Oral Vitamin D Supplements Increase Serum 25-Hydroxyvitamin D in Postmenopausal Women and Reduce Bone Calcium Flux Measured by $^{41}$Ca Skeletal Labeling


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Abstract

Background: Ensuring adequate vitamin D status in older adults may reduce the risk of osteoporosis. Serum 25-hydroxyvitamin D [25(OH)D] concentration is the recommended biomarker of vitamin D status, but the optimal serum 25(OH)D concentration for bone health in postmenopausal women remains unclear.

Objective: The aim of this study was to apply the highly sensitive $^{41}$Ca skeletal labeling technique and the measurement of urinary $^{41}$Ca:$^{40}$Ca ratios to determine the serum 25(OH)D that has greatest benefit on bone calcium flux in postmenopausal women.

Methods: We administered a mean intravenous $^{41}$Ca dose of 870 pmol to healthy postmenopausal women ($n = 24$, age (mean ± SD): 64 ± 6.0 y) without osteoporosis. After 6 mo, at the nadir of their wintertime serum 25(OH)D status, each of the women sequentially consumed daily oral cholecalciferol supplements of 10, 25, and 50 mg/d (in this order), each for 3 mo. We assessed serum 25(OH)D concentrations monthly and urinary $^{41}$Ca:$^{40}$Ca ratios biweekly. $^{41}$Ca:$^{40}$Ca ratios were measured with low-energy accelerator mass spectrometry. With the use of pharmacokinetic analysis, we determined the effect of varying serum 25(OH)D concentrations on $^{41}$Ca transfer rates.

Results: At baseline, the mean (95% CI) serum 25(OH)D concentration was 16.2 (13.5, 18.8) μg/L. After the first, second, and third intervention periods, mean (95% CI) serum 25(OH)D increased to 29.8 (27.2, 32.4), 36.9 (34.2, 39.7), and 46.6 (41.2, 52.0) μg/L, respectively. Supplementation was associated with a downward shift in the urinary $^{41}$Ca:$^{40}$Ca ratio compared with the predicted $^{41}$Ca:$^{40}$Ca ratio without vitamin D supplementation. In the model, the most likely site of action of the increase in serum 25(OH)D was transfer from the central compartment to a fast exchanging compartment. At this transfer rate, predicted values were a concentration at half-maximal effect of 2.33 μg/L and an estimate of the maximal effect of 31.7%. After the first, second, and third intervention periods, the mean changes in this transfer rate were +18.0%, +25.7%, and +28.5%, respectively.

Conclusion: In healthy postmenopausal women, increasing serum 25(OH)D primarily affects calcium transfer from the central compartment to a fast exchanging compartment; it is possible that this represents transfer from the extracellular space to the surface of bone. A serum 25(OH)D concentration of ~40 μg/L achieves ~90% of the expected maximal effect on this transfer rate. This trial was registered at clinicaltrials.gov as NCT01053481.

Keywords: $^{41}$Ca, calcium, bone health, postmenopausal women, vitamin D, serum 25(OH)D vitamin D, skeletal labeling

Introduction

Osteoporosis-related bone fractures are an increasingly important cause of disability in older adults (1), and their treatment costs are a major burden on health care systems in many countries (2). Dietary and lifestyle interventions may be effective in preventing bone loss, but assessing their subtle impact on bone structure is challenging. Current techniques, such as measuring bone mineral density (BMD) and serum...
biomarkers of bone metabolism, are limited by their low sensitivity and/or high variability (3). Labeling the skeleton with $^{41}$Ca to monitor bone calcium flux is a promising technique to evaluate bone metabolism (3, 4). This method can detect small changes in bone calcium flux with high sensitivity and precision over a short period of time, making it an ideal tool for assessing dietary and lifestyle interventions in small groups (3, 4). We have previously demonstrated the high sensitivity of the $^{41}$Ca method to assess a bisphosphonate intervention on bone calcium flux (4).

One of the main regulators of calcium homeostasis is vitamin D, specifically, the biologically active metabolite 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) (5). Vitamin D deficiency is common in older adults and is a risk factor for osteoporosis (6, 7). The concentration of serum 25-hydroxyvitamin D [25(OH)D] is the recommended biomarker of vitamin D status (8), and oral vitamin D supplementation can increase serum 25(OH)D concentrations and reduce fracture risk (7, 9). However, the optimal concentration of serum 25(OH)D for bone health remains unclear. After lengthy debate, the US Institute of Medicine proposed an estimated average requirement and RDA for vitamin D of 10 and 20 µg/d, or 400 and 800 IU/d, respectively (10). The basis for those recommendations was the assumption that a serum 25(OH)D concentration of $\geq$20 µg/L is sufficient for nearly all the adult population (10). Other experts argue that a serum 25(OH)D concentration of $\geq$30 µg/L is required for bone health and a recommended dietary intake higher than the current estimated average requirement and RDA (11, 12).

