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The Effects of Vitamin D and Its Receptor in Normal Subjects and Kidney Stone Formers

By

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A thesis submitted in conformity with the requirements
For the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathology
University of Toronto

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Abstract

Vitamin D exists in two major forms, as the D2 and D3 form. It is accepted that vitamin D2 and vitamin D3 exert different biological effects in all animal species tested. Yet in humans, vitamin D2 and D3 are presumed to have equivalent biological effects. To test this assumption in humans, I compared the ability of equimolar quantities of ingested vitamin D2 or D3 to increase serum 25-hydroxyvitamin D (25(OH)D), the clinical measure of vitamin D nutrition.

Vitamin D may also play an important role in hypercalciuric calcium stone formation because calcitriol, the hormonal form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)2D), controls calcium homeostasis. Available data support the hypothesis that disordered 1,25(OH)2D production is one cause of idiopathic hypercalciuria (IH). I tested the hypothesis that patients with idiopathic hypercalciuria may have an exaggerated inverse relationship between serum 1,25(OH)2D and 25(OH)D in the physiologic range. IH patients challenged with a vitamin D supplement should therefore exhibit a larger fall in serum 1,25(OH)2D than controls.

For 1,25(OH)2D to exert its effects, it must bind to the vitamin D receptor (VDR). Genetic VDR polymorphisms have been reported to predict bone mass. Since vitamin D plays an important role in calcium metabolism, I investigated whether these VDR polymorphisms are associated with hypercalciuria.

The results showed that vitamin D3 is more potent than vitamin D2 at elevating serum 25(OH)D. Thus, the assumption of D3 and D2 equivalence may be erroneous and should be reconsidered. In addition, only a small sub-group of IH stone formers have an inverse relationship between serum 25(OH)D and 1,25(OH)2D. Finally, I found no association between VDR polymorphism and stone formers in comparison to controls. However, there was an association between VDR polymorphism and urinary calcium excretion, suggesting that VDR may exert a weak effect on renal calcium metabolism.
Introduction

Historical Perspective

Vitamin D is best known for its ability to prevent and cure rickets. Whistler and Glisson gave the first scientific descriptions of rickets back in 1650 (1) but the connection between rickets and vitamin D was not uncovered until the 1900’s. In northern European cities, the incidence of rickets increased to epidemic proportions as industrialization and urban development expanded, and sun exposure declined. The causative factor of rickets was not elucidated until the development of the nutrition sciences and the appreciation of the existence of vitamins.

Sniadecki provided the first insight into a possible cause of rickets in 1822. He noticed that children living in narrow urban streets and poorly lit dwellings of Warsaw had very high incidence of rickets whereas children in rural areas did not. He concluded that exposure to sunshine could cure rickets (2). Almost a century later in 1918, Mellanby experimentally induce rickets in beagles by keeping them indoors and feeding them a diet of oatmeal. He reversed rickets in the dogs with cod liver oil and concluded that rickets was a nutritional disease that could be cured by another property of the fat soluble vitamin A in the cod oil (3). However, McCollum demonstrated that the anti-rachitic activity, which he called vitamin D, was separable from vitamin A when he heated and aerated the cod oil to destroy the vitamin A activity (4). At the same time, working on the conclusion of Sniadecki, Huldschinsky exposed rachitic children to radiation from a mercury arc lamp. He reported dramatic reversal of rickets within 4 months after ultraviolet (UV) exposure (5).

In further experiments with UV radiation, Steenbock and Black (6), and Hess and Weinstock (7) found that UV irradiation could impart anti-rachitic activity to food. This concept led to addition of pro-vitamin D to milk and subsequent irradiation to impart anti-rachitic
activity. It also provided the means for Askew and colleagues (8) to produce large quantities for structural determination of vitamin D$_2$ in 1931. Later in 1936, Windaus and co-workers (9) isolated and identified the structure of vitamin D$_3$. After the structure of vitamin D was determined, it was synthesized and directly added to food.

The original anti-rachitic substance termed vitamin D$_1$ was a mixture of ergocalciferol and other sterols and is no longer used. There are two major active forms, vitamin D$_2$ and vitamin D$_3$. (figure 1). Vitamin D$_2$, also known as ergocalciferol, is produced by UV irradiation of the fungal steroid, ergosterol. Vitamin D$_2$ differs from vitamin D$_3$, also known as cholecalciferol, by an extra double bond between the 22-23 carbon and an additional 24-methyl group. Because of their similar structure and potency, it has long been assumed that equal molecular weights of vitamin D$_2$ and D$_3$ have equal effects in humans.
Figure 1

The structure of vitamin D₃ and vitamin D₂. Vitamin D₂ has an extra double bond between the 22-23 carbon and an additional 24-methyl group.
Formation and Metabolism of Vitamin D

In 1978, Esvelt et al (10) demonstrated that vitamin D₃ is produced naturally in the skin by UV activation of 7-dehydrocholesterol. Ultraviolet B photons with wavelengths between 290 and 315 nm penetrate into the skin, where they are absorbed by epidermal and dermal stores of 7-dehydrocholesterol. The UV rays cause photolysis of the 9-10 carbon bond of 7-dehydrocholesterol to form a 9,10-secosterol called previtamin D. Previtamin D is inert and must undergo isomerization to form vitamin D (11).

By definition, a vitamin is a substance the body requires but does not produce. Vitamin D is therefore a misnomer because it is produced in the skin by ultraviolet irradiation. Exposure to sunlight maintains vitamin D status because few foods contain significant amounts of vitamin D naturally. Clothing, sunscreen, increased air pollution, and migration to temperate latitudes limits the amount of UV radiation reaching the skin to generate vitamin D (12). Thus, vitamin D production is severely impaired in much of the world’s temperate populations. When UV rays are not intense enough, dietary supplements are required to overcome inadequate endogenous production of vitamin D. Under these conditions calciferol becomes a vitamin.

Once formed in the skin, vitamin D enters the dermal capillary bed where it is bound to vitamin D binding protein (DBP) and enters the circulation (13). Absorption of dietary vitamin D requires emulsification with bile salts before transport across intestinal epithelium (14). After absorption, it is incorporated into chylomicrons and passed via the lymphatics into the circulation (15).

Whether it is produced by the skin or ingested from the diet, the liver hydroxylates vitamin D to 25-hydroxyvitamin D (25(OH)D) (16). This hydroxylation is carried out by both microsomal and mitochondrial fractions of cytochrome P-450 (17-18). However, only the mitochondrial fraction of P450 is thought to be active in vivo (19). 25(OH)D-hydroxylation in the from microsomes is sex-dependent in rats; 25(OH)D hydroxylase from female rat liver microsome has only one fifth the activity of the male (20). The activity of vitamin D hydroxylase
in humans may also be sex dependent. 25(OH)D is the predominant circulating form of vitamin D and its circulating level reflects vitamin D nutritional status (21). 25(OH)D is transported by DBP to the kidney where it is hydroxylated again to form 1,25-dihydroxyvitamin D (1,25(OH)₂D), the active hormonal form of vitamin D (22). Renal 25(OH)D-1α-hydroxylase is also a cytochrome P-450 mixed-function oxidase that is localized in the inner mitochondrial membrane of the proximal renal tubules. It is composed of three proteins that are integral components of the mitochondrial membrane: ferredoxin reductase, ferredoxin, and a cytochrome P-450 (23).

Regulation of the vitamin D endocrine system occurs through the stringent control of the renal 1-hydroxylase activity, keeping 1,25(OH)₂D in strict homeostatic range despite varying amounts of 25(OH)D substrate. The main regulatory factors are serum concentrations of calcium and phosphate, parathyroid hormone (PTH), and 1,25(OH)₂D₃ itself. Low serum calcium may activate renal 1α-hydroxylase, independent of PTH (24). The increase in PTH in response to hypocalcemia stimulates 1α-hydroxylase to increase synthesis of 1,25(OH)₂D (25). To prevent excess synthesis, 1,25(OH)₂D suppresses its own biosynthesis in the kidney and PTH synthesis by the parathyroid gland (26).

1,25(OH)₂D helps maintain serum calcium levels in a narrow range to support proper function of nerve and muscle tissues. It is also needed to maintain a calcium-phosphate concentration product high enough to allow mineralization of type I collagen matrix in bone (27). 1,25(OH)₂D supports these functions by increasing intestinal absorption of dietary calcium, promoting renal retention of calcium and mobilizing calcium from skeletal stores to restore extracellular calcium concentrations. In addition to this classic mechanism of calcium homeostasis, 1,25(OH)₂D also exerts other effects such as inhibition of cell proliferation, and modulation of cell differentiation and the immune system (28-30).
The Vitamin D Receptor

*Discovery, Isolation and Cloning of VDR*

For the hormonal form of vitamin D to exert its effects, it must bind to its target receptor. Low abundance hindered early attempts to identify the receptor for vitamin D. Haussler et al (31) provided the first evidence of a receptor for vitamin D. They identified a polar metabolite of vitamin D that was associated with the chromatin fraction of intestinal mucosa. Holick et al later identified the metabolite as 1,25(OH)$_2$D$_3$ (22). Further in vitro studies demonstrated that a 50-60 kD protein facilitated 1,25(OH)$_2$D$_3$ binding to chromatin. The protein also displayed saturable binding of 1,25(OH)$_2$D$_3$ and its specificity for other metabolites matched their in vivo potency (32). These studies demonstrated the existence of a receptor for vitamin D and that it mediated the biological actions of vitamin D.

Development of molecular techniques in the 1980's facilitated the isolation of the vitamin D receptor (VDR). Using purified monoclonal avian VDR antibodies, Pike et al (33) screened and isolated avian VDR complementary DNA (cDNA) from an expression library. Baker et al (34) then used the partial avian sequence to isolate human VDR from an intestinal library. The full length human VDR cDNA is 4605 base pairs (bp) long containing a 115 bp noncoding leader sequence, a 1,281 bp open reading frame, and a 3209 bp 3' noncoding sequence. The VDR protein is 427 amino acids in length with a molecular mass of approximately 48 kD. Analysis of the deduced amino acid sequence revealed that the VDR protein belonged to the superfamily of trans-acting transcriptional regulatory factors, including the glucocorticoid, mineralocorticoid, progesterone, estrogen, and androgen receptors, and the thyroid hormone receptors. VDR is most closely related to the thyroid hormone receptors (35).

The VDR gene is comprised of 11 exons that, together with intervening introns, span approximately 75 kb. The noncoding 5'-end of the gene includes exons 1A, 1B, and 1C. Eight
additional exons (exons 2-9) encode the structural portion of the VDR gene product. The structure of VDR can be divided into several domains: a small amino terminal domain whose function is unknown, a DNA binding domain between amino acids 20-115 (36-37), a hinge region between 115-220 (38), and a vitamin D binding domain at the carboxy-terminal (220-427) (39).

Upon binding by 1,25(OH)₂D, VDR becomes hyperphosphorylated and recruits the retinoic acid receptor (RXR) into a heterodimeric complex that binds strongly to DNA (40). The 1,25(OH)₂D-RXR-VDR heterocomplex selectively recognizes vitamin D response elements (VDRE) in the promoter regions of regulated genes such as osteocalcin (41). Transcription factors TFIIB and TFIID join the RXR-VDR complex to alter conformation of DNA strands to affect transcription of genes influenced by vitamin D (42).

**VDR Polymorphisms**

In 1989, Faraco et al (43) discovered a polymorphism in VDR recognized by the ApaI restriction enzyme and reports of polymorphisms at BsmI and TaqI restriction enzymes followed (44). Morrison et al (45) gave the first hint of the importance of VDR polymorphism and its influence on vitamin D metabolism when they reported that VDR polymorphisms in normal subjects at both the BsmI and TaqI restriction sites were associated with significant differences in circulating osteocalcin levels. Since vitamin D is a potent regulator of osteocalcin, the most abundant non-collagen protein in bone and a marker of bone turnover, their results hinted a possible role of VDR in the development of bone disorders. The same group later showed that VDR polymorphism was associated with bone mineral density. Individuals without the BsmI restriction site in VDR had significantly lower bone mineral density (44). Since then, other investigators have found that VDR polymorphisms are associated with other disorders such as prostate cancer, osteoarthritis, diabetes, and hyperparathyroidism (46-49). These reports suggest that VDR polymorphisms may affect metabolic processes that can lead to disease states.
vitamin D plays an important role in calcium metabolism, it is reasonable to question whether VDR polymorphisms disturb calcium handling in a manner that can increased the risk for kidney stone disease.
Kidney Stones in General

Pathogenesis of Kidney Stone Disease

Nephrolithiasis, or kidney stone disease, is a complex disorder where urinary constituents precipitate to form macroscopic crystals. The crystalline components of renal stones are usually held together by a matrix composed of urinary proteins such as albumin, glycosaminoglycan, and gamma-carboxyglutamic acid (50). Stones can occur anywhere along the collecting system of the urinary tract, obstructing urine flow and causing extreme pain. It can also cause bleeding and local erosion into kidney tissue. North Americans have a 12% lifetime chance of developing a kidney stone, men being more prone to the disease than women. Middle-age white males have a 1% annual incidence of the disease (51).

Uric acid, struvite or cystine stones are the major non-calcareous stones (50). Together they make up only 25% of all stones. Formation of uric acid stones usually results from increased urinary uric acid excretion in primary gout. Urea-splitting bacteria that infect the urinary tract produce struvite stones. These bacteria split urea to form ammonium and hydroxyl ion thereby reducing the solubility of struvite and promoting its precipitation. Cystine stones are caused by inborn errors of cystine transport leading to high concentration of urinary cystine. Cystine then precipitates in renal tubules because of its low solubility.

The remaining 75% are calcareous stones composed of calcium in combination with oxalate and/or phosphate (52). The main factors that contribute to aggregation and growth of calcium oxalate within the urinary tract are: i) increased concentration of urinary constituent above its solubility, and ii) the lack of powerful inhibitor of crystal formation and growth in the urine. Furthermore, epithelial damage of renal tubules may also allow crystals to adhere to cell surfaces and initiate stone growth (53). Specific risk factors include hypercalciuria, hyperoxaluria, hypomagnesiuria, hypocitraturia, urine pH and low urine volume. These factors act to promote stone formation by allowing greater amounts of free ion to aggregate. Higher calcium and oxalate excretion frequently observed in stone formers increase the supersaturation
of calcium oxalate, resulting in a higher probability of calcium oxalate formation. Magnesium competes with calcium for absorption at the intestine and thereby lowers calcium absorption (54). It may also help decrease calcium oxalate crystal formation (55). Citrate acts as an inhibitor of stone formation by forming a soluble complex with calcium. Thus, low citrate excretion increases the likelihood of stone formation (56). More alkaline urine pH affects the solubility of phosphate while more acidic urine affects the solubility of uric acid to promote its precipitation. Most importantly, low urine output will concentrate urine constituents, so increasing urine volume helps dilute urinary constituent to prevent stone formation.

**Role of Vitamin D Endocrine System in Calcium Stones**

Vitamin D has been implicated in kidney stone formation because stone formers generally excrete of high amounts of calcium in their urine. Flocks first made the association between high urinary excretion of calcium and kidney stones in 1939 (57). It was not until 1953 that Albright used the term “idiopathic hypercalciuria” to describe persistent hypercalciuria without hypercalcemia (58). Half of all stone formers have idiopathic hypercalciuria (IH), a condition where affected individuals excrete more than 4 mg of calcium per kg body weight per day. These individuals exhibit high circulating 1,25(OH)2D despite normal serum calcium, PTH, and the absence of any systemic illness (59). The hypercalciuria and high 1,25(OH)2D observed in IH suggest a possible role of the vitamin D endocrine system in the pathogenesis of stone formation.

It has been proposed that two distinct types of IH exist, absorptive hypercalciuria and renal hypercalciuria. In absorptive hypercalciuria, the primary abnormality is said to be intestinal hyperabsorption of calcium (60). This excessive absorption of dietary calcium increases serum calcium concentrations, which in turn suppresses PTH and increases renal filtered load. When the kidney excretes the excess filtered calcium to maintain homeostasis, hypercalciuria results (61). In renal hypercalciuria, the primary defect is believed to be
impairment of renal tubular reabsorption. Excessive loss of calcium at the kidney lowers serum calcium. This stimulates PTH secretion to release skeletal calcium in an effort to restore normal balance. In addition, PTH activates renal 25(OH)D-1α-hydroxylase to increase 1,25(OH)₂D synthesis which enhances calcium absorption. (24). These two groups have been distinguished by an oral calcium test. Absorptive hypercalciurics show an increase in urinary calcium after calcium loading while resorptive hypercalciurics show high PTH which normalizes after calcium loading (62).

Supporting evidence exists for each of the possible causes of hypercalciuria. However, renal calcium leak is uncommon and occurs mostly in medulary sponge kidney disease (63). Excessive intestinal absorption of calcium should suppress both PTH and 1,25(OH)₂D (64) yet high levels of 1,25(OH)₂D are frequently seen (65-66). Broadus et al (67) have observed elevated serum 1,25(OH)₂D in IH that was suppressible with a dose of calcium. However, the suppressed 1,25(OH)₂D rebounded to its initial high levels after two weeks. This observation suggests disordered regulation of 1,25(OH)₂D is a likely cause of IH.


Experiment #1

Rationale:

Although 1,25(OH)₂D is the most potent vitamin D metabolite, it is not the only active metabolite. There is evidence that, 25(OH)D also exerts biologic effects (68-69). The potency of a vitamin D preparation is based on its ability to increase 25(OH)D, the accepted measure of vitamin D status.

It has long been assumed that equimolar amounts of vitamin D₂ and vitamin D₃ have equal potency. The evidence for this assumption about vitamin D nutrition is based upon crude bioassays such as the "rat line test" (70) and upon the comparisons of a variety of infant antirachitic studies done in the 1930’s (71). Today, the “unit” of a vitamin D preparation is based on conversion of gram quantities, where 1 International Unit (IU) equals 25 ng of either form of the vitamin (72). Yet, the effect of one IU of vitamin D₂ does not equal one IU of vitamin D₃ since there is a fixed molecular weight difference (396 daltons for D₂ and 384 daltons for D₃).

In addition, all non-human species tested show differences in response to vitamin D₂ and D₃. In birds, vitamin D₂ is less than one tenth as effective as vitamin D₃ at increasing 25(OH)D (73), and in monkeys, vitamin D₃ is far more effective than vitamin D₂ (74). In rats, surprisingly, vitamin D₂ is more effective than vitamin D₃ (75). Human studies comparing the efficacy of

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* In the "rat line test" bioassay, test rats are placed on a high calcium and low phosphorus diet for three to four weeks to induce rickets. Substances containing unknown quantities of vitamin D are fed to test rats for ten days. After the test period, the rats were sacrificed and sections of the forearm bone was stained with silver phosphate to visualize newly deposited calcium in the metaphysis. The recently laid down bone forms a line after staining and the line’s thickness is proportional to the dose of vitamin D. The unknown substance containing vitamin D can be compared to known standards to determine vitamin D potency.
vitamin D₂ and D₃ have yielded conflicting results. Two studies found no difference in the ability of vitamin D₂ and vitamin D₃ to raise 25(OH)D (76-77), while one study found that vitamin D₃ was more effective than vitamin D₂ (78). In all the human studies, the sample size (<10 subjects per group) did not provide enough statistical power for conclusive results. Furthermore, these studies did not consider the confounding effects of solar exposure on background levels of vitamin D. To help resolve the issue of equivalence, I compared the ability of equimolar quantities of vitamin D₂ and vitamin D₃ to elevate serum 25(OH)D in 2 large groups of healthy volunteers in Toronto. The study was done between February and early May when vitamin D levels and sun exposure are low.
**Hypothesis:**

Since all animal species showed differences in their response between vitamin D\(_2\) and D\(_3\), there should also be a difference between the efficacy of vitamin D\(_2\) and D\(_3\) in humans in raising serum 25(OH)D.

**Materials and Methods**

Each vitamin was purchased in crystalline form from Sigma (St Louis, MO) and dissolved in U. S. Pharmacopoeia grade ethanol. Appropriately blanked UV absorption spectra before and after the study remained identical. Molar concentration of vitamin D\(_2\) and D\(_3\) was adjusted to 260 nmol per 0.6 mL of ethanol, based on absorbance at 265 nm (7.90 AU, and using the extinction coefficient 18,300 AU mol\(^{-1}\) L\(^{-1}\) (79) on a Hewlett Packard 8452A diode array spectrophotometer. In addition, chromatographic analysis (80) consistently indicated only the one peak appropriate for each vitamin D preparation.

**Study Population**

The protocol was carried out between February and early May, when serum 25(OH)D is at its annual low concentration in Toronto. There were 72 volunteer subjects taking vitamin D. Mean age was 38 ± 9 (SD) years. Of these, 34 were randomly assigned in a double-blind manner, to take either vitamin D\(_3\) or vitamin D\(_2\). The rest of the subjects were given vitamin D\(_3\) only. The subjects took 260 nmol (~100 μg, 4000 IU) of vitamin D per day for 14 consecutive days. The vitamin D\(_2\) treated group consisted of 5 males and 12 females, while the vitamin D\(_3\) group consisted of 19 males and 36 females. A third group consisted of 17 untreated subjects...
who did not wish to take the vitamin D supplement, but who agreed to have blood drawn at the appropriate times. None of the subjects had been or were taking vitamin D supplements in excess of the recommended nutritional intake. Individuals who had recently taken or were intending to take a winter vacation in southern latitudes were excluded from the study. This protocol was approved by a University of Toronto Ethics Committee, and each subject signed a consent form.

**25(OH)D and 1,25(OH)\(_2\)D Assays**

25(OH)D and 1,25(OH)\(_2\)D concentrations were determined with the Incstar RIA kit (Stillwater, MN) and by thymus receptor assay respectively. Serum samples from each patient (before and after dosing) were analyzed together in the same run to eliminate inter-assay variations. Intra-assay coefficient of variation (CV) for 25(OH)D and 1,25(OH)\(_2\)D were 13% and 15% respectively, and inter-assay CV for 25(OH)D and 1,25(OH)\(_2\)D 22% and 26%.

**25(OH)D – RIA Assay**

The lipid fraction in 50 µL of serum was extracted with 500 µL of acetonitrile. After thorough mixing, the lipid fraction was separated by centrifugation at 2000 g for 30 minutes at 4 °C. Then 25 µL of lipid fraction was incubated with 50 µL of \(^{125}\)I labelled 25(OH)D, 1 ml of non-specific binding buffer and 1 ml of 25(OH)D antiserum for 90 minutes at room temperature.

The 25(OH)D-antibody complex was precipitated with 500 µL of donkey-anti-goat antibody for 20 minutes at room temperature. The 25(OH)D-antibody complex was separated by centrifugation at 2000 g for 30 minutes at room temperature. The supernatant was decanted and the \(^{125}\)I radioactivity in the pellet was measured in a gamma counter. Unknown values were determined from standard curves constructed by plotting \(^{125}\)I radioactivity against known concentration of 25(OH)D.
1,25(OH)$_2$D - Thymus Receptor assay

1,25(OH)$_2$D was measured following the procedure of Hollis (81). In summary, one ml of acetonitrile was used to extract the lipid fraction from 1 ml of serum. The lipid fraction was separated by centrifugation at 2000 g at 4 °C for 30 minutes. The pH of the lipid containing supernatant was adjusted to 10.6 with 1.0 ml of 0.4 M K$_2$HPO$_4$.

1,25(OH)$_2$D in the lipid extracted fraction was purified by chromatography on C18-OH Sepak cartridges (Varian Associate, Harbor City, CA). Cartridges were preconditioned with 5 mL acetone, followed by 3 mL methanol and 2 mL water. Samples were applied and eluted by sequentially applying 2 mL water, 5mL (70:30) methanol:water, 5 mL (90:10) hexane:methylene chloride, 5 mL (99:1) hexane:isopropanol, and 5 mL (97:3) hexane:isopropanol. The hexane:isopropanol (97:3) eluant containing 1,25(OH)$_2$D was evaporated under nitrogen and low heat. The partially purified 1,25(OH)$_2$D was reconstituted in 125 μL absolute ethanol and stored at -20°C until the assay.

For the assay, 30 μL of 1,25(OH)$_2$D reconstituted in ethanol was incubated with 500 μL of HEKM [HEPES-12 g/L, EDTA-0.56 g/L, KCl-37.2 g/L, Na Molybdate-2.06 g/L (Sigma, St. Louis)] buffer containing 0.77 g/L of dithiothreitol buffer and thymus receptor for 1 hour at room temperature. 20 μL of $^3$H-labelled 1,25(OH)$_2$D binding tracer (Amersham, UK) was added and allowed to equilibrate for 1 hour at room temperature. 400 μL of dextran charcoal (BHD Inc, Toronto) solution was added and allowed to incubated at room temperature for 1 hour to remove excess tracer. Charcoal was removed by centrifugation at 2000 g for 30 minutes at 4°C. Supernatant was transferred to scintillation vials and 10 mL of scintillation fluid (Beckman, Fullerton, CA) was added before counting in scintillation counter. Unknown values were determined from standard curve by $^3$H radioactivity against known concentrations.
Results

Despite the random allocation of subjects, the ratio of males to females in the two vitamin D treatment groups was essentially the same - for vitamin D2, 5/12 (42%); for vitamin D3, 18/36 (50%). There were no significant differences between males and females, in terms of the basal serum 25(OH)D, or in the changes observed with vitamin D dosing. Both vitamin D2 and D3 supplementation significantly increased serum 25(OH)D (p<0.02) (Table 1.1). Vitamin D2 supplement increased 25(OH)D by an average of 13.7 nmol/L, while the vitamin D3 supplement increased it by 23.3 nmol/L above baseline values. The mean difference between the increases was 9.6 nmol/L, with a 95% confidence interval of 1.4 nmol/L to 17.8 nmol/L. Mean concentrations of 1,25(OH)2D were not affected by either supplement (data not shown). There was no change in 25(OH)D during the study period in the 17 untreated subjects.

The plot of basal 25(OH)D concentration against the increase in 25(OH)D for the vitamin D3 treated group shows a significant inverse linear correlation (r= -0.472, p<0.001, Figure 1.1). A similar inverse relation also existed in the vitamin D2 group (r= -0.681, p=0.003, Figure 1.2). For the vitamin D3 supplemented group, the regression equation between the change in 25(OH)D (change) and baseline was: change = [(-0.418) X (baseline)] + 40.6, and for the vitamin D2 treated group: change = [(-0.440) X (baseline)] + 33.0.

Data from the vitamin D3 treated group was divided into tertiles, based on the subjects' baseline 25(OH)D concentration, to test for the effect of prior vitamin D nutrition on the response to vitamin D supplementation. The first tertile exhibited the largest increase in 25(OH)D while the third tertile showed less than half of that increase (Table 1.2). One-way ANOVA and Tukey's honest significant difference indicated that the 25(OH)D increase in the third tertile was smaller than the increase seen in each of the first or second tertile.

Since the increase in 25(OH)D after dosing was affected by baseline concentration, the baseline concentration was used as a covariate in ANCOVA to adjust for the slight difference
between the two vitamin D treated groups. After accounting for the slight differences in baseline concentrations between vitamin D$_3$ and D$_2$ supplemented groups, the increase in 25(OH)D with vitamin D$_3$ supplementation remained significantly higher than vitamin D$_2$ (p=0.03).
Table 1.1

25(OH)D concentrations before and after vitamin D supplementation.

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D$_2^{a}$</th>
<th>Vitamin D$_3^{a}$</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>43.7 (17.7)</td>
<td>41.3 (17.7)</td>
<td>39.8 (18.7)</td>
</tr>
<tr>
<td>Final</td>
<td>57.4 (13.0)$^{a}$</td>
<td>64.6 (17.2)$^{b}$</td>
<td>42.8 (20.7)</td>
</tr>
<tr>
<td>Change</td>
<td>13.7 (11.4)</td>
<td>23.3 (15.7)$^{c}$</td>
<td>3.0 (8.1)</td>
</tr>
</tbody>
</table>

$^{a}$ The vitamin D$_2$ and the untreated group each had 17 subjects while the vitamin D$_3$ group had 55 subjects.

$^{b}$ Paired t-test indicated that both vitamin D$_2$ and D$_3$ supplementation increased serum 25(OH)D (p<0.02), and that it was unchanged without supplementation in the control group (p>0.05).

$^{c}$ Vitamin D$_3$ supplement increase 25(OH)D significantly more than vitamin D$_2$ supplement by unpaired t-test (p=0.03). Baseline 25(OH)D concentration for all groups were not significantly different by one-way ANOVA.
Table 1.2

25(OH)D increase in Vitamin D$_3$ treated group stratified by baseline 25(OH)D concentration (mean ± SD)$^a$

<table>
<thead>
<tr>
<th>25(OH)D range (nmol/L)</th>
<th>Average baseline 25(OH)D (nmol/L)</th>
<th>Average increase (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Tertile</td>
<td>(10-34)</td>
<td>22.3 (7.9)</td>
</tr>
<tr>
<td>2nd Tertile</td>
<td>(35-49)</td>
<td>41.1 (4.1)</td>
</tr>
<tr>
<td>3rd Tertile</td>
<td>(50-86)</td>
<td>61.5 (8.5)</td>
</tr>
</tbody>
</table>

$^a$ Based on the 25(OH)D baseline values, subjects were divided into tertiles to test for the effects of prior vitamin D status on the increase in 25(OH)D. The first tertile had 19 subjects while the second and third each had 18 subjects.

$^b$ One-way ANOVA indicated a difference in the increase of 25(OH)D based on different vitamin D status prior to vitamin D intake (p=0.002). The third tertile had a significantly lower increase in 25(OH)D than either the 1st (p=0.001) or 2nd tertile (p=0.03), as determined by Tukey's honest significant difference test.
Figure 1.1.
A plot of baseline 25(OH)D against the increase in 25(OH)D after vitamin D₃ supplementation in normal volunteers. The data showed a significant inverse relationship (r=-0.472, p<0.001). This indicated that the increase in 25(OH)D after vitamin D₃ supplementation is associated with prior vitamin D status. Dotted lines indicate 95% C.I. of the regression.
Figure 1.2.
Baseline 25(OH)D versus the change in 25(OH)D after vitamin D₂ supplementation. The results show an inverse relationship similar to the vitamin D₃ supplemented group ($r = -0.681$, $p=0.003$).
Discussion

Reports showing that vitamin D\textsubscript{2} and D\textsubscript{3} are equi-potent (76-77), or that vitamin D\textsubscript{3} is more effective may have been influenced by a number of factors (78), especially the relative stability of the vitamin D preparations used.

First, vitamin D preparations can be highly variable. Before the present study, I tested the vitamin D preparations made for us by the pharmacy departments of two local hospitals. At both institutions it was conventional to prepare the vitamin D in "simple syrup", an aqueous sugar solution. Analysis of UV absorption by vitamin D showed significant decomposition. The singular peak UV absorbance at 265 nm decreased significantly after a few days. Furthermore, the peak and valley at 265 nm and 220 nm in the UV absorption spectrum disappeared. Consistent with the observation of vitamin D breakdown, Whyte and Haddad determined the potency of intra-muscular vitamin D\textsubscript{2} and D\textsubscript{3} preparations, by bioassay and by biochemical methods. They found that vitamin D content differed significantly from the manufacturer's labelled claim, in some cases by as much 50% (77). Vitamin D\textsubscript{2} and D\textsubscript{3} have long been known to degrade differently (79), particularly when exposed to varying temperatures, humidity, or even storage in certain containers (82). Moreover, different "inert" constituents in vitamin D formulations can substantially affect vitamin D stability (83). There is no indication in earlier studies comparing vitamin D\textsubscript{2} and D\textsubscript{3} in humans that vitamin D stability was checked. For this study, I prepared vitamin D doses in U.S.P. grade ethanol in collaboration with the hospital pharmacy. I validated vitamin D preparations before by UV spectrophotometry and found that the spectra were unchanged.

Second, endogenous production of vitamin D may have confounded earlier studies because of variable UV light exposure in the subjects. In one study the time of dosing was not specified (76). Two studies specify early summer, or "from April to November" (77-78) when solar exposure would have increased endogenous production of vitamin D. My study was
conducted between February and early May when basal concentrations of 25(OH)D are at their lowest. The untreated subjects exhibited no change in serum 25(OH)D, indicating that endogenous production of vitamin D did not influence outcome. Finally, previous studies were hampered by insufficient statistical power (76-78).

Endogenous control of 1,25(OH)_{2}D was inferred by the fact that neither vitamin D_{2} nor D_{3} supplementation affected serum 1,25(OH)_{2}D levels. As expected, supplementation with vitamin D_{2} and vitamin D_{3} both elevated serum 25(OH)D. However, with vitamin D_{3}, the 25(OH)D increase was 70% greater than the increase obtained with vitamin D_{2}. In rats, sex hormones may influence vitamin D-25-hydroxylase (84). The same effect may be present in humans but is unlikely to affect these results because the male-to-female ratio in the two treated groups were similar.

I also found that the increase in serum 25(OH)D after vitamin D supplementation was dependent on prior vitamin D nutrition. Above and beyond 50 nmol/L of basal 25(OH)D, the effect of vitamin D_{2} and D_{3} at increasing serum 25(OH)D diminished progressively. This inverse relationship between basal 25(OH)D and the rise in 25(OH)D with vitamin D supplement was detected because the dosing was carried out at the annual nadir for 25(OH)D. In addition, these Toronto subjects exhibited lower 25(OH)D levels (mean of 40 nmol/L) (85) than the mean of 75 nmol/L reported for US cities (86). Comparison between my results and the U.S. results is valid because both groups now use the same method to measure 25(OH)D, and both participate in the EQAS proficiency survey, sharing samples (85-86). At the higher basal 25(OH)D concentrations in US cities, the inverse relation shown in Figure 1.1 and Table 1.2 could go undetected unless normal subjects were pre-selected for lower 25(OH)D concentrations than the regional norms. With basal 25(OH)D levels higher than 50 nmol/L, the phenomenon may reach a plateau which is not quite evident from the data presented here. Additional studies would be required to exclude the unlikely circumstance that extra vitamin D would ever cause a decrease in 25(OH)D.
An increase in serum 25(OH)D relating inversely to basal 25(OH)D levels has been observed before. In individuals exposed to ultraviolet light treatment sessions, both Mawer et al (87) and Snell et al (88) found similar results. MacLennan and Hamilton (89) also described a similar response to vitamin D treatments, where 25(OH)D increased more in those with lower initial 25-(OH)D concentrations. All of these studies attributed the phenomenon to product inhibition of liver vitamin D-25-hydroxylase. In rats, vitamin D supplementation had been shown to have a marked effect on lowering 25-hydroxylase activity, both in vitro and in vivo (90). My results show that product inhibition applies to 25-hydroxylation of both vitamin D₂ and vitamin D₃ in humans equally.

Several mechanisms could contribute to the greater capacity of vitamin D₃ to increase 25(OH)D. Although intestinal absorption of vitamin D was not determined, studies of tritium-labelled vitamin D₂ and vitamin D₃ in healthy subjects show similar fecal recovery after oral dosing (77). This suggests that differential intestinal absorption is not the reason for the different effects of vitamin D₂ and D₃ on serum 25(OH)D. The relative affinity for vitamin D-binding protein (DBP) and substrate affinity for vitamin D₃ by 25-hydroxylase is a second possibility to be considered. Nilsson and associates (91) measured vitamin D affinity for purified human DBP and reported higher association constants for vitamin D₃ (2.8X10⁻⁸ M⁻¹) than vitamin D₂ (1.3X10⁻⁸ M⁻¹), respectively. After measuring vitamin D and its metabolites, Hollis and associates (81) analyzed human milk versus plasma concentration by regression analysis. They found higher quantities of vitamin D₂ and its major metabolite 25(OH)D₂ in milk than vitamin D₃ and its metabolite 25(OH)D₃. This suggested that vitamin D₂ and 25(OH)D₂ had lower affinity for DBP, and thus existed in relatively greater free amounts available for transport into milk. In rats, 25-hydroxylase is known to exist in both microsomal and mitochondrial fractions. However in human, the mitochondrial fraction of 25-hydroxylase converts vitamin D₃ to 25(OH)D₃ five times faster than vitamin D₂ to 25(OH)D₂ (19). Similarly, when human liver mitochondrial P-450 vitamin D hydroxylase cDNA is transfected into COS-1 kidney cells, these cells metabolized vitamin D₃ but showed no vitamin D₂ hydroxylating ability (92). It is therefore
possible that vitamin D$_3$ is a better substrate for mitochondrial 25-hydroxylase, while vitamin D$_2$ is preferred by the microsomal fraction. Thus, the higher affinity to DBP would reduce the clearance rate of vitamin D$_3$ compared to vitamin D$_2$ and the more efficient 25-hydroxylation by mitochondrial fraction should increase the production rate of 25(OH)D$_3$ compared to 25(OH)D$_2$. The observed differences in vitamin D$_3$ and vitamin D$_2$ effects in my experiments could therefore be explained by the differences in the in-vitro binding to DBP and the enzymology of vitamin D 25-hydroxylase. The results are also consistent with findings in other primate species (75).

Perhaps it should not be surprising that vitamin D$_2$ is less effective per mole than vitamin D$_3$. Although vitamin D$_2$ can be manufactured through the ultraviolet radiation of lipid extracted from yeast (93), it is not a natural product of human biology (94). Its existence in our food supply is due to artificial supplementation with food additives that are used because of ease of synthesis and industrial convenience.


**Experiment #2**

**Rationale:**

The cause of the altered vitamin D metabolism in calcareous kidney stone formers remain unclear. However, levels of vitamin D metabolites provide insights into possible etiology. The pattern of vitamin D metabolites in IH is similar to what may be seen in nutritional rickets and some osteomalacias, low 25(OH)D, yet elevated 1,25(OH)2D (95-96). Under conditions of low 25(OH)D, extra renal tissue cannot stimulate increased synthesis of 1,25(OH)2D (97). Local production of 1,25(OH)2D is also required for regulating other metabolic processes, including promoting cell differentiation, inhibiting cell proliferation, and other non-genomic effects, as well as, its classic effects on calcium metabolism (28-30,98).

When circulating 25(OH)D is insufficient for peripheral production of 1,25(OH)2D, the kidney may compensate by increasing synthesis of 1,25(OH)2D to increase availability at peripheral sites. The kidney can synthesize 1,25(OH)2D at low levels of 25(OH)D because it is one of the sites where DBP is removed (99). The release of 25(OH)D intracellularly increases its availability for further metabolism by the kidney.

When the concentration of 25(OH)D is high, there is potentially more substrate for 1,25(OH)2D synthesis. However, observations in rats suggest that vitamin D supplementation suppresses rather than elevates 1,25(OH)2D levels. Moderate supplementation in rats with vitamin D3 increased 1,25(OH)2D transiently for one day. However, at the end of one week the 1,25(OH)2D levels dropped below that observed before dosing (100).

Thus, excessive production of 1,25(OH)2D when serum 25(OH)D is low may compensate for diminished peripheral production of 1,25(OH)2D. However, this response may result in the disregulation of 1,25(OH)2D seen in IH. If renal synthesis is over-compensating for low peripheral levels of 25(OH)D, then an increase in 25(OH)D may down-regulate renal 1,25(OH)2D production in IH stone formers, in a manner similar to that seen in rats.
**Hypothesis:**

IH stone formers have an exaggerated inverse relationship between 1,25(OH)$_2$D and 25(OH)D. Moreover, increasing serum 25(OH)D will lower 1,25(OH)$_2$D in IH subjects to a greater extent than normal subjects.

**Methods and Materials**

Kidney stone formers with idiopathic hypercalciuria attending the regional lithotripsy clinic at the Wellesley Central Hospital and healthy volunteers participated in the study after giving informed consent. Both subjects and healthy volunteers took 260 nmol (~100 µg, 4000 IU) of vitamin D$_3$ per day for 14 consecutive days. None of the subjects had been or were taking vitamin D supplements in excess of the recommended nutritional intake. The protocol was carried out between February and May, when serum 25(OH)D is at its annual low concentration in Toronto. The study protocol was approved by a University of Toronto Ethics Committee.

The previous experiment established that vitamin D$_3$ was more potent than vitamin D$_2$ in raising 25(OH)D concentrations. Vitamin D$_3$ was prepared in the same manner as the first experiment. 25(OH)D concentrations were determined by the Incstar RIA kit (Stillwater, MN) and 1,25(OH)$_2$D concentrations were determined by calf thymus receptor assay as in experiment #1. Serum samples for each patient (before and after dosing) were analyzed together in the same run. Serum and urine metabolites where measured in the clinical laboratory at Mount Sinai Hospital, Toronto using standard analytic methods for these analytes (101). Assays for vitamin D metabolites were described in experiment #1.

Intact PTH was assayed with the immunoradiometric assay (IRMA) kit from Diagnostic Systems Laboratories (Webster, Texas). Briefly, 200 µL of standards, controls and samples were
added to 12x75 mm plastic test tubes. The inner walls of the test tubes were coated with immobilized anti-PTH antibodies directed against amino acids 39-84 of the PTH peptide. 100 µL of ¹²⁵I-labelled anti-PTH antibodies which recognize amino acids 1-34 of PTH was immediately added to all tubes and mixed. The tubes were incubated at room temperature for 24 hours. Tubes were decanted and washed twice with 2 ml of diluted (1:10) wash buffer. The PTH sandwiched between the immobilized and ¹²⁵I labelled antibodies were counted for 1 minute in a gamma counter. Unknown values were derived from standard curves constructed by plotting gamma emission against known PTH concentrations.
**Results**

As shown in table 2.1, controls had basal vitamin D nutritional status that was not significantly different from stone formers. After taking the vitamin D supplement, both groups exhibited similar increases in serum 25(OH)D. A plot of the basal 25(OH)D concentration against the increase in 25(OH)D for the controls showed a significant inverse linear correlation (r=-0.472, p<0.001, with 95% C.I. of the regression, figure 2.1).

Vitamin D supplementation did not affect 1,25(OH)2D concentrations in control subjects. However, they did show a significant inverse relationship between the baseline 25(OH)D level and the change in 1,25(OH)2D (r = -0.345, p=0.02, figure 2.2). Controls showed an inverse relationship between basal PTH and basal 25(OH)D (r=-0.422, p=0.01, figure 2.3). Vitamin D supplements suppressed PTH in controls but not in stone formers (table 2.1). Furthermore, normal subjects with baseline 25(OH)D less than 50 nmol/L generally had greater decrease in PTH after vitamin D dosing, although the result does not reach significance (r=0.382, p=0.06 one tail).

In stone formers, the relationship between basal 25(OH)D and the subsequent increase in 25(OH)D after dosing showed the same trend as the normal population (figure 2.4). The slope of the regression line between baseline 25(OH)D and the subsequent 25(OH)D increase was less than zero (p=0.03, one tail test vs zero slope). Prior to starting the vitamin supplement, the stone formers had significantly higher 1,25(OH)2D concentration (97.6 ±35.0 pmol/L) than the control subjects (74.6 ± 32.2 pmol/L, p=0.01). High 1,25(OH)2D may have been driven by PTH levels that were significantly higher than controls (3.84 ±2.1 vs 2.62 ±1.5 pmol/L, p=0.02 by Mann-Whitney U test). Stone formers exhibited an inverse relation between the baseline 1,25(OH)2D and 25(OH)D levels (stones r=-0.662, p=0.001, figure 2.5) after removal of one data point that was outside the 95% CI for the distribution of points around the least squares regression line. If the points were included, that relationship did not quite reach significance. In addition, 1,25(OH)2D, increased significantly from baseline after supplementation regardless of basal
25(OH)D (figure 2.6). Extra vitamin D intake did not affect urinary calcium or PTH in the stone formers or any other measured parameters (table 2.2).
Table 2.1. Effect of Vitamin D Supplementation in Controls and Stone Formers

<table>
<thead>
<tr>
<th></th>
<th>25(OH)D(^a) (nmol/L)</th>
<th>1,25(OH)(_2)D (pmol/L)</th>
<th>PTH (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Stones</td>
<td>Controls</td>
</tr>
<tr>
<td>Baseline</td>
<td>41.3 (17.7)(^b)</td>
<td>38.6 (16.7)</td>
<td>74.6 (32.2)</td>
</tr>
<tr>
<td>Final</td>
<td>64.6 (17.2)(^b)</td>
<td>62.5 (18.4)(^b)</td>
<td>79.9 (36.2)</td>
</tr>
<tr>
<td>Change</td>
<td>23.3 (15.7)</td>
<td>23.9 (16.2)</td>
<td>5.3 (24.7)</td>
</tr>
<tr>
<td>N</td>
<td>55</td>
<td>21</td>
<td>44</td>
</tr>
</tbody>
</table>

