Randomized Clinical Trial of Vitamin D₃ Doses on Prostatic Vitamin D Metabolite Levels and Ki67 Labeling in Prostate Cancer Patients

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Context: Vitamin D₃ might benefit prostate cancer (PCa) patients because prostate cells can locally synthesize the active hormone calcitriol.

Objective: Our objective was to determine the effects of oral vitamin D₃ on vitamin D metabolites and PCa proliferative activity in prostate tissue.

Design and Setting: We conducted a double-blind randomized clinical trial at surgical oncology clinics in Toronto, Canada.

Patients: PCa patients (Gleason 6 or 7) participated in the study. Of 66 subjects who were enrolled, 63 completed the dosing protocol.

Intervention: Vitamin D₃ (400, 10 000, or 40 000 IU/d) was orally administered before radical prostatectomy.

Main Outcome Measures: We evaluated vitamin D metabolite levels and Ki67 labeling in surgical prostate tissue. Safety measures, PTH, and prostate-specific antigen (PSA) were also assessed.

Results: Prostate tissue and serum levels of vitamin D metabolites, including calcitriol, increased dose dependently ($P < .03$) and were significantly higher in the 40 000-IU/d group than in every other dose group ($P < .03$). Prostate vitamin D metabolites correlated positively with serum levels ($P < .0001$). Ki67 measures did not differ significantly among vitamin D dose groups. However, cross-sectional analysis indicated that the calcitriol level attained in prostate was inversely associated with Ki67 intensity and Ki67 (3+) percent positive nuclei in PCa and benign tissue ($P < .05$). Safety measures did not change adversely with dosing. Compared with the 400-IU/d group, serum PTH and PSA were lower in the combined higher-dose groups at the end of the study ($P < .02$).

Conclusions: Oral vitamin D₃ raised prostate calcitriol levels (level 1 evidence) and modestly lowered both PSA and PTH. Although Ki67 expression did not differ among dose groups, its levels correlated inversely with prostate calcitriol. These suggestions of clinical benefit justify continued clinical research. (J Clin Endocrinol Metab 98: 1498–1507, 2013)
The circulating concentration of the hormone precursor 25-hydroxyvitamin D \([25(\text{OH})D, \text{calcidiol}]\), is the direct result of both oral intake of vitamin D\(_3\) (cholecalciferol) and skin exposure to UV light. Prostate cancer (PCa) risk is lower in men with a life history of greater sun exposure (1). Furthermore, survival rates of breast cancer, colorectal cancer, and PCa are 25% higher if they are diagnosed and treated in summer compared with winter (2). There is also seasonality in the rate of rise in prostate-specific antigen (PSA) in patients monitored for untreated low-grade PCa, with the slowest rate of increase during spring and summer (3). Consistent with that, vitamin D\(_3\) supplementation has been shown to slow the rate of PSA increase in men being evaluated and monitored for failed PCa surgery or radiation (4).

Virtually all previous clinical trials addressing the relevance of vitamin D in PCa have used the active metabolite calcitriol (1,25-dihydroxyvitamin D) or its analogs (5–10). The disadvantage of the systemic administration of calcitriol or its analogs as a chemotherapeutic agent is that supraphysiological doses are required that rely on spill-over of the active agent from blood into prostate tissue (5, 7). Calcitriol and its analogs have narrow margins of safety with high risk of hypercalcemia, and because these agents have short half-lives, they are present at target tissues only intermittently if given at the commonly used weekly dosing interval (11). In contrast, the inactive precursor vitamin D\(_3\) has a long half-life and a wide margin of safety (12, 13) and should in theory generate a sustained therapeutic action. Indeed, a recent open-label clinical trial with historical controls showed that vitamin D\(_3\) treatment (4000 IU/d) for 1 year led to a decrease in the number of positive cores (or decrease in Gleason score) in PCa patients under active surveillance (14).

A clinical strategy designed to achieve higher serum 25(OH)D concentrations is supported by reports that physiologic concentrations of 25(OH)D exhibit antiproliferative effects on cultured prostate cells equivalent to a 100-fold supraphysiologic excess of calcitriol (15–17). Moreover, physiological levels of vitamin D\(_3\) have been reported to inhibit growth and induce differentiation of prostate epithelial cells (18–20). These effects are possible because prostate cells possess both of the enzymes (ie, 25-hydroxylase and 1α-hydroxylase) needed to convert vitamin D\(_3\) to the active paracrine hormone, calcitriol (19). Therefore, we hypothesized that oral vitamin D\(_3\) will increase intraprostate calcitriol concentration and affect prostate gland biology.

Direct measurement of vitamin D metabolites in human prostate tissue has not been reported, and vitamin D metabolism within the prostate remains poorly understood. Furthermore, the effects of oral vitamin D\(_3\) dosing on prostate cell proliferative activity in clinical samples have not been evaluated in a clinical trial. Recently, we developed and validated a novel method to measure calcitriol concentration in human tissues (21). Here, we describe the results from our randomized clinical trial (RCT) on the tissue effects of various doses of vitamin D\(_3\) given orally to PCa patients scheduled to undergo radical prostatectomy. The primary objectives were to characterize the clinical effects of vitamin D dosing on prostate tissue levels of vitamin D metabolites and on prostate cell proliferation.

Patients and Methods

The study protocol was approved by the Research Ethics Boards of the University Health Network, Sunnybrook Health Sciences Centre, and affiliates. All patients signed a form indicating their informed consent. The trial was registered with [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT00741364).

Patients

Patients with localized PCa scheduled to undergo radical prostatectomy were recruited from the urologic oncology clinics of Princess Margaret Hospital (at the University Health Network) and Sunnybrook Health Sciences Centre. Patients were considered eligible for the study if they met the following criteria: diagnosis of Gleason score 6 or 7 adenocarcinoma of the prostate biopsy, plasma calcium <2.62 mmol/L, urine calcium to creatinine ratio <1.0, plasma phosphate <1.40 mmol/L, serum PTH <6.9 pmol/L, plasma creatinine <200 μmol/L, plasma alanine transaminase (ALT) <40 U/L, and plasma alkaline phosphatase (ALP) <150 U/L. Patients were excluded for previous treatment of PCa or benign prostatic hyperplasia (eg, 5α-reductase inhibitors), regular use of vitamin D supplementation >2000 IU/d, significant sunlight exposure during the study (eg, travel to a tropical destination without regularly using sunscreen), duration for intervention <3 weeks, and history of hypercalcemia, sarcoidosis, or urolithiasis. Consented patients who could not participate in the study due to insufficient duration for intervention (<3 weeks) but otherwise met all other eligibility criteria were enrolled (without randomization) into the control (untreated) arm of the study. Data from the control arm was used only in cross-sectional (ie, correlation) analyses.

Study design

Our study was a multicenter double-blind RCT of vitamin D in PCa patients. Eligible patients were randomly allocated to 1 of 3 vitamin D\(_3\) doses: 1) 400 IU (10 μg), 2) 10 000 IU (250 μg), and 3) 40 000 IU (1000 μg). Patients in the control (nonrandomized) arm of the study did not receive any supplemental vitamin D (0 IU). Treated patients consumed the vitamin D orally, once per day, for a 3- to 8-week period ending the day before radical prostatectomy. Up to an additional 2 weeks of treatment were permitted if surgery was delayed for unrelated reasons. Overall, the protocol did not affect or delay primary treatment in any way. Analysis was intention-to-treat and involved all patients randomized to groups.
Materials

Liquid vitamin D doses were prepared by dissolving crystalline U.S. Pharmacopoeia (USP)-grade vitamin D₃ (Sigma, St Louis, Missouri) into USP-grade ethanol (Commercial Alcohols Inc, Brampton, Canada) as described previously (22). Quality control was performed by spectroscopy and also independently by a licensed pharmaceutical testing laboratory using USP method 31 (Quality Compliance Laboratories, Markham, Canada). Vitamin D concentrations were tested bimonthly and remained unchanged. Vitamin D doses were physically identical, unidentifiable, and consumed daily by each patient by mixing 1 mL of the ethanolic solution into juice or water before drinking it.

Assessments and sampling

Treated patients were evaluated during a screening (baseline) clinic visit, biweekly follow-up assessments, and on the day of surgery before entering the operating room (ie, final visit). At baseline, the following information was obtained: eligibility criteria, use of concomitant therapies (medications, supplements, or herbal preparations), biopsy Gleason score, and anthropometrics (age, height, weight, and ethnicity). During intervention, patients were interviewed biweekly via telephone to record any adverse events or changes in concomitant therapies since the previous assessment. Venous blood and urine samples were collected from each treated patient at the baseline and final visits for biochemical testing. Serum aliquots were stored at −80°C until analyses. A urine sample was provided 2 weeks after the start of intervention for additional safety monitoring. Compliance was monitored by measuring the remaining volume of liquid in the vials returned at the final visit.

Upon radical prostatectomy, the resected prostate specimen was weighed, measured, and inked to indicate margins. Fresh tissue (100–500 mg) from the peripheral zone (PZ) and the transition zone (TZ) of prostate was excised by a pathologist during gross examination and stored at −80°C until analyses. The remaining prostate specimen was fixed in formalin for >48 hours and submitted in toto for paraffin embedding and sectioning according to routine pathology practice. The formalin-fixed paraffin-embedded prostate blocks were stored at ambient temperature until immunohistochemical analyses.

Outcome measures

One primary outcome was the serum and prostate tissue levels of vitamin D metabolites attained in the patients after oral vitamin D₃ administration. These metabolites included 25(OH)D₃, calcitriol, and 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃]. The other primary outcome was the expression of the proliferation marker Ki67 (MIB-1) in normal and PCa tissue. The key safety indicator was urinary calcium excretion (ratio of millimolar concentrations of urine calcium and urine creatinine), measured at baseline, 2 weeks into vitamin D treatment, and at the final visit. Other safety-related measures were plasma levels of calcium, phosphate, creatinine, ALT, and ALP, measured at the baseline and final visits. Secondary outcomes included serum PSA and PTH.

Biochemical methods

Serum 25(OH)D₃ and 24,25(OH)₂D₃ were measured by liquid chromatography-tandem mass spectrometry (23). Serum calcitriol was determined by enzyme immunoassay (Immunodiagnostic Systems, Scottsdale, Arizona). Prostate tissue vitamin D metabolite concentrations were measured separately in prostate PZ and TZ and involved an initial tissue pre-extraction procedure reported elsewhere by our group (21). In the tissue extracts, calcitriol was measured by enzyme immunoassay, whereas 25(OH)D₃ and 24,25(OH)₂D₃ were quantified by liquid chromatography-tandem mass spectrometry.

Vitamin D-binding protein (DBP) was measured in a subset of patient serum (n = 12, 12, and 11 for 400, 10 000, and 40 000 IU/d, respectively) and prostate TZ homogenates (n = 12, 10, and 10 for 400, 10 000, and 40 000 IU/d, respectively; 100 mg) by ELISA (Immunodagnostik, Bensheim, Germany). Measures of free 25(OH)D₃, calcitriol, and 24,25(OH)₂D₃ were calculated as the ratio between the molar concentrations of the respective vitamin D metabolite and DBP (21). Calcium, creatinine, phosphate, ALT, ALP, PSA, and PTH were measured by routine hospital laboratory methods.

Table 1. Baseline Demographics of Vitamin D-Treated Study Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>400 IU/d</th>
<th>10 000 IU/d</th>
<th>40 000 IU/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>57.4 ± 6.8</td>
<td>58.9 ± 6.2</td>
<td>55.9 ± 7.3</td>
<td>57.6 ± 6.7</td>
</tr>
<tr>
<td>Ethnicity, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>54 (83)</td>
<td>18 (90)</td>
<td>17 (77)</td>
<td>19 (83)</td>
</tr>
<tr>
<td>Black</td>
<td>4 (6)</td>
<td>1 (5)</td>
<td>3 (14)</td>
<td>0</td>
</tr>
<tr>
<td>Other</td>
<td>7 (11)</td>
<td>1 (5)</td>
<td>2 (9)</td>
<td>4 (17)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>27.8 ± 4.0</td>
<td>27.9 ± 4.5</td>
<td>27.8 ± 3.5</td>
<td>27.8 ± 4.2</td>
</tr>
<tr>
<td>Gleason score, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>33 (52)</td>
<td>10 (50)</td>
<td>9 (42.9)</td>
<td>14 (60.9)</td>
</tr>
<tr>
<td>7</td>
<td>31 (48)</td>
<td>10 (50)</td>
<td>12 (57.1)</td>
<td>9 (39.1)</td>
</tr>
<tr>
<td>Serum PSA, µg/L</td>
<td>6.99 ± 4.56</td>
<td>7.08 ± 4.55</td>
<td>7.02 ± 4.75</td>
<td>6.87 ± 4.59</td>
</tr>
<tr>
<td>Serum 25(OH)D₃, nmol/L</td>
<td>65.4 ± 23.9</td>
<td>59.4 ± 13.9</td>
<td>65.2 ± 26.5</td>
<td>71.4 ± 27.7</td>
</tr>
<tr>
<td>Serum 25(OH)D₃, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;100 nmol/L</td>
<td>50 (91)</td>
<td>17 (100)</td>
<td>18 (90)</td>
<td>15 (83)</td>
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<tr>
<td>&lt;75 nmol/L</td>
<td>39 (71)</td>
<td>15 (88)</td>
<td>14 (70)</td>
<td>10 (56)</td>
</tr>
<tr>
<td>&lt;50 nmol/L</td>
<td>13 (24)</td>
<td>4 (23)</td>
<td>5 (25)</td>
<td>4 (22)</td>
</tr>
<tr>
<td>&lt;25 nmol/L</td>
<td>2 (4)</td>
<td>0</td>
<td>2 (10)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Does not include untreated control patients (n = 10) for which only vitamin D metabolites and DBP were measured at the final visit; these patients had similar demographic characteristics as treated patients. Baseline values did not differ among treatment groups (P > .05). Results are shown as mean ± SD if not noted otherwise.
Validation of prostate tissue vitamin D metabolite assay

The prostate tissue vitamin D metabolite assays were evaluated as previously reported for colon calcitriol (21). In bovine tissue free of endogenous vitamin D, mean recoveries (percent coefficient of variation) of exogenously added 25(OH)D₃, calcitriol, and 24,25(OH)₂D₃ were 94.4 ± 6.3, 95.8 ± 8.3, and 91.5 ± 14.9, respectively. A subset of prostate (n = 8) and serum (n = 14) samples was assayed in duplicate to determine method precision. Total within-run precision (percent coefficient of variation) of 25(OH)D₃, calcitriol, and 24,25(OH)₂D₃ measurement were similar in prostate tissue (13.0%, 11.1%, and 14.3%, respectively) and serum (7.0%, 10.2%, and 9.4%, respectively).

Ki67 labeling and digital immunoscoring

Tissue microarrays (TMAs) were created from pathologist-annotated formalin-fixed paraffin-embedded blocks from each of the study cases. Three benign normal (N) and 3 PCa cores (1 mm diameter) were obtained from each case. TMA sections were immunostained with a polyclonal antibody (1:1000 dilution) specific for Ki67 (Novus Biologicals, Littleton, Colorado). TMA slides were scanned onto a computer using an Aperio scanner and segmented using TMAlab. Genie tissue pattern recognition software (Aperio, Vista, California) was trained to recognize glands with a sensitivity of over 90% and was then merged with an adjusted nuclear algorithm. Genie analysis was conducted on 32 whole cores (24 PCa cores) and correlated with results of the adjusted nuclear algorithm on manually annotated cores and subsequently on immunostained TMA sections, rejecting cores with <300 nuclei as recognized by Genie analysis. Atrophy and high-grade prostatic intraepithelial neoplasia were also rejected. Each core was analyzed individually by a trained pathologist. After evaluation of each core, 356 cores of 438 (81.3%) were available for evaluation.

The quantitative digital immunoscoring platform provided the following Ki67 measures: 1) mean percent positive nuclei = positive nuclei (1+, 2+, or 3+)/total nuclei; 2) intensity score = 1+ (weak), 2+ (moderate), or 3+ (intense); 3) histoscore = percent positive nuclei × intensity score; and 4) mean (3+) percent positive nuclei = positive nuclei (3+ only)/total nuclei.

Statistical analyses

The randomization sequence was generated using computer software to produce randomly permuted blocks of 6 (ie, 2 of each dose in each sequence of 6 study ID numbers), stratified by center [Random Allocation Software version 1.0 (2004), by M. Saghaei]. All data were analyzed with SPSS software (version 20). Graphs were created with GraphPad Prism version 4 for Windows. Within-group changes in biochemical variables over time were analyzed with paired 2-tailed t tests. Between-group differences in biochemical variables were analyzed with 1-way ANOVA followed by Bonferroni post hoc testing. Associations between biochemical measures were examined by means of the Spearman r correlation coefficient. The criterion for significance was set at P < .05.

Results

Patients

Between October 2008 and July 2011, a total of 66 of 94 screened patients were randomized to intervention, whereas 10 additional patients were enrolled as untreated control subjects (Supplemental Figure 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org). Overall, the mean duration of vitamin D₃ treatment was 33.6 ± 9.5 days, without differences among
treatment groups ($P = .53$). Compliance to vitamin D treatment was 97%. Four patients withdrew prematurely from the study for reasons unrelated to intervention. Demographics and baseline characteristics of study patients were similar among treatment groups (Table 1).

**Vitamin D metabolite analyses**

Serum 25(OH)D$_3$ and 24,25(OH)$_2$D$_3$ increased significantly from baseline to final in all 3 vitamin D$_3$-treated groups ($P < .01$) in a dose-response manner such that the highest levels were attained in the 40 000-IU/d group ($P < .0001$) (Figure 1). Serum calcitriol levels rose significantly with dosing in the 10 000- and 40 000-IU/d groups ($P < .0001$), and the highest serum calcitriol concentration was achieved in the highest-dose group ($P < .0001$).

Prostate tissue levels of 25(OH)D$_3$, calcitriol, and 24,25(OH)$_2$D$_3$ (in PZ and TZ) increased dose-dependently [$P$ (linear trend) < .03]. Prostate tissue concentrations of each of the 3 vitamin D metabolites were significantly higher in the 40 000-IU/d group than in every other dose group ($P < .03$). Furthermore, prostate tissue 25(OH)D$_3$, calcitriol, and 24,25(OH)$_2$D$_3$ concentrations did not differ significantly between PZ and TZ regions ($P > .16$) in any dose group.

Serum levels of 25(OH)D$_3$, calcitriol, and 24,25(OH)$_2$D$_3$ were strongly correlated with their mean levels in prostate tissue (Figure 2). The relationships between serum and prostate vitamin D metabolite levels were best characterized by nonlinear (quadratic) regression, all of them showing significantly positive intercepts at 0 values of serum 25(OH)D$_3$ (95% confidence interval, 56.9–99.1 nmol/kg), calcitriol (7.9–51.0 pmol/kg), and 24,25(OH)$_2$D$_3$ (12.4–19.9 nmol/kg) ($P < .05$). Furthermore, 25(OH)D$_3$ levels in serum and in prostate were positively correlated with prostate calcitriol. Lastly, there was no significant relationship between circulating or prostatic concentrations of vitamin D metabolites and the duration of vitamin D dosing ($P > .39$).

Because it could be argued that vitamin D metabolites detected within the prostate were an artifact of residual blood within the tissue, we measured the extracellular transport protein for vitamin D in the plasma, DBP, in homogenates of prostate tissue used for the metabolite assays. In the prostate tissue, average DBP concentrations were 890.9 ± 207.7 nmol/kg, which was much lower than serum levels, 7029.4 ± 1108.3 nmol/L ($P < .001$) (Supplemental Figure 2). Accordingly, free levels of 25(OH)D$_3$, calcitriol, and 24,25(OH)$_2$D$_3$ in prostate tissue increased dose-dependently to levels that were highest in the 40 000-IU/d group ($P < .05$). Free levels of every vitamin D metabolite were substantially higher in prostate than in serum ($P < .0001$).

**Ki67 proliferation measures**

To assess the validity of the digital immunohistochemistry data, we compared the various Ki67 measures between N and PCa glands. As expected, these measures were significantly higher in PCa compared with benign tissue: Ki67 percent positive nuclei (PCa, 0.67%; N, 0.26%; $P < .0001$), Ki67 histoscore (PCa, 1.36; N, 0.56, $P < .0001$), and Ki67 (3+) percent positive nuclei (PCa, 0.20%; N, 0.10%; $P = .002$).

In PCa and benign tissue, Ki67 measures did not differ significantly
among vitamin D dose groups \( (P > .46) \) (Supplemental Figure 3). However, there were inverse correlations between the prostate tissue level of calcitriol and Ki67 intensity as well as Ki67 \((3+\) percent positive nuclei both in PCa and in benign tissue \( (P < .05) \) (Figure 3). Furthermore, prostate and serum calcitriol levels correlated inversely with Ki67 histoscore in benign tissue and with Ki67 intensity in PCa, respectively \( (P < .05) \) (Supplemental Table 1). Apart from calcitriol, no other vitamin D metabolite in serum or prostate was significantly related to any of the Ki67 proliferation measures (Supplemental Table 1). Upon stratifying by the attained prostate calcitriol level (Supplemental Figure 4), patients at the highest quartile of prostate calcitriol \( (\geq 37 \text{ pmol/kg}) \) showed significantly lower Ki67 intensity \( \text{PCa, } P = .048 \) and Ki67 \((3+) \) percent positive nuclei \( \text{PCa, } P = .01; \text{N, } P = .049 \) compared with those with lower levels.

**Safety measures and adverse events**

Plasma calcium concentrations did not change with dosing from baseline to final and remained well within the normal reference range \( (2.20–2.60 \text{ mmol/L}) \) throughout the study (Table 2). In all 3 treatment groups, the urine calcium to creatinine ratio decreased significantly from baseline \( (P < .01) \) to a level that was similar among groups at the final visit \( (P = .40) \). At week 2, the urine calcium to creatinine ratio was equivalent among dosing groups \( (P = .16) \). Urinary calcium excretion remained well within normal values (urine calcium to creatinine ratio \( < 1.0 \)) in all patients throughout the study. There were no cases of hypercalcemia or hypercalciuria.

Plasma creatinine, ALT, and ALP levels did not change with dosing \( (P > .16) \). Plasma phosphate and ALP decreased slightly over time only with 400 IU/d \( (P = .001) \) and 40 000 IU/d \( (P = .04) \), respectively. A small number of patients experienced marginal and asymptomatic hypophosphatemia and ALT elevation. These cases were distributed evenly among dose groups and were likely attributed to differences in blood sampling conditions (ie, baseline, fed; final, fasting).