Dietary intake of calcium is low in older adults in many countries, and this may increase both the requirement for vitamin D and the optimal serum 25(OH)D concentration needed for bone health (13). Dairy products are a staple of the Swiss diet, and calcium intake in Swiss women meets or exceeds the RDA (14), but because of Switzerland’s northern latitude, many Swiss women have low serum 25(OH)D concentrations at the end of winter. Therefore, the aim of this study was to apply the $^{41}$Ca technique in postmenopausal Swiss women with adequate dietary calcium intake but poor wintertime vitamin D status in order to investigate the effects of gradually increasing serum 25(OH)D concentrations beginning during the late winter nadir. Our hypothesis was that a serum 25(OH)D concentration of 20 µg/L would be sufficient to maximize calcium transfer rates to bone in these subjects.

**Methods**

**Subjects.** Enrolled women living in Switzerland and meeting the following inclusion criteria: 1) female; 2) age 50–80 y; 3) at least 5 y postmenopausal; 4) no chronic diseases; 5) nonsmoking; 6) BMI between 18 and 30 kg/m$^2$; 7) not taking calcium or vitamin D supplements or any medication affecting bone metabolism; 8) not following a vegetarian or other restricted diet; and 9) T-scores for BMD by DXA greater than $-2.5$ at both the femoral neck and lumbar spine. Subjects were recruited from the program for elderly citizens at the University of Zurich and by local newspaper advertisements. Interested volunteers were invited to an information session at the Human Nutrition Laboratory, ETH Zurich. Blood draws and medical examinations of all study participants were performed at the Center on Aging and Mobility at the University Hospital Zurich. We estimated that a sample size of 20 would be sufficient to detect a 10% difference in the urinary ratio of $^{41}$Ca:$^{42}$Ca; this was based on an SD of 10% in the $^{41}$Ca:$^{42}$Ca ratio observed in our previous study (4), with 80% power and a level of significance of 0.05. Anticipating a dropout rate of 20–25%, we aimed to recruit 25 subjects.

**Study design.** Total study duration was 16 mo (August 2009–December 2010) and the study was divided into 2 main periods: a labeling period of $\sim$6 mo and an intervention period of 9 mo. We labeled the subjects with $^{41}$Ca in August/September 2009, and began the intervention in March/April 2010, at the nadir of yearly vitamin D status in Swiss women. We chose a 6 mo labeling period based on our previous study, in which urinary $^{41}$Ca was shown to originate mainly from bone 6 mo after administration of the $^{41}$Ca isotope (3). During the intervention period, all subjects consumed daily oral cholecalciferol supplements (DSM Nutritional Products). These were given at increasing doses of 10, 25, and 50 µg/d, each for 3 mo, to progressively increase serum 25(OH)D concentrations over the 9 mo intervention period, in combination with increasing sunlight exposure. During the intervention period, we instructed the subjects to apply sunscreen before any prolonged sunlight exposure. Compliance with the supplementation regimen was monitored monthly by tablet count.

The primary outcome measure of this study was the urinary $^{41}$Ca:$^{42}$Ca ratio throughout the 9 mo intervention determined in urine samples collected biweekly (as described later), which were used in the pharmacokinetic modeling. Secondary outcome measures were several markers of bone metabolism [parathyroid hormone (PTH), deoxypyridinoline, and bone alkaline phosphatase (BALP)] measured at the beginning of the intervention, as well as after 3, 6, and 9 mo.

**Screening examination.** At enrollment, we performed a brief medical history and clinical examination (including blood pressure, heart rate, and electrocardiogram) and obtained venous blood samples to measure standard hematologic parameters, serum 25(OH)D concentration, luteinizing hormone (LH), follicle-stimulating hormone (FSH), PTH, and plasma concentrations of calcium and creatinine. BMD at the spine (lumbar spine L1–L4) and total hip by DXA (Discovery QDR 4500, Hologic) was measured at the Triemli Hospital Zurich. For quality control, phantom scans with the use of a spine phantom were conducted before each measurement (4–5 times/wk). Moreover, the hospital was participating in the quality control system QUALIM Swiss-QC, in which all control measurements were centrally checked and archived.

**Isotopic labeling.** We prepared the $^{41}$Ca labeling solution as described previously (3). For this study, we prepared $^{41}$Ca doses (0.87 nmol $^{41}$Ca per 5 mL vial) from the original dosing solution, IRMM-3703, by dilution with physiologic saline, which was purified by sterile filtