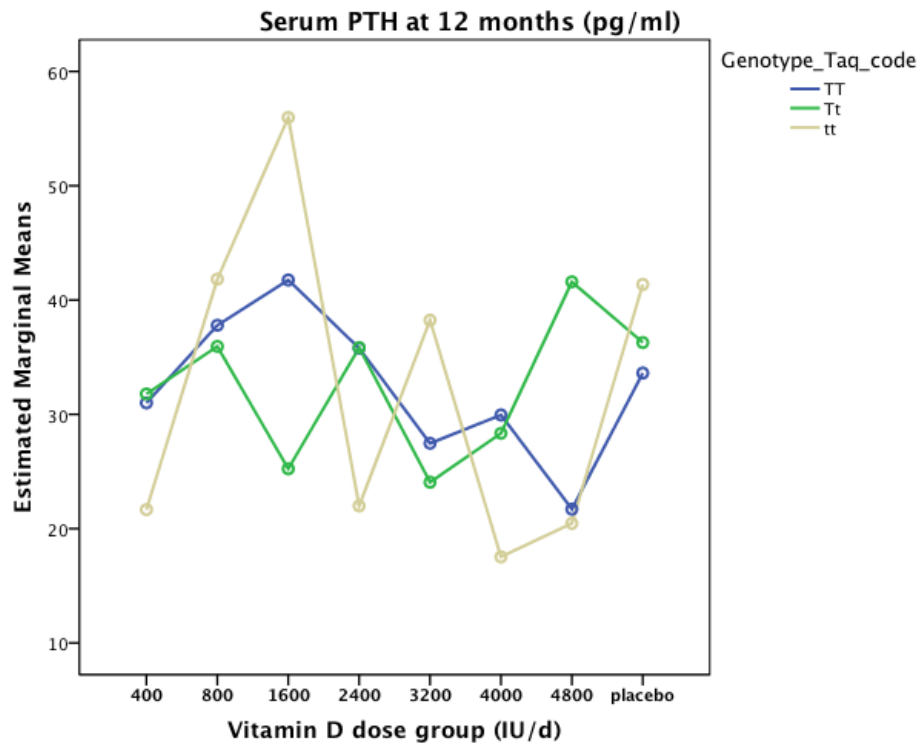


Figure 16 – Serum PTH (parathyroid hormone) at the end of study according to dose group and TaqI genotypes

4. Discussion

The primary finding of this first intervention study to test the effect of VDR and DBP SNPs on serum 25OHD in response to vitamin D supplementation in older Caucasian women was that there was no significant effect of genotypes based on VDR and DBP SNPs on final serum 25OHD and PTH levels at the end of one year. However there was significant effect of vitamin D dose, baseline serum 25OHD and BMI on final serum 25OHD levels at the end of one year. A higher baseline serum 25OHD is responsible for the smaller increase in serum 25OHD with increasing doses of vitamin D as found in previous studies (90). This study also confirms the findings of previous studies where obese subjects had a lower serum 25OHD level (25,26).

There was no effect of vitamin D receptor (ApaI and TaqI) and vitamin D binding protein genotypes (HaeIII and StyI) based on SNPs in these genes on the dose response to vitamin D supplementation in terms of serum 25OHD and serum PTH at 12 months. Most of the studies done previously that have found association between serum 25OHD levels and VDR and/or DBP SNPs were cross-sectional studies, although some of them had large sample sizes (71-82). In a cross-sectional study involving 1530 Hispanics and African Americans with mean age 40 years, BMI 28.9 and mean serum 25OHD of 14.6 ng/ml, the investigators found that serum 25OHD was associated with HaeIII and StyI SNPs with the small s (lysine) allele having less serum 25OHD and 1,25(OH)₂D as compared to S (threonine) allele (72). Similar findings in terms of serum 25OHD were reported in a case-control study involving 4010 breast cancer patients and controls (74). In another trial of prostate cancer, HaeIII was associated with serum 25OHD in 780 control subjects with ~ 10% difference between homozygous wild and rare alleles ($p = 0.0004$) (75). These results as found in this prostate cancer trial are comparable to that found in 152 control patients of another COPD trial (76). In another study of 143 healthy Bangladeshi adults, the researchers found no association between VDR SNPs (ApaI, TaqI, Fok and BsmI) and serum

25OHD levels (77). In a study involving 741 premenopausal Caucasian women, both HaeIII and StyI were associated with serum 25OHD levels after adjustment for BMI, age, vitamin D and calcium intake, education, smoking status, season at the time of blood draw and leisure time physical activity (serum 25OHD was 60.8 nmol/L in HH vs 67.5 in hh, $p = 0.0003$ and 25OHD = 67.2 in SS and 58.4 nmol/L in ss genotypes, $p < 0.0001$) (82). The results of our study are different from some of these studies. This might be due to small sample size but our study represents the first study where both VDR and DBP SNPs were analyzed in an interventional trial. Also, since this was an intervention trial over 1 year, we did not need to adjust for season of blood draw. All other covariates were adjusted in the multivariate analysis. Our results are supported by another recent study that found no association between rs7041 (HaeIII) and rs4588 (StyI) SNPs and serum 25OHD levels (91). This study was done in 156 Caucasian subjects (66 males with mean age 44 yrs and 90 females with mean age 61 yrs) and then the findings were confirmed in a replication cohort of 340 Caucasian post-menopausal women (91). One small study involving 98 subjects (90 females) followed for one year did find an effect of DBP SNP (StyI) on the response to vitamin D supplementation (83). However, this was not a placebo-controlled trial and the population characteristics were heterogeneous - men and premenopausal women were included and mean age was 52 years.

These findings might suggest that the effect of VDR and DBP SNPs on serum 25OHD levels might have been overestimated in literature due to multiple reasons – cross sectional design, lack of adjustment for all relevant confounders, different assays of serum 25OHD used and other factors. Most previous studies of vitamin administration used a single dose and were often of short-term duration. Also exact comparisons among studies have to be tempered by the fact that serum 25OHD assays vary over the years according to the methodology and even using the same kit. But the results of our study should be interpreted with caution, as this might well be due to small sample size.

Strengths and Limitations:

Strengths of ViDOS include the study design with adequate power to detect differences in terms of final serum 25OHD across a broad range of dose groups. Most vitamin D studies have been single dose studies and did not utilize a dose response design. This is the first multiple-dose response randomized double blind placebo controlled trial to be conducted in any population. There are some limitations of the current study. These negative findings apply to the SNP's chosen and it is possible that other SNPs may show positive results. Serum 25OHD was measured by Diasorin RIA which is subject to assay to assay and laboratory to laboratory variation (92) and not by liquid chromatography tandem Mass Spectrometry (LC-MS/MS) measurement procedure that some consider to be the gold standard due to less variation (29). Despite this weakness, inter and intra-assay variations were low and the Bone metabolism laboratory also participates in DEQAS program. Furthermore, as this study was conducted in healthy postmenopausal women, these findings may not be generalizable to other groups. The highest dose of vitamin D used in ViDOS was 4800 IU/d and as such it is difficult to predict the dose response curve beyond this dose.

5. Conclusion:

In a one-year intervention trial utilizing various doses of vitamin D in postmenopausal Caucasian women, there was no significant effect of genotypes based on VDR and DBP SNPs on final serum 25OHD and PTH levels at the end of one year. There was a significant effect of vitamin D dose, baseline serum 25OHD and BMI on final serum 25OHD levels at the end of one year. However, the findings of our genotype study are preliminary in nature and need to be confirmed in large, prospective studies with adequate sample size for sufficient power.

6. Appendix

a. Method for Agarose gel electrophoresis

1. Add 9 grams of agarose gel to beaker
2. Add 450 ml of TAE 1x buffer
3. Microwave for 4 minutes
4. Stir with spatula
5. Microwave again for 1 minute
6. Let it stand for 10 minutes in hood
7. Add 50 μ l of Lonza gel stain
8. Mix it completely
9. Let stand again for 2-4 minutes or if already cool pour it on the plate
10. Keep the gel combs at the positions taking into consideration the negative pole
11. Allow solidifying.
12. Take the combs out and put DNA ladder at each of 4 ends
13. Put PCR product in 4 wells alongside DNA ladder
14. Put DNA samples in other wells
15. Fill the gel plate with TAE1x buffer
16. Start electrophoresis for 2 hours at 200 volts, 150 mill amperes and 50 kilowatts
17. Take the gel out and take photographs under UV light under ethidium bromide filter
18. Record in lab book and store the file with specific name

b. Inclusion and Exclusion Criteria

Exclusion Criteria for screening:

1. Cancer within the last 10 years or terminal illness
2. Previous hip fracture
3. Hemiplegia
4. Uncontrolled type I diabetes \pm significant proteinuria or fasting blood sugar >140 mg in type II.
5. Kidney stones- active disease or kidney stones > 2 times in lifetime
6. Chronic renal failure (serum creatinine >1.4 mg/dl)
7. Evidence of chronic liver disease, including alcoholism
8. Previous treatment with bisphosphonates for more than 3 months in the past
9. Fluoride, PTH or PTH derivatives e.g. teriparatide treatment in the last 6 months
10. Previous treatment within the last 6 months with calcitonin or estrogen
11. Chronic high dose corticosteroid therapy (> 10 mg/d) for over 6 months
12. Physical conditions severe enough to prevent reasonable physical activity like rheumatoid arthritis, osteoarthritis and heart failure
13. Currently on anticonvulsants (Dilantin, Phenobarbital), high dose thiazide therapy (> 37.5 mg/d) and any drugs interfering with vitamin D metabolism
14. Serum calcium > 10.6 mg/dl or > 0.3 mg/dl more than upper limit of normal on 2 baseline tests
15. 24 hour urine calcium > 290 mg/dl on 2 baseline tests
16. Bone mineral density T-score < -3 on spine or hip

Inclusion Criteria for Screening:

1. Minimum age 57 years (at least 7 years postmenopause), maximum age 90 years

2. BMI < 45
3. Serum 25OHD > 5 ng/ml and < 20 ng/ml
4. Willing to give signed informed consent form
5. If in the last 6 months taking vitamin D supplements including multivitamins, 3 month wash out period

c. Data Tables

Supplementary Table 1 – List of Reagents used for DNA genotyping

Sr No.	Reagent
1	DNA Primers – Integrated DNA technologies
2	PCR Master Mix – Go Taq Green Master Mix (Promega Corporation)
3	Nuclease free water – Promega Corporation
4	Restriction Endonucleases – Apa I, Taq I, Hae III and Sty I – New England Biolabs
5	100 base pair DNA ladder – Promega Corporation
6	GenePure LE Quick Dissolve Agarose - Fischer
7	Ethidium bromide
8	Lonza Gel Star Nucleic Acid Stain - Fischer

Supplementary Table 2 – Serum 25-hydroxyvitamin D and parathyroid hormone levels at

12 months in genotypes based on HaeIII SNP – Descriptive statistics

	Genotype HaeIII	Dose group of vitamin D (IU/d)	Mean	Std. Deviation
Serum 25OHD at 12 months (ng/ml)	HH	400	32.4800	11.77842
		800	25.9025	13.67527
		1600	34.6850	6.61952
		2400	42.2775	9.35257
		3200	35.3000	19.15658
		4000	44.3025	18.13369
		4800	42.8150	12.52293
		Placebo	15.6429	5.68446
		Hh	400	30.3314
	800		27.2971	5.10495
	1600		36.3900	4.31151
	2400		39.0617	6.95106
	3200		36.8240	4.52764
	4000		45.8000	10.34482
	4800		58.6420	6.10498
	Placebo		19.1383	7.04776
	hh		400	22.7367
		800	38.5200	6.33568
		1600	40.5760	8.37161
		2400	44.4433	7.95548
		3200	36.2400	.

		4000	52.8533	4.72890	
		4800	43.1725	13.81178	
		Placebo	23.7175	3.50929	
Serum PTH at 12 months (pg/ml)	HH	400	26.3333	9.45163	
		800	32.2500	7.41058	
		1600	31.0000	11.47170	
		2400	28.2500	5.33854	
		3200	24.3333	8.38650	
		4000	36.5000	19.07005	
		4800	21.1667	7.52108	
		Placebo	38.0000	7.23418	
		Hh	400	37.7143	15.05229
			800	36.1429	11.06690
1600	41.6000		16.92040		
2400	34.6667		15.04216		
3200	26.4000		8.79204		
4000	25.8571		15.00476		
4800	27.0000		14.08900		
Placebo	40.8333		11.16094		
hh	400		31.0000	3.00000	
	800	35.5000	.70711		
	1600	29.0000	2.73861		
	2400	42.6667	25.42309		
	3200	36.0000	.		
	4000	19.0000	7.00000		
	4800	44.0000	14.07125		
	Placebo	28.2500	5.31507		

Supplementary Table 3 - Serum 25-hydroxyvitamin D and parathyroid hormone levels at 12 months in genotypes based on StyI SNP – Descriptive statistics

	Genotype StyI	Dose group of vitamin D (IU/d)	Mean	Std. Deviation	
Serum 25OHD at 12 months (ng/ml)	SS	400	24.0367	3.62133	
		800	36.4600	14.00624	
		1600	38.3067	6.81038	
		2400	42.0714	9.65750	
		3200	37.3267	3.19189	
		4000	47.7429	12.07550	
		4800	45.0783	11.11275	
		Placebo	18.5386	6.17209	
	Ss	400	29.8211	5.31562	
		800	25.3678	6.06255	
		1600	40.1667	5.34057	
		2400	40.3900	6.56951	
		3200	36.2525	10.82525	
		4000	45.6300	13.58910	
		4800	61.7500	2.36291	
		Placebo	19.1710	7.20773	
	ss	400	46.0000	.	
		1600	29.6050	8.50649	
		2400	42.2700	9.30075	
		4000	48.3900	.	
		4800	35.5275	3.21468	
	Serum PTH at 12 months (pg/ml)	SS	400	36.0000	11.35782
			800	35.0000	7.54983
			1600	31.5556	11.09179
2400			37.1429	16.56732	
3200			30.6667	6.80686	
4000			25.7143	15.94485	
4800			41.6667	14.50057	
Placebo			33.0000	5.32291	
Ss		400	33.0000	13.78405	
		800	34.7778	10.15847	
		1600	38.6667	18.47521	
		2400	31.1667	15.15806	
		3200	25.6250	8.08769	
		4000	24.6667	11.75868	
		4800	21.2500	5.31507	
		Placebo	40.3000	9.84378	
ss		400	23.0000	.	
		1600	43.0000	14.14214	
		2400	28.7500	5.56028	
		4000	56.0000	.	
		4800	19.2500	8.77021	

Supplementary Table 4 - Serum 25-hydroxyvitamin D and parathyroid hormone levels at 12 months in genotypes based on ApaI SNP – Descriptive statistics

Genotype ApaI		Dose group of vitamin D (IU/d)	Mean	Std. Deviation	
Serum 25OHD at 12 months (ng/ml)	AA	400	22.5550	3.61332	
		800	27.3550	9.45402	
		1600	33.2800	.	
		2400	42.1675	9.51023	
		3200	36.5900	2.99379	
		4000	52.2833	10.31152	
		4800	49.7250	17.35947	
		Placebo	13.2933	3.24008	
	Aa	400	33.3386	7.57397	
		800	30.8100	9.16850	
		1600	38.0992	7.49509	
		2400	43.6522	6.98545	
		3200	36.3300	11.74714	
		4000	48.9050	10.61241	
		4800	44.5267	11.51536	
		Placebo	19.9246	6.44314	
	aa	400	25.4633	1.53637	
		800	20.1333	4.13812	
		1600	33.7350	.37477	
		2400	36.0950	8.65881	
		3200	32.7000	.	
		4000	36.0933	13.12675	
		4800	53.1300	16.42074	
		Placebo	22.0400	.	
	Serum PTH at 12 months (pg/ml)	AA	400	29.5000	2.12132
			800	43.5000	12.02082
			1600	58.0000	.
			2400	22.2500	5.18813
3200			28.0000	7.70281	
4000			19.0000	6.24500	
4800			17.5000	2.12132	
Placebo			31.3333	11.93035	
Aa		400	34.0000	15.62050	
		800	33.8889	8.08462	
		1600	30.6667	8.32666	
		2400	39.5556	16.01648	
		3200	28.1429	6.41427	
		4000	25.5000	15.00476	
		4800	33.6667	16.86713	
Placebo		36.9231	10.35585		
aa		400	35.0000	13.11488	

	800	37.6667	13.86843
	1600	41.0000	26.87006
	2400	29.2500	4.57347
	3200	14.0000	.
	4000	41.0000	18.02776
	4800	24.6667	8.73689
	Placebo	27.0000	.

Supplementary Table 5 - Serum 25-hydroxyvitamin D and parathyroid hormone levels at 12 months in genotypes based on TaqI SNP – Descriptive statistics

	Genotype TaqI	Dose group of vitamin D (IU/d)	Mean	Std. Deviation
Serum 25OHD at 12 months (ng/ml)	TT	400	32.8780	10.30838
		800	27.4514	9.29062
		1600	39.6033	7.85867
		2400	38.5438	7.48918
		3200	31.5260	9.04514
		4000	47.5414	14.31950
		4800	47.1217	12.19307
		Placebo	19.6111	5.67918
	Tt	400	28.4133	2.49078
		800	30.1060	10.91194
		1600	35.8900	7.01533
		2400	42.7617	8.92330
		3200	39.9167	8.34822
		4000	46.8983	10.58057
		4800	50.3200	11.40067
		Placebo	18.9371	7.36105
	tt	400	20.0000	.
		800	28.5700	7.00842
		1600	35.6200	.
		2400	47.0000	6.55744
		3200	36.2400	.
4000		42.1900	.	
4800		41.7500	18.47911	
Placebo		11.8800	.	
Serum PTH at 12 months (pg/ml)	TT	400	33.6000	11.21606
		800	32.5714	10.26088
		1600	39.0000	16.45600
		2400	35.7500	15.85425
		3200	25.4000	8.23408
		4000	29.5714	15.77822
		4800	24.0000	9.85901
		Placebo	33.3333	8.03119
	Tt	400	34.3333	16.21933
		800	35.8000	8.92749
		1600	28.4286	3.30944
		2400	34.6667	13.07925
		3200	26.6667	6.94742
		4000	26.6667	17.09581
tt	400	39.6000	19.02104	
	Placebo	38.0000	13.66260	
	tt	400	28.0000	.

800	41.6667	9.07377
1600	53.0000	.
2400	22.6667	6.65833
3200	36.0000	.
4000	17.0000	.
4800	23.3333	10.21437
Placebo	35.0000	.

7. Glossary

1,25(OH) ₂ D	1,25-dihydroxyvitamin D
24,25(OH) ₂ D	24,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D
AF-2	Activation factor 2
AI	Adequate intake
BMD	Bone mineral density
BMI	Body mass index
bp	base pair
bsa	Bovine serum albumin
cAMP	Cyclic adenosine mono phosphate
CBP	Cyclic adenosine mono phosphate binding protein
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CYP24	25-hydroxyvitamin D-24-hydroxylase
CYP27B1	25-alpha-Vitamin D hydroxylase
DBP	Vitamin D binding protein
DEQAS	Vitamin D external quality assessment scheme
DNA	Deoxyribonucleic acid
DRI	Dietary Reference Intake
DRIP	Vitamin D receptor interacting protein
EAR	Estimated average requirement
HAT	Histone acetyl transferase
HIPAA	Health Insurance Portability and Accountability Act
IOM	Institute of Medicine
IRB	Institutional Review Board
IU	International Units
LBD	Ligand binding domain
ml	Milliliter
MN	Minnesota
NC	North Carolina
ng	Nanogram
NHANHES	National Health and Nutrition Examination Survey
NIA	National Institute on Aging
OR	Oregon
PCR	Polymerase Chain Reaction
PTH	Parathyroid hormone
RBC	Red blood corpuscles
RDA	Recommended Dietary Allowance
RFLP	Restriction Fragment Length Polymorphism
RIA	Radio-immunoassay
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
SD	Standard Deviation
SE	Standard Error
SNP	Single nucleotide polymorphism
SRC-1	Steroid receptor coactivator - 1
T _m	Melting temperature
UVB	Ultraviolet B radiation
ViDOS	Vitamin D supplementation in older subjects
VDR	Vitamin D receptor
WHO	World Health Organization

β
 μ

Beta
Micro

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