All reported side effects were classified as grade 1 (mild), evenly balanced among treatment groups, and judged by the attending uro-oncologist to be unrelated to intervention.

**Serum PTH and PSA**

As anticipated, serum PTH decreased over time within the highest-dose group \( (P < .0001) \), but this was not statistically significant in the other dose groups \( (P > .32) \) (Figure 4). Final serum PTH was also lower in the 40 000-IU/d group than in the other groups \( (P < .0001) \). Combining the higher treatment doses \( (10 000 \text{ and } 40 000 \text{ IU/d}) \) for increased statistical power indicated a decline in serum PTH from baseline to final \( (P = .0001) \). Serum PSA decreased over time in the groups receiving 10 000 IU/d \( (P = .04) \) or 40 000 IU/d \( (P = .19) \), whereas the level remained unchanged in the 400-IU/d group \( (P = .81) \) (Figure 4). The change in serum PSA over time was not significantly different between the randomization groups \( (P = .60) \). Reductions in serum PSA (final vs baseline) were observed in 61%, 70%, and 81% of patients treated with 400, 10 000, and 40 000 IU/d of vitamin D3, respectively.
positive when their respective serum levels were extrapo-
ses indicated that prostate 25(OH)D3, calcitriol, and 24,25(OH)2D3 metabolites were partly reflective of their serum concentrations; however, the vitamin D metabolites measured in prostate were not artifacts from blood. Finally, the free fraction of calcitriol results using muscle tissue (21). Although nonspecific background or matrix effects of the method validation had demonstrated detection limits for calcitriol results using muscle tissue (21). The present study extends these preclinical findings into the clinical setting by providing some evidence of the biological effects of increased calcitriol levels in prostate. Through quantitative digital immunohistochemistry, we measured Ki67.

Discussion

Skepticism that vitamin D taken orally could produce a significant increase in prostatic calcitriol is why previous clinical trials focused on oral calcitriol and its analogs (5–10). Here we report the first direct clinical trial providing evidence that 1) oral consumption of the nutrient vitamin D can modulate tissue levels of vitamin D metabolites, 2) vitamin D metabolism occurs in vivo within prostate, and 3) higher levels of prostate tissue calcitriol may affect prostate gland biology.

We found that supplementation with 40 000 IU/d of vitamin D3 resulted in the highest accumulation of 25(OH)D3, calcitriol, and 24,25(OH)2D3 metabolites within prostate tissue. Prostate levels of these metabolites were partly reflective of their serum concentrations; however, our data also provide some evidence of local vitamin D metabolism within prostate in vivo. Regression analyses indicated that prostate 25(OH)D3, calcitriol, and 24,25(OH)2D3 concentrations remained significantly positive when their respective serum levels were extrapolated to zero. This implies a basal level of vitamin D metabolism within prostate that is independent of serum. Although nonspecific background or matrix effects of prostate tissue on the immunoassays cannot be excluded, the method validation had demonstrated <10 pmol/L (detection limit) calcitriol results using muscle tissue (21). Furthermore, the carrier protein of vitamin D metabolites in the circulation, DBP, was virtually absent in the prostate tissue, indicating that the vitamin D metabolites measured in prostate were not artifacts from blood. Finally, the free indices of vitamin D metabolites were much higher in prostate compared with serum, suggesting increased accessibility and biological activity of these metabolites at the tissue level (24).

GROUPING THE HIGHER VITAMIN D DOSES (10 000 AND 40 000 IU/d) also showed a significant decrease in serum PSA (P = .017). Serum PSA and PTH levels were not correlated (r = 0.03, P = .83).

Table 2. All Safety-Related Biochemical Measures and Side Effects in Vitamin D-Treated Study Patients

<table>
<thead>
<tr>
<th>Measure</th>
<th>400 IU/d</th>
<th>10,000 IU/d</th>
<th>40,000 IU/d</th>
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</thead>
<tbody>
<tr>
<td>Plasma calcium, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypercalcemia (&gt;2.60 mmol/L)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Urine calcium, mmol/L</td>
<td>2.62 ± 0.28b</td>
<td>1.79 ± 1.17b</td>
<td>3.09 ± 2.64b</td>
</tr>
<tr>
<td>Urine calcium (mmol/L to creatinine (mmol/L) ratio)</td>
<td>0.24 ± 0.16b</td>
<td>0.11 ± 0.07c,d</td>
<td>0.25 ± 0.17b</td>
</tr>
<tr>
<td>Week 2</td>
<td>0.06 ± 0.23b,d,e</td>
<td>0.25 ± 0.14b</td>
<td>0.25 ± 0.10b,e</td>
</tr>
<tr>
<td>Hypercalciuria (ratio &gt; 1.0), n (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plasma creatinine, μmol/L</td>
<td>88.2 ± 14.6b</td>
<td>83.7 ± 13.9b</td>
<td>82.4 ± 13.9b</td>
</tr>
<tr>
<td>Creatinine elevation (&gt;110 μmol/L), n (%)</td>
<td>0</td>
<td>0</td>
<td>82.0 ± 14.2b</td>
</tr>
<tr>
<td>Plasma phosphatase, mmol/L</td>
<td>1.03 ± 0.14b</td>
<td>0.92 ± 0.15c,d</td>
<td>1.06 ± 0.18b</td>
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<td>Hypophosphatemia (&lt;0.80 mmol/L), n (%)</td>
<td>0</td>
<td>5 (27.8)</td>
<td>0 (4.9)</td>
</tr>
<tr>
<td>Plasma ALP, U/L</td>
<td>25.0 ± 8.8b</td>
<td>24.1 ± 7.4b</td>
<td>30.7 ± 12.5b</td>
</tr>
<tr>
<td>ALT elevation (&gt;40 U/L), n (%)</td>
<td>0</td>
<td>1 (5.6)</td>
<td>0 (4.9)</td>
</tr>
<tr>
<td>Plasma ALP, U/L</td>
<td>73.4 ± 12.6b</td>
<td>69.7 ± 12.3b</td>
<td>70.4 ± 13.2b</td>
</tr>
<tr>
<td>ALP elevation (&gt;150 U/L), n (%)</td>
<td>0</td>
<td>0</td>
<td>70.2 ± 15.1b</td>
</tr>
<tr>
<td>Adverse events, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Constipation</td>
<td>0</td>
<td>1 (4.5)</td>
<td>0</td>
</tr>
<tr>
<td>Muscle weakness</td>
<td>1 (4.8)</td>
<td>1 (4.5)</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1 (4.8)</td>
<td>0</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>Itchy skin</td>
<td>2 (9.5)</td>
<td>0</td>
<td>2 (8.7)</td>
</tr>
</tbody>
</table>

Values are means ± SD, where applicable. All adverse events were grade 1 (mild) and judged to be unrelated to intervention by attending oncologist.

For each time point, values in a row without a common superscript letter differ significantly (P < 0.05, between-group).

Significantly different from baseline (P < 0.05, within-group).

* Significantly different from final (P < 0.05, within-group).

GROUPING THE HIGHER VITAMIN D DOSES (10 000 AND 40 000 IU/d) also showed a significant decrease in serum PSA (P = .017). Serum PSA and PTH levels were not correlated (r = 0.03, P = .83).

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protein, a cellular marker of proliferation that is present during all active phases of the cell cycle (G1, S, G2, and mitosis) but is absent from resting cells (G0) (27). Analysis by dose group revealed no significant differences in Ki67 measures among the doses per se, likely because Ki67 is expressed at low levels and with considerable tissue heterogeneity, thereby requiring higher statistical power than in the present study to detect significant differences. However, the level of calcitriol attained in prostate tissue correlated negatively with Ki67 intensity and histoscore. In fact, those patients that achieved higher prostate calcitriol levels (in particular, >37 pmol/kg) showed a substantially lower proportion of nuclei stained strongly positive (3+) for Ki67. Because Ki67 staining intensity, reflective of protein expression (28), has been shown to generally increase during growth phases of the cell cycle (27, 29–31), these findings suggest a possible cell-cycle effect of calcitriol on Ki67, whereby higher prostatic levels of calcitriol reduce Ki67 expression. Indeed, a reduction in Ki67 expression with higher tissue calcitriol may correspond to previous in vitro findings of calcitriol-induced growth arrest of PCa cells at G1 (25, 26). Our results are also in line with those of a recent study by Kovalenko et al (32), who demonstrated that mice fed a low-vitamin D diet, or had their VDR deleted, exhibited higher proliferation (i.e., increased Ki67 labeling index) and lower apoptosis in their prostate. Nevertheless, the relationship between tissue calcitriol and Ki67 requires further study and validation.

The observed interindividual variability in prostate calcitriol production and lack of vitamin D dose effect on Ki67 measures suggest that other physiological determinants of calcitriol may also be relevant to PCa. Calcium might be one such determinant of tissue calcitriol levels. Circulating calcitriol normally increases in response to a low dietary intake of calcium to stimulate the calcium-absorptive efficiency of the gut (33). A high dietary intake of calcium suppresses calcitriol, and this suppression may account for the epidemiological association between high calcium intakes and PCa risk (34). Indeed, low serum calcitriol levels have been related to increased risk of PCa, although this finding has not been consistent across studies (35). Furthermore, polymorphisms in genes involved in the vitamin D metabolic pathway have been associated with vitamin D status as well as risk of oral and prostate cancers (36, 37). Thus, genetic polymorphism in vitamin D metabolic enzymes could also contribute to the observed interindividual variability in prostate calcitriol production and in biological responses to vitamin D therapy. Future studies should identify and elucidate the physiological regulators of calcitriol production in tissues.

Even though both serum 25(OH)D3 and calcitriol levels had increased from baseline with the higher oral vitamin D3 doses, the classic safety indices for vitamin D excess, namely plasma and urine calcium concentrations, were unaffected. Furthermore, kidney and liver function were not impaired by vitamin D dosing, and all reported side effects were minor and unrelated to study intervention. Similarly, an excellent safety profile for high-dose vitamin D supplementation has been demonstrated in

![Figure 4](image-url). Serum PTH (A) and PSA (B) responses to vitamin D3 dosing. Values are means ± SE. Means without a common letter differ significantly between groups (P < .05). *Significant difference from baseline to final within groups (P < .05). doi: 10.1210/jc.2012-4019 jcem.endojournals.org 1505
Because calcitriol is thought to be a regulated paracrine between local tissue concentrations of calcitriol and Ki67.ings did show a significant cross-sectional relationship between local tissue concentrations of calcitriol and Ki67. Nonetheless, our findings do not serve as a basis to advocate higher doses of vitamin D for the prevention or treatment of PCa.

The results also show that treatment with high-dose vitamin D₃ (≥10 000 IU/d) should be studied further as a way to moderate serum PSA and PTH responses in PCa patients. Although the declines we observed in serum PSA were small, they occurred over a very brief course of vitamin D₃ treatment (~1 month) and are consistent with previous reports showing that oral vitamin D₃ (2000 IU/d) or calcitriol (7) can slow PSA rise in PCa patients. Furthermore, a recent clinical trial demonstrated that vitamin D₃ supplementation can slow PCa progression in patients under active surveillance without significant changes in PSA (14). The declines in serum PTH reported in our study are also of interest. An oncogenic role for PTH in the prostate is supported by preclinical studies and clinical evidence showing that elevated PTH levels correlated with reduced survival in patients with androgen-independent PCa (41). Therefore, the suppression of PTH by vitamin D may not only reduce PCa mortality but could also decrease skeletal morbidity in PCa (eg, fracture risk and bone pain), which is often exacerbated by androgen deprivation therapy (42).

This clinical trial had limitations. What was missing was a clinically meaningful outcome, but because this was a phase II RCT, the focus was on the surrogate markers studied. Despite achieving our first primary outcome of a dose-dependent increase in prostate calcitriol, this trial did not demonstrate a significant effect of vitamin D dose on Ki67 measures. This may be due to a lack of statistical power. Compared with prostatic calcitriol, the response of prostatic Ki67 expression is mechanistically distant from oral vitamin D, and therefore, the principles of metabolomics predict a weaker statistical relationship with the oral dose (11). Given the low expression and wide variability of Ki67 percent positive nuclei observed in this study (SD, 0.6%; effect size, 0.5) and other studies, a retrospective power analysis indicates that a greater number of patients (~128 patients) would be required to detect a significant dose response in Ki67. Nonetheless, our findings did show a significant cross-sectional relationship between local tissue concentrations of calcitriol and Ki67. Because calcitriol is thought to be a regulated paracrine signaling molecule, it is appropriate that the supply of vitamin D is but only one of a number of factors that can potentially determine calcitriol within the prostate. Hence, a cross-sectional analysis relating prostatic calcitriol and Ki67 should be expected to be statistically more powerful than the originally planned comparison across vitamin D dose groups. Although it is a weakness of the present analysis that the demonstration of statistical significance in Ki67 results was by cross-sectional analysis and included data from untreated patients, the analysis was appropriate, but it provides a lower level of evidence than the planned dose-response effect would have achieved.

In conclusion, our clinical trial data support the hypothesis that prostatic in vivo vitamin D metabolism can be modulated by high oral vitamin D dosing. Furthermore, the decrease in Ki67 labeling and modest declines in serum PSA and PTH with higher prostate calcitriol achieved with vitamin D doses (10 000 and 40 000 IU/d) suggest a potential clinical benefit. Lastly, the vitamin D doses (400–40 000 IU/d) were well tolerated by PCa patients without signs of toxicity. Further studies are needed to validate the potential utility of moderate-dose vitamin D₃ supplementation in PCa prevention and of higher doses of vitamin D as part of the treatment for PCa.

Acknowledgments

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