\(^b\) statistical significant increase from baseline values (p<0.001 for 25(OH)D and p=0.01 for 1,25(OH)\(_2\)D);
\(^c\) statistically different baseline 1,25(OH)\(_2\)D values in stone formers compared to normal subjects (p=0.01);
\(^d\) greater increase in 1,25(OH)\(_2\)D in stone formers compared to normal subjects (p=0.04)
\(^e\) significant decrease in serum PTH in normal subjects after vitamin D intake (p=0.02)
\(^f\) Stone formers have higher PTH than normal subjects (p=0.02 by Mann-Whitney U test)
<table>
<thead>
<tr>
<th>Serum Analytes</th>
<th>Before</th>
<th>After</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ionized Ca</td>
<td>1.24 (0.09)</td>
<td>1.25 (0.05)</td>
<td>20</td>
</tr>
<tr>
<td>Ca</td>
<td>2.42 (0.13)</td>
<td>2.41 (0.10)</td>
<td>21</td>
</tr>
<tr>
<td>Creatinine</td>
<td>85.8 (17.9)</td>
<td>84.8 (15.4)</td>
<td>20</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.02 (0.13)</td>
<td>1.11 (0.25)</td>
<td>21</td>
</tr>
<tr>
<td>Urea</td>
<td>5.40 (1.14)</td>
<td>5.50 (1.08)</td>
<td>21</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>340.9 (94.1)</td>
<td>337.9 (95.8)</td>
<td>21</td>
</tr>
<tr>
<td>K</td>
<td>4.31 (0.43)</td>
<td>4.12 (0.26)</td>
<td>21</td>
</tr>
<tr>
<td>Na</td>
<td>138.9 (3.1)</td>
<td>138.5 (2.5)</td>
<td>21</td>
</tr>
<tr>
<td>Mg</td>
<td>0.82 (0.08)</td>
<td>0.81 (0.11)</td>
<td>20</td>
</tr>
<tr>
<td>PTH</td>
<td>3.84 (2.1)</td>
<td>3.45 (1.7)</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Urine Analytes</th>
<th>Before</th>
<th>After</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Volume</td>
<td>2218 (795)</td>
<td>2252 (794)</td>
<td>21</td>
</tr>
<tr>
<td>Ca</td>
<td>5.78 (3.35)</td>
<td>5.83 (2.57)</td>
<td>20</td>
</tr>
<tr>
<td>Creatinine</td>
<td>14.6 (5.3)</td>
<td>14.8 (6.3)</td>
<td>20</td>
</tr>
<tr>
<td>Phosphate</td>
<td>30.5 (18.0)</td>
<td>27.0 (12.9)</td>
<td>21</td>
</tr>
<tr>
<td>Urea</td>
<td>437.1 (180.9)</td>
<td>418.3 (170.0)</td>
<td>19</td>
</tr>
<tr>
<td>Uric Acid</td>
<td>3.95 (1.55)</td>
<td>3.78 (1.47)</td>
<td>20</td>
</tr>
<tr>
<td>K</td>
<td>97.0 (46.0)</td>
<td>88.5 (29.4)</td>
<td>21</td>
</tr>
<tr>
<td>Na</td>
<td>229.9 (106.5)</td>
<td>212.6 (85.1)</td>
<td>21</td>
</tr>
<tr>
<td>Mg</td>
<td>4.51 (1.92)</td>
<td>4.02 (1.92)</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.2 shows serum and urine biochemistry results in stone formers before and after taking daily doses of 4000 IU of vitamin D₃ for two weeks. Values indicate the mean (± SD). All serum values are in mmol/L except for creatinine and uric acid (μmol/L), and PTH (pmol/L). All urine values are in mmol/day except volume which is in mL. There were no significant differences in any biochemical parameters (before vs after by paired t-test).
Figure 2.1.
A plot of baseline 25(OH)D against the increase in 25(OH)D after vitamin D₃ supplementation in normal volunteers. The data showed a significant inverse relationship (r=-0.472, p<0.001). This indicated that the increase in 25(OH)D after vitamin D₃ supplementation is associated with prior vitamin D status. Dotted lines indicate 95% C.I. of the regression.
After vitamin D dosing, normal subjects showed a significant inverse relationship where those with low 25(OH)D had higher increases in 1,25(OH)₂D ($r = -0.345 \ p=0.02$).
Normal subjects with lower 25(OH)D tended to have higher PTH ($r = -0.422$, $p=0.01$).
Figure 2.4
Plot of initial 25(OH)D levels and the increase in 25(OH)D after dosing in stone formers. Similar to the normal subjects, an inverse trend is also evident ($r=-0.376$) because the slope of the regression line is less than zero ($p=0.05$, one tail test against zero slope).
Figure 2.5
Plot of initial 25(OH)D levels against 1,25(OH)$_2$D exhibit a significant an inverse relation between the baseline 1,25(OH)$_2$D and 25(OH)D levels in stone formers ($r=-0.662$, $p=0.001$). Open circle indicates outlier (outside 95% CI of samples) which were omitted from analysis.
Graph of Baseline 25(OH)D versus the 1,25(OH)$_2$D increase after vitamin D supplementation in stone formers. In contrast to normal subjects, the 1,25(OH)$_2$D increase was not dependent on baseline 25(OH)D. Arrow indicates patient with marked increase in 1,25(OH)$_2$D.
Figure 2.7
Vitamin D supplementation had the greatest effect in subjects who were relatively vitamin D deficient. After dosing, subjects with baseline 25(OH)D less than 50 nmol/L had greater decrease in PTH ($r=0.382$, $p=.06$, one tail regression of subjects with baseline 25(OH)D less than 50 nmol/L).
Discussion

Both in normal subjects and stone formers, daily supplementation of vitamin D at 4000 IU for two weeks increased mean serum 25(OH)D by about 50%, from approximately 40 nmol/L to 65 nmol/L. Similar to the effect observed for the vitamin D$_3$ treated group in experiment #1, the initial vitamin D status predicted the change in 25(OH)D levels after supplementation. In stone formers as in controls, those with the lowest vitamin D status had the highest increase in 25(OH)D after dosing. These observation are consistent with the results of Mawer et al. They used ultraviolet radiation and reported that the 25(OH)D increase from dermal synthesis was inversely related to basal vitamin D status (87). This inverse correlation suggests that during periods of moderate vitamin D deficiency, vitamin D stores are preserved either through slower production and/or slower clearance. Normal subjects also exhibited an inverse relationship between basal 25(OH)D and PTH; individuals with higher 25(OH)D tended to have lower PTH. In addition, there was a significant decrease in PTH concentration in the normals after a relatively short vitamin D dosing protocol of only two weeks. Several other studies have reported this effect of PTH suppression after vitamin D administration (102-104). Takeuchi et al found that daily intake of 800 IU of vitamin D per day can suppress secondary hyperparathyroidism in a healthy population of young and elderly Japanese males and females (104). Recently, Thomas et al (105) showed a similar elevation in PTH at 25(OH)D levels below 40 nmol/L in unselected patients. Dawson-Hughes et al demonstrated the same relationship in elderly (106). Kinyamu et al found that PTH correlated significantly with calcium intake from milk, but not with other sources of dietary calcium. Further, calcium was probably not a factor in suppressing PTH because calcium absorption was similar between groups taking and not taking vitamin D supplements (107). This suggested that vitamin D added to milk or its metabolites may be able to suppress PTH directly. Long term vitamin D sufficiency may control mild hyperparathyroidism, thus potential preventing bone loss (108).
Extra vitamin D intake did not affect the mean 1,25(OH)₂D concentration in controls. The active vitamin D hormone is tightly regulated in normal subjects. In those with higher initial vitamin D status, 1,25(OH)₂D production is decreased after vitamin D dosing. At 50 nmol/L of 25(OH)D or higher, 1,25(OH)₂D remained unchanged after vitamin D challenge (mean decrease of 5.4 ± 20.7 pmol/L). However, in those subjects with a serum 25(OH)D below 50 nmol/L the 1,25(OH)₂D level increased after vitamin D supplementation (mean increase 10.8 ± 25.1 pmol/L). Taken together, these physiological changes suggest that subjects with 25(OH)D below 50 nmol/L are vitamin D “deficient”; when more vitamin D became available, 1,25(OH)₂D levels increased and PTH declined.

The results confirm earlier studies showing that vitamin D metabolism in stone formers is different than the normal population. In this study, stone formers had significantly higher 1,25(OH)₂D levels than controls at similar concentrations of serum 25(OH)D (109) (67). The baseline vitamin D status suggest that IH stone formers may have an exaggerated relationship between 25(OH)D and 1,25(OH)₂D because those with low 25(OH)D had higher 1,25(OH)₂D levels (Figure 2.5). This relationship was not found in normal subjects. However, vitamin D supplementation revealed differences between 1,25(OH)₂D levels observed before and after vitamin D dosing. Before treatment, the serum 1,25(OH)₂D level was highest in those with lower serum 25(OH)D levels. When extra vitamin D was given, serum 1,25(OH)₂D increased significantly for the group. If IH stone formers do in fact have disordered 1,25(OH)₂D regulation that manifests as an exaggerated inverse relationship between 25(OH)D and 1,25(OH)₂D, then stones formers should show a greater decrease in 1,25(OH)₂D after vitamin D supplementation. In addition, stone formers should have a steeper regression relationship between 25(OH)D and 1,25(OH)₂D compared to normal subjects.

Most stone formers demonstrated increased 1,25(OH)₂D levels after vitamin D dosing regardless of basal 25(OH)D concentration. One patient exhibited a particularly striking response to vitamin D with a marked increase in 1,25(OH)₂D. His low vitamin D status (25(OH)D=17 nmol/L) may account for the remarkable increase in 1,25(OH)₂D because of
vitamin D deficiency. The reason for the increase is unclear because three other stone formers with similar 25(OH)D concentrations prior to vitamin D dosing showed more moderate increase in 1,25(OH)₂D. A few patients exhibited the hypothesized drop in 1,25(OH)₂D after vitamin D dosing. In most cases, however, increased 25(OH)D following supplementation resulted in a higher rather than lower 1,25(OH)₂D. The 1,25(OH)₂D increase during the the two week protocol may be a short term response to vitamin D dosing because the baseline data reflect the long-term equilibrium and show a relationship which is the opposite of the treatment response.

These results reveal another aspect of 1α-hydroxylase function, first elucidated when Broadus et al studied the response of hypercalciuric subjects to an oral calcium load (67). The high 1,25(OH)₂D levels in IH were suppressible with daily dosing of 1000 mg of calcium. However after two weeks, the 1,25(OH)₂D rebounded to its initial levels, suggesting “disorder vitamin D metabolism”.

Several investigators have questioned the classifications of IH stone formers into absorptive and renal hypercalciurics, suggesting that absorptive and renal hypercalciuria are not distinct derangements of calcium metabolism but rather a continuous spectrum of the same disorder (110-111). Under a low calcium diet, the distribution of PTH and urine calcium of IH stone formers should show two distinct subgroups but instead the distribution was continuous with no separation (112). Aladjem et al (111) found that in IH subjects who were initially classified as absorptive hypercalciurics switched to renal hypercalciurics when tested several years later. These results suggest that division of IH into absorptive and renal hypercalciuria may be artificial.

My results show that there is one cluster of patients showing an increased 1,25(OH)₂D response following vitamin D supplementation and a few patients exhibiting large increases or decreases. The small group size may be inadequate to differentiate two distinct groups or a clear spectrum. The small number of patients showing the hypothesized response suggests that disordered vitamin D regulation due to excessive compensatory synthesis of 1,25(OH)₂D may only constitute a small part the overall dysfunction characterizing IH.
In the stone formers, there was no change in PTH levels after vitamin D dosing even though they had higher basal PTH compared to normal subjects. Although the PTH level was in the upper range of normal, higher PTH levels in stone formers may account for differences in 1,25(OH)₂D between normal subjects and stone formers. The higher PTH may drive the production of 1,25(OH)₂D resulting in a higher basal level. Higher PTH in stone formers may be secondary to renal calcium loss, triggering compensatory increases in PTH. However renal hypercalciuria is rare and is unlikely to represent the majority of the stone formers. Most likely, the higher PTH levels are secondary to low calcium diet of stone formers taken in the belief that it can prevent further stone formation (66,112).

The observed results in the stone formers and normal subjects following 4000 IU of vitamin D are in agreement with those seen after exposure to ultraviolet radiation, another method of increasing vitamin D nutrition. Stamp et al (113) previously reported that one UV treatment increased 25(OH)D levels by the same amount as an oral dose of 10,000 IU of vitamin D. Varghese et al treated normal subjects and stone formers with UV light daily for two weeks (114). By increasing vitamin D supplementation by 10,000 IU/day, Varghese et al elevated 25(OH)D in their subjects three fold greater than what I achieved with 4000 IU (an increase of 70 nmol/L for UV treatment vs 23 nmol/L for oral dosing). Furthermore, normal subjects who were vitamin D replete decreased their 1,25(OH)₂D levels, while stone formers generally had increased 1,25(OH)₂D after irradiation. Qualitatively, cutaneously derived vitamin D results in a gradual and sustained increase in 25(OH)D after 7 days. Ingested supplements cause a rapid increase to peak serum levels within 12 hours, followed by a rapid decline due to hepatic metabolism (115). Since daily doses were given for 14 days, vitamin D levels would likely have plateaued at the end of the dosing period, comparable to the effects achieved with UV exposure.

Vitamin D nutrition might have benefited almost half the normal subjects because it raised 25(OH)D, suppressed PTH in vitamin D depleted subjects, and lowered serum 1,25(OH)₂D in the vitamin D replete group. Stone formers also benefit from extra vitamin D nutrition when 25(OH)D is increased. Even though 1,25(OH)₂D increased, the total intake of
56,000 IU of vitamin D over two weeks did not result in any detectable effects on either serum calcium or urinary calcium in the stone formers. Varghese found that after two weeks of UV irradiation over urinary calcium increased slightly but not significantly (114). Previous evidence and my data showed that moderate short-term vitamin D supplementation is unlikely to promote stone formation. In normal subjects, it took eight weeks of supplementation at 4000 IU per day of vitamin D3 to increase urinary calcium significantly, even though 1,25(OH)2D was unaffected (76). In a similar study, daily dosing with 4000 IU of vitamin D3 in normal subjects for 6 months increased urinary calcium. However, the same treatment with vitamin D2 did not significantly elevate urinary calcium. Since vitamin D2 is about half as effective as vitamin D3, a prolonged daily dose less than 2000 IU is unlikely to affect normal subjects or kidney stone formers (78).

The amount of vitamin D used in this study was probably too low to adversely influence calcium metabolism. No doubt, excessive vitamin D intake can produce hypercalciuria and increase the risk of stone disease as well as soft tissue calcification. However, moderate hypercalciuria alone does not necessarily cause stone formation. Other conditions must be present such as low urine volume, extreme urine pH, lack of urinary inhibitors of stone formation, and renal epithelial damage that allows retention of nucleated crystals to promote growth of macroscopic calcium stones (51). My data suggest that moderate supplementation is unlikely to elevate 1,25(OH)2D to a degree where increased calcium excretion can exacerbate stone formation. Thus, stone formers may actually benefit from increased serum 25(OH)D and decrease serum PTH after moderate vitamin D supplementation without increasing the risk of stone formation. Because the dosing protocol lasted only two weeks, the answer as to whether moderate long-term vitamin D supplementation is detrimental to stone formers would require longer-term studies.

Although the controls and stone formers in this study were on a free diet, stone formers tended to avoid calcium in the hope that lowered calcium intake will reduce stone formation (110,116). A calcium deficient diet can induce mild hyperparathyroidism that can lead to bone
loss. Stone formers on low calcium diet have been reported to show decreased bone mineral density. (117,118). Although IH stone formers did not show a significant reduction in PTH levels after vitamin D intake, long-term supplementation may suppress PTH to help slow bone loss. Contrary to the intuitive view, there is evidence that high calcium diets can actually reduce the chance of stone formation and improve calcium balance in bone tissue. Burtis et al found that a high calcium diet (1000 mg/day) decreases calcium excretion in calcium oxalate stone formers, and demonstrated a positive effect of calcium on reduction of stone formation (101). Conceivably, the extra calcium binds free oxalate in the intestine, thus decreasing oxalate absorption. The oxalate anion is known to have a greater effect on calcium oxalate supersaturation than the calcium cation and decreased oxalate absorption decreases the risk of stone formation (119).

In summary, feedback inhibition of 1,25(OH)2D in stone formers may be temporarily impaired production when 25(OH)D supplies are increased because I observed no decrease in 1,25(OH)2D after vitamin D challenge. Perhaps a higher vitamin D dose or a longer follow up time would have been required to show decreases in 1,25(OH)2D. Of note are a quarter of both healthy young to middle age adults and stone formers demonstrating vitamin D insufficiency (designated serum 25(OH)D less than 25 nmol/L). However, 25(OH)D levels below 25 nmol/L may, in fact, not be the true measure of vitamin D “deficiency”, because controls with vitamin D levels below 50 nmol/L of 25(OH)D had greater increase in 25(OH)D, greater rise in 1,25(OH)2D and a decline in PTH in response to the vitamin D supplement. These responses constitute criteria for true vitamin D insufficiency. Above 50 nmol/L of 25(OH)D, further intake of vitamin D will not appreciably increase 25(OH)D or 1,25(OH)2D, nor is PTH appreciably suppressed.

Therefore, if 25(OH)D levels below 50 nmol/L are considered indicative of vitamin D deficiency, then over half the subjects in this study had inadequate vitamin D nutrition warranting supplementation, regardless of stone forming status. Kinyamu et al has suggested that serum 25(OH)D in the elderly should be at least 122 nmol/L because only at this level are
PTH concentrations reduced those considered normal in young adults. Dawson-Hughes has further proposed that in the elderly population, the lower level for the optimal range for 25(OH)D should be set as high as 100 nmol/L, because at this level, extra vitamin D no longer suppresses PTH secretion (106). My results suggest that PTH are not supressable when baseline 25(OH)D are above 50 nmol/L in young adults. For adults with less than 50 nmol/L of 25(OH)D, there was a trend towards those with lower vitamin D status having the greatest PTH suppression after dosing. PTH levels were unchanged if basal 25(OH)D was around 50 nmol/L. Taken together, these results suggests that vitamin D status in normal healthy adults of least 50 nmol/L minimize the risk of vitamin D insufficiency.

The reason for elevated 1,25(OH)\textsubscript{2}D in IH is still unclear. Kidney stone disease is multifactorial and complex. It is possible that factors affecting 1,25(OH)\textsubscript{2}D production such as interleukins, cytokines and the prostaglandins (120-122) promote 1,25(OH)\textsubscript{2}D production independent of PTH. In this case high rates of 1,25(OH)\textsubscript{2}D synthesis may be outside of the normal regulatory control of the vitamin D endocrine system. However, a definitive causal relation between various biological factors as a mechanistic explanation of the IH remains hypothetical at this time.
Experiment #3

Rationale

Half of all stone formers have idiopathic hypercalciuria, characterized by high circulating 1,25(OH)$_2$D despite normal serum calcium, PTH levels and the absence of any systemic illness. As indicated in experiment #2, vitamin D is thought to play a prominent role in kidney stone formation because abnormal 1,25(OH)$_2$D regulation is a prominent feature.

Surveys have established that individuals with a family history of kidney stones tend to have higher incidence of the disease (123-124). Based on the patterns of transmission, several investigators have suggested that IH follows an autosomal dominant mode of inheritance (125-127). A possible explanation for linking abnormalities in vitamin D function and the inheritance of kidney stones and vitamin D is by examining the function of the vitamin D receptor (VDR). Recently identified polymorphisms in the VDR gene have been reported to affect VDR function (44), perhaps by altering VDR mRNA transcription regulation. Changes in VDR expression could amplify the effects of 1,25(OH)$_2$D and thereby influence calcium metabolism. Since, 1,25(OH)$_2$D up-regulates its own receptor, any small increase in its synthesis combined with subtle increases in the quantity of VDR could conceivably amplify the effects of the hormone at the intestines to stimulate intestinal absorption of calcium. More importantly, VDR have been reported to be a predictor of both bone mineral density and calcium absorption (44). The identified VDR polymorphisms may also point to VDR as a predisposing factor for kidney stones. In light of these findings, I examined whether genetic polymorphisms in VDR, identified by an improved direct haplotyping method, are associated with stone disease (128).
Hypothesis

Stone formers should have different allelic frequencies of VDR polymorphisms compared to controls. Thus, calcium excretion in stone formers may be associated with specific VDR polymorphisms.

Methods and Materials

Study Population

224 calcium stone forming patients (159 men and 65 women, average age 52.4 ± (SD) 12.6 years) referred to the Lithotripsy Clinic at The Wellesley Central Hospital (Toronto, Canada) for treatment of kidney stones donated blood for haplotyping. Urine and serum biochemistry assayed by standard laboratory methods were obtained from a metabolic profile of patients referred for treatment of kidney stones. Controls were unselected, young women (age 18-35) enrolled in a study at Women’s College Hospital in Toronto, Ontario. These studies were approved by the Women’s College Hospital Research Ethics Board and the University of Toronto Ethics Committee.

DNA Extraction

DNA was extracted from whole blood by salt precipitation (129). Five to ten mL of whole blood was lysed with red blood cell (RBC) lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM Na₂EDTA, pH 7.4) and placed on ice for 15 minutes. The solution was centrifuged at 1000 g for 10 minutes and the supernatant decanted. The pellet containing extracted nuclei was washed twice in 10 ml of RBC lysis buffer and recentrifuged and the supernatant decanted. The
white blood cell (WBC) pellet was resuspended in 4 mL of Nuclei Lysis Buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na₂EDTA, pH 8.2) by vortexing. 0.3 mL of 100 g/L SDS solution and 0.1 mL of Proteinase K (20 mg/mL) was added and the solution left to incubated overnight at 37 °C in a water bath. Digested proteins were precipitated by adding 1 mL of saturated NaCl solution and shaken vigorously for 30 seconds. The resultant mixture was centrifuged at 3200 g for 30 minutes. Supernatant was decanted and another 0.5 mL of saturated NaCl solution was added, shaken and centrifuged at 3200 g for 30 minutes. Two volumes of absolute ethanol (~10 mL) were added and the tubes gently inverted to precipitate the DNA. The DNA was spooled out with a sterile disposal loop and washed in 70% ethanol, allowed to dry at room temperature and resuspended in water. The dissolved DNA was stored at 4 °C.

Genotyping was performed by a direct haplotyping method (130), which allow for an accurate assignment of genotype. This method can detect any deviation from the expected linkage disequilibrium between the three restriction sites that could potentially uncover novel VDR associations found in different populations. Briefly, the forward primer 5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3' and the reverse primer 5'-AACCAGCGGGAAGAGGTCAAGGG-3' were used to amplify a 2229 base pair region containing the three polymorphic restriction enzyme recognition sites at the 3' end of the VDR. 200 ng of extracted DNA was amplified in a 100 μL volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTP, 150 ng of each primer and 0.5 unit of Taq polymerase (Pharmacia). The polymerase chain reaction (PCR) was carried out on a Perkin Elmer 4800 model for 30 cycles (97 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min) followed by final extension at 72 °C for 5 minutes. 15 μL of PCR products were incubated in a water bath and digested with 5 units of the restriction enzymes BsmI, ApaI and TaqI simultaneously in a buffer containing 150 mM Tris-HCl pH 7.5, 25 mmol NaCl and 35 mmol MgCl₂ for 1 hour at 37 °C, and then 1 hour at 65 °C. A restriction map of the amplicon is shown in figure 3.1.

Digested fragments were separated in 5-20% polyacrylamide TBE precast gels using the Xcell II Mini-Cell apparatus (Novex, San Diego, USA). Electrophoresis was performed at
constant voltage (125 V) for 2 hours in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na2EDTA), then at 100 V for 3 hours. Separated DNA fragments were visualized with a silver staining kit from Pharmacia, Sweden (figure 3.2). Markers were constructed by mixing digested PCR products containing all possible RFLP fragments. All possible combinations of the three restriction sites can be determined from the resultant fragments visualized after digestion to assign haplotype and genotype.

Serum and urine biochemistries were measured by standard methods in the routine clinical laboratory. TmP/GFR was calculated from monographs of Walton and Bijvoet (131) Calcium excretion was calculated using an excretion index (CEI) where CEI = (urine calcium/urine creatinine)/(serum calcium/serum creatinine). CEI values were grouped according to VDR genotype (table 5). VDR alleles were named as previously defined. Capital letters denote the absence and lower case letters denote the presence of restriction sites for the three enzymes, BsmI (B/b), Apal (A/a) and TaqI (T/t). VDR haplotypes with different combinations of the three restriction sites were coded as follows: allele 1 represented the baT haplotype, allele 2 - BAt, allele 3 - bAT, allele 4 - BAT, and allele 5 - bAt. Allele 6 (rare) found in the control group is the Bat haplotype (See Figure 3.1). Differences in distribution of genotype and haplotype were assessed by chi-square test. Comparisons of variables between genotype and haplotypes were made by ANOVA. Multiple regression was used to determine the relationship of genotype to urinary calcium excretion.
Schematic Presentation of the VDR region covering BsmI, ApaI and TaqI polymorphic sites

Figure 3.1
Schematic diagram of DNA fragments produced for the various haplotypes after restriction enzyme digestion. Arrows indicate restriction sites recognized by indicated the restriction enzymes. Taq* is an invariant restriction site present in all subjects. Reproduced with permission from Peltekova et al (130).
Genotypes. Asterisks indicate rare haplotypes. Reproduced with permission from Pelcova et al. Polyacrylamide gel. Base pair markers in lane two were constructed by mixing known fragments. Both panels A and B show the RFLP pattern after resolving digested DNA fragments on an agarose gel.

Figure 3.2
**Results**

Genotyping were determined for all 224 patients entered into the study. The distribution of the three polymorphisms (*BsmI*, *ApaI*, or *TaqI*) did not differ from that predicted by the Hardy-Weinberg equilibrium ($\chi^2 < 1$ for all three polymorphic sites, 2 df, $p > 0.5$). VDR genotype frequencies of kidney stone formers are shown in table 3.1 and the composite haplotype frequencies in table 3.2. There was a 97% association between *BsmI* and *TaqI* polymorphisms (b associated with T and B associated with t) compared to 98% for the control group, confirming the extensive, but not complete linkage disequilibrium between these two sites. There was no difference in distribution of *BsmI*, *TaqI* and *ApaI* genotypes or haplotypes between the stone formers and controls. Haplotype frequencies for stone formers and controls were similar and haplogroup distribution was not significantly different between stone formers and control. The rare alleles (alleles 4, 5, 6 and 8) constitute less than 5% of total haplotypes and these were combined for statistical analysis.

There were also no differences found for serum calcium and phosphate concentrations, urine calcium or urine phosphate, in comparing the stone-formers by genotype or haplogroup (tables 3.3 & 3.4). However, the calcium excretion index (CEI) showed a significant difference by *ApaI* genotype (table 3.3). Stone-formers with the 'AA' genotype had a lower CEI than those with either 'aa' or 'Aa' (figure 3.3). In examining haplotypes, the CEI was lower in patients with two copies of allele 2 ('BAt') than those with none. When the top and bottom quintiles of calcium excretion were compared by genotype frequency, no differences in genotype distribution were found for *BsmI* or *TaqI* RFLP. However, at the *ApaI* site, the top quintile had more 'a' than 'A' alleles (table 3.5). No significant trends was observed for CEI grouped according to haplogroup, nor was any allelic dose effect observed for haplogroup alleles 1, 2 or 3. Table 3.6 summarizes the multiple regression of different predictors on urine calcium. Urine creatinine was the best predictor of urinary calcium, accounting for approximately 19% of the variation. On the other hand, *ApaI* genotype only accounted for 2% of the observed variation in urine calcium excretion.
The table shows the frequencies and the percentages (in brackets) of BsmI, ApaI, and TaqI polymorphism along with frequencies for various detected haplogroups.
## Table 3.2.

RFLP allele frequencies in controls and stone-former groups (% in brackets)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Stone formers*</th>
<th>Controls formers*</th>
<th>Haplotype</th>
<th>Stone formers*</th>
<th>Controls formers*</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>270 (60.3)</td>
<td>803 (59.2)</td>
<td>[1]</td>
<td>211 (49.3)</td>
<td>611 (45.1)</td>
</tr>
<tr>
<td>B</td>
<td>178 (39.7)</td>
<td>553 (40.8)</td>
<td>[2]</td>
<td>168 (37.5)</td>
<td>535 (39.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[3]</td>
<td>57 (12.7)</td>
<td>190 (14.0)</td>
</tr>
<tr>
<td>a</td>
<td>211 (47.1)</td>
<td>612 (45.1)</td>
<td>[4]</td>
<td>10 (2.2)</td>
<td>17 (1.3)</td>
</tr>
<tr>
<td>A</td>
<td>237 (52.9)</td>
<td>744 (54.9)</td>
<td>[5]</td>
<td>2 (0.4)</td>
<td>2 (0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>[6]</td>
<td>0 (0.0)</td>
<td>1 (0.07)</td>
</tr>
<tr>
<td>t</td>
<td>170 (37.9)</td>
<td>538 (39.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>278 (62.1)</td>
<td>818 (60.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The table shows the allelic frequencies for VDR genotypes and haplogroups. Numbers indicate the frequencies and brackets indicate percentages.

* Not significantly different from controls, by $\chi^2$ test.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of subjects</th>
<th>Serum Calcium (mmol/L)</th>
<th>Serum phosphate (mmol/L)</th>
<th>Urinary calcium (mmol/24 h)</th>
<th>Urinary phosphate (mmol/24 h)</th>
<th>Calcium excretion index</th>
<th>TmP/GFR (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bb</td>
<td>85</td>
<td>2.32 (0.11)</td>
<td>1.03 (0.17)</td>
<td>5.54 (3.39)</td>
<td>26.2 (10.1)</td>
<td>14.72 (7.42)</td>
<td>0.91 (0.17)</td>
</tr>
<tr>
<td>Bb</td>
<td>100</td>
<td>2.31 (0.18)</td>
<td>1.05 (0.16)</td>
<td>5.32 (2.83)</td>
<td>27.7 (10.8)</td>
<td>14.92 (8.13)</td>
<td>0.91 (0.19)</td>
</tr>
<tr>
<td>BB</td>
<td>39</td>
<td>2.31 (0.12)</td>
<td>1.05 (0.16)</td>
<td>5.07 (2.80)</td>
<td>27.5 (10.5)</td>
<td>13.26 (6.26)</td>
<td>0.93 (0.22)</td>
</tr>
<tr>
<td>Aa</td>
<td>51</td>
<td>2.33 (0.10)</td>
<td>1.03 (0.16)</td>
<td>5.46 (3.65)</td>
<td>25.1 (9.8)</td>
<td>14.78 (7.81)</td>
<td>0.91 (0.17)</td>
</tr>
<tr>
<td>Aa</td>
<td>109</td>
<td>2.31 (0.17)</td>
<td>1.05 (0.16)</td>
<td>5.68 (2.94)</td>
<td>27.2 (10.2)</td>
<td>15.69 (8.16)*</td>
<td>0.92 (0.18)</td>
</tr>
<tr>
<td>AA</td>
<td>64</td>
<td>2.32 (0.12)</td>
<td>1.05 (0.15)</td>
<td>4.73 (2.58)</td>
<td>28.4 (10.9)</td>
<td>12.45 (5.75)*</td>
<td>0.92 (0.21)</td>
</tr>
<tr>
<td>Tt</td>
<td>33</td>
<td>2.33 (0.11)</td>
<td>1.06 (0.16)</td>
<td>5.11 (2.91)</td>
<td>27.6 (8.5)</td>
<td>13.34 (6.46)</td>
<td>0.92 (0.20)</td>
</tr>
<tr>
<td>Tt</td>
<td>104</td>
<td>2.31 (0.17)</td>
<td>1.05 (0.17)</td>
<td>5.32 (2.84)</td>
<td>27.7 (11.2)</td>
<td>14.85 (8.13)</td>
<td>0.91 (0.20)</td>
</tr>
<tr>
<td>TT</td>
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<td>2.32 (0.11)</td>
<td>1.04 (0.16)</td>
<td>5.51 (3.33)</td>
<td>26.2 (10.0)</td>
<td>14.68 (7.27)</td>
<td>0.92 (0.17)</td>
</tr>
</tbody>
</table>

Means and (SD) of serum and urine biochemistries of analytes affected by the vitamin D endocrine system were grouped according to genotypes.

* significant difference of means between Aa and aa genotypes, by Kruskal Wallis 1-Way ANOVA, p=0.043
Table 3.4.
Means for serum and urine chemistries grouped by haplogroup

<table>
<thead>
<tr>
<th>Haplogtype</th>
<th>Number of subjects</th>
<th>Serum calcium (mmol/L)</th>
<th>Serum phosphate (mmol/L)</th>
<th>Urinary calcium (mmol/24 h)</th>
<th>Urinary phosphate (mmol/24 h)</th>
<th>Calcium excretion index</th>
<th>TmP/GFR (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1,2]</td>
<td>74</td>
<td>2.31 (0.19)</td>
<td>1.06 (0.17)</td>
<td>5.63 (2.91)</td>
<td>27.4 (10.4)</td>
<td>15.98 (8.71)</td>
<td>0.91 (0.19)</td>
</tr>
<tr>
<td>[1,1]</td>
<td>51</td>
<td>2.33 (0.10)</td>
<td>1.03 (0.16)</td>
<td>5.46 (3.65)</td>
<td>25.1 (9.8)</td>
<td>14.78 (7.81)</td>
<td>0.91 (0.17)</td>
</tr>
<tr>
<td>[2,2]</td>
<td>33</td>
<td>2.33 (0.11)</td>
<td>1.06 (0.16)</td>
<td>5.11 (2.91)</td>
<td>27.6 (8.5)</td>
<td>13.34 (6.46)</td>
<td>0.92 (0.20)</td>
</tr>
<tr>
<td>[1,3]</td>
<td>31</td>
<td>2.31 (0.12)</td>
<td>1.04 (0.18)</td>
<td>5.48 (2.85)</td>
<td>27.4 (10.4)</td>
<td>14.39 (6.70)</td>
<td>0.92 (0.16)</td>
</tr>
<tr>
<td>[2,3]</td>
<td>22</td>
<td>2.33 (0.13)</td>
<td>1.04 (0.14)</td>
<td>4.25 (2.11)</td>
<td>29.9 (12.3)</td>
<td>11.36 (4.82)</td>
<td>0.91 (0.20)</td>
</tr>
<tr>
<td>[2,4]</td>
<td>6</td>
<td>2.22 (0.11)</td>
<td>1.03 (0.20)</td>
<td>4.83 (2.29)</td>
<td>26.8 (17.8)</td>
<td>12.84 (5.51)</td>
<td>0.96 (0.30)</td>
</tr>
<tr>
<td>[1,4]</td>
<td>3</td>
<td>2.25 (0.03)</td>
<td>1.04 (0.09)</td>
<td>6.93 (3.63)</td>
<td>23.3 (7.2)</td>
<td>17.84 (6.12)</td>
<td>0.94 (0.11)</td>
</tr>
<tr>
<td>[3,3]</td>
<td>2</td>
<td>2.38 (0.12)</td>
<td>1.13 (0.25)</td>
<td>5.05 (2.76)</td>
<td>36.0 (8.5)</td>
<td>11.60 (4.17)</td>
<td>0.95 (0.21)</td>
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<tr>
<td>[1,5]</td>
<td>1</td>
<td>2.31</td>
<td>0.94</td>
<td>12.30</td>
<td>20.0</td>
<td>28.40</td>
<td>0.89</td>
</tr>
<tr>
<td>[4,5]</td>
<td>1</td>
<td>2.29</td>
<td>0.88</td>
<td>1.60</td>
<td>15.0</td>
<td>6.75</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Means and (SD) of serum and urine biochemistries of analytes affected by the vitamin D endocrine system were grouped according to haplogroups.
Table 3.5.  
Differences in genotype distribution between top and bottom quintiles for CEI

<table>
<thead>
<tr>
<th></th>
<th>BsmI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ApaI</th>
<th>TaqI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottom</td>
<td>15</td>
<td>21</td>
<td>9</td>
</tr>
<tr>
<td>Quintile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top</td>
<td>17</td>
<td>24</td>
<td>4</td>
</tr>
</tbody>
</table>

Subjects were ranked according to CEI and the distribution of VDR frequencies of the top quintile was compared against the bottom quintile.

<sup>a</sup>Numbers indicated are counts

<sup>*</sup>Significant difference in distribution of ApaI genotype by $\chi^2$ test, p=0.02
Table 3.6.
Parameter estimates of urine calcium in multivariate regression

<table>
<thead>
<tr>
<th>Variable</th>
<th>Parameter</th>
<th>$R^2$</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Creatinine</td>
<td>-0.044</td>
<td>0.050</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine Creatinine</td>
<td>0.171</td>
<td>0.195</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine Magnesium</td>
<td>0.669</td>
<td>0.146</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine Sodium</td>
<td>0.010</td>
<td>0.016</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine Oxalate</td>
<td>-0.004</td>
<td>0.039</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apa1</td>
<td>-0.582</td>
<td>0.019</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Various predictor variables were used in multivariate regression to determine their influence on urinary calcium excretion.

Statistical calculations for all three experiments were performed on SPSS version 7.5 (SPSS Inc., Chicago, IL) with the assistance of George Tomlinson.
Calcium excretion index (CEI) grouped by genotype
Number indicate group size

* "AA" genotype had significantly lower CEI compared to "Aa" by Kruskal-Wallis 1-way ANOVA (p=0.043)
Disordered regulation of the vitamin D endocrine system in idiopathic hypercalciuric kidney stone formers is thought to contribute to high levels of 1,25(OH)\textsubscript{2}D (59,132). Elevated 1,25(OH)\textsubscript{2}D can promote excessive calcium absorption and increase the risk of stone formation because of hypercalciuria. Since VDR polymorphisms have been shown to be associated with disorders of calcium metabolism (133-135) it is possible that allelic variants of VDR are associated with the elevated levels of 1,25(OH)\textsubscript{2}D seen in idiopathic hypercalciuria.

In this study, distribution of VDR alleles in both the control and stone forming populations did not deviate from the Hardy-Weinberg equilibrium. The frequencies of BsmI, ApaI, and TaqI alleles in these groups were also similar to previously published reports for Caucasian populations (136-137). I found no difference in VDR genotype or haplotype distribution between kidney stone formers and controls. When grouped by genotype or haplotype, there was no significant differences in serum or urine calcium and phosphate. These results indicate that the effect of VDR polymorphism on calcium metabolism, is small. Cooper and Umbach (138) reached a similar conclusion in their meta-analysis of 16 published studies examining VDR and BMD. In the combined data, ‘BB’ genotype at the hip, spine and radius had a modest but significant association with a 2% reduction in BMD compared to the ‘bb’ genotype. In my study, ApaI genotype could only account for 2% of the variability of urinary calcium excretion in a multivariate regression (Table 3.6). This weak effect of VDR polymorphisms may be masked by environmental factors such as diet and lifestyle, as well as other calcium regulating genes such as the calcium sensing receptor (139).

Since 1,25(OH)\textsubscript{2}D acts to increase calcium absorption, a compensatory increase in urinary calcium excretion should be evident if homeostasis is maintained. Given the original association of the 'B' allele of BsmI polymorphism and lower bone mineral density reported by Morrison et al (44), one might have expected lower calcium absorption in those with the 'B' allele, and lower calcium absorption would contribute to their negative calcium balance. However, I did not detect any association of urine calcium excretion \textit{per se} with any of the 3
RFLP's. However, after calculating a calcium excretion index (CEI) a significant association was detected with the ApaI polymorphism. The CEI may be a better indicator of urinary calcium excretion because it corrects for inter-individual variation in calcium absorption, and provides a true estimate of renal calcium reabsorption. The CEI in individuals with an 'a' allele was higher than those homozygous for the 'A' allele. In addition, ApaI genotype distribution between low, compared to the high excretors was also significantly different, with the highest calcium excretors having an increased frequency of the 'a' allele (Table 3.3 & 3.5). This suggests that the ApaI VDR genotype is associated with variability in excretion of urinary calcium.

I did not observe a dose effect of the ApaI locus as reported for the linked BsmI site by Morrison(44) and the 'bAT' haplogroup (140). When 'aa' and 'Aa' genotype are combined and compared to the 'AA' genotype, those with the 'aa' and 'Aa' genotype had higher calcium excretion, suggesting a quasi-dominant effect of the 'a' allele. This is consistent with linkage studies suggesting that kidney stone disease is transmitted in an autosomal dominant fashion (126-127).

Several previously reported studies are in accordance with my results, assuming a coupling between intestinal absorption and renal reabsorption. Wishart et al (141) reported that the 'bbaaTT' haplotype had higher radiocalcium absorption in premenopausal women. This result is consistent with my observation that the 'aa' genotype had higher calcium excretion. Gennari et al reported significant differences in fractional calcium absorption between different haplotypes. They showed that the [1,1] ('bbaaTT') genotype had significantly higher fractional calcium absorption than the [2,2] ('BBAAtt') genotype (142). Several other studies presented contrasting observations. Francis et al examined calcium absorption in 48 men but found no association between radiocalcium absorption and TaqI VDR polymorphism (143). However, their small group size may have prevented the detection of a small effect. In 92 Caucasian females, Kinyamu et al (107) found no difference in calcium absorption or VDR protein expression in association with BsmI or TaqI polymorphic alleles. However, they did not determine whether an association existed with calcium absorption at the ApaI site. I also did not observe a significant association calcium excretion at either the BsmI or the TaqI site.
Two studies in different ethnic populations yielded results contrary to my findings. In a group of African-American women, Zmuda et al (144) found no significant association between calcium absorption and any of the three VDR RFLP. However, the African-American women in the study had an allelic distribution of Apal RFLP that was substantially different than ours, with few 'aa' genotypes, a racial difference that has been described before (145). In another study of 84 post-menopausal Thai women, 'bb' BsmI genotype was associated with higher 24 hour urinary calcium excretion. However, the same effect was not present for Apal or TaqI genotypes (146). In the Asian population, differences in urinary excretion resulted from altered intestinal absorption not renal reabsorption, because individuals with different BsmI genotypes had the same fractional calcium excretion. This suggested that in these individuals vitamin D receptor effects at the intestine may predominate over those at the kidney (64).

Both African and Asian populations have different VDR allelic distributions compared to Caucasians (146-148). Furthermore, comparison between different ethnic populations might generate discrepant results as a results of different degrees of linkage disequilibrium between these 3' VDR polymorphisms and other linked functional sites (145,149). This may account for some of the disparate results between African and Asians compared to my Caucasian group of stone formers.

In studies of Caucasians, Zerwekh et al found no difference in the distribution of BsmI polymorphisms between stone formers (n= 33) and controls (n=36). The small sample sizes in this study limited the detection of any minor effects (150).

Two larger studies of Caucasian population in the Toronto area have also shown effects of VDR on BMD. In a group of 678 young 18-35 year old normal females and 72 primary biliary cirrhosis patients, the baT allele was associated with lower bone mineral density (151,152). Comparison of my results and the Toronto area studies are important because these groups derive from the same highly admixed genetic background in an urban geographic location with a relatively homogenous environment.

Calcium intake may be an important external factor that could obscure the effects of VDR polymorphism. Dawson-Hughes et al (153) found that the 'B' allele had significantly lower
calcium absorption than the 'b' allele on a low calcium diet. However, the effect disappeared on a high calcium diet. Kiel et al (154) also found similar dietary interaction where women with the 'bb' genotype taking greater than 800 mg of calcium per day had significantly higher BMD. High calcium intake can obscure any effect of vitamin D on intestinal absorption, because at high concentration of calcium, passive paracellular flux of calcium across intestinal epithelium occurs concurrently with active transport mechanisms requiring 1,25(OH)$_2$D (155).

Low calcium intake has been shown to exacerbate vitamin D deficiency by depleting 25(OH)D as the body attempts to generate 1,25(OH)$_2$D to restore calcium balance. Low 25(OH)D levels may also affect free levels of 1,25(OH)$_2$D that could result in functional differences at the receptor level because both metabolites are transported by the same binding protein (DBP). So far only the early results reports of Morrison et al and Howard et al have demonstrate an association of differences in 1,25(OH)$_2$D levels and BsmI polymorphism (44,156). Subsequent investigations have not confirmed this association (107,157). Wishart et al (141) demonstrated an association of the ApaI site with calcium absorption without strict dietary control prior to testing. Although limited data exist concerning dietary habits of stone formers, they tend to avoid calcium in the belief this will reduce the likelihood or severity of attacks, often based on advice from physicians (158,159). Avoidance of calcium can lead to osteomalacia which has been found in stone formers on a low calcium diet (117-118). Moreover, various studies have indicated that increased dietary calcium may reduce the risk of calcium stones formation (124,158). It is conceivable that lower calcium consumption in stone patients helps unmask the observed effect.

It is not yet clear how intronic changes in DNA sequence, such as those at the ApaI restriction site, ultimately affect VDR function. Most likely, these markers are in linkage disequilibrium with critical functional sequences in or nearby the VDR gene itself. One candidate is the polymorphic poly-adenine (poly A) stretch in the 3' untranslated region (3'UTR) of VDR. Ingles et al (145) have reported that the long poly-A sequence (18-24 residues) is in strong linkage with the 'b' allele in a Caucasian population. Studies in eukaryotic cells have shown that the longer polyA sequence may allow for easier recruitment of mRNA into
ribosomes, resulting in increased VDR protein translation efficiency. Differences in the length of the poly-A sequence may therefore result in a greater VDR accumulation that would, in turn, amplify the effect of any given amount of 1,25(OH)₂D. A polymorphic FokI restriction site in exon 2 of VDR that alters the protein sequence by three amino acids has been associated with BMD in several different populations (160-162). A change in VDR protein length, even if minor, is more likely to result in functional differences than intronic or silent exonic variations. This start codon polymorphism has not yet been found to be linked to disorders of calcium metabolism or indeed in any change of VDR-mediated actions \textit{in vitro}.

Increased intestinal calcium absorption and increased urinary excretion could result from small changes in VDR receptor function or number. Since, 1,25(OH)₂D up-regulates its own receptor (98), any small increase in its synthesis combined with subtle increases in the quantity of VDR could potentially amplify the effects of this hormone at the intestine to stimulate increased intestinal calcium absorption.

In summary, my results show an association of Apal genotype with calcium metabolism in a population of stone formers, where those homo or heterozygous for the 'a' genotype ('aa', 'Aa') had a higher calcium excretion index than those homozygous for the 'A' genotype ('AA'). Since increased calcium excretion is a known risk factor for kidney stones, further investigation that begins with more precise dietary control should reveal whether the 'a' allele of the VDR polymorphism is truly a marker for increased risk of calcium stone formation.
My results show that, on a molar basis vitamin D3 is more effective at raising serum 25(OH)D than vitamin D2. The long-standing assumptions concerning the equivalence of vitamin D2 and D3 were based on 60-year-old studies (71) using crude anti-rachitic effects in infants as criteria. The assumption of vitamin D2 and D3 equivalence used to express vitamin D nutrition should be reconsidered.

This study also detected widespread vitamin D insufficiency. The increase in 25(OH)D, 1,25(OH)2D, and decrease in PTH following vitamin D supplementation in individuals with baseline 25(OH)D below 50 nmol/L suggest that vitamin D insufficiency may occur at 25(OH)D levels twice as high as currently proposed levels. 1,25(OH)2D levels are strictly regulated and remain relatively unchanged even when 25(OH)D levels are extremely high.

In contrast to normal subjects, stone formers exhibit a decreased feedback inhibition, as there was no suppression of 1,25(OH)2D after moderate vitamin D supplementation. The nature of the 1,25(OH)2D deregulation and the unusual response to vitamin D supplementation in stone formers remains elusive. However, further studies that eliminate baseline variability or follow patients over longer periods may delineate the fixed difference in the response of 1,25(OH)2D to additional vitamin D intake.

Because VDR allelic frequencies in stone formers were similar to normal subjects any effect of VDR polymorphisms on calcium stone formation is probably small. More likely, the association of VDR polymorphisms with calcium excretion is linked to a nearby functional polymorphism such as the highly polymorphic 3’ untranslated region of the VDR. Screening of DNA sequence near the VDR locus on chromosome 12 may clarify this. Despite the lack of a demonstrable association between VDR polymorphisms and kidney stone formation, I detected an association of ApaI genotype with adjusted calcium excretion. Those with 'a' genotype excreted more calcium than the 'A' genotype. Investigations with subjects pre-selected for genotypes and calcium dosing under stricter dietary controls should reveal whether the 'a'
genotype of the VDR polymorphism is truly a marker of calcium excretion and hence increased risk of calcium stone formation.
References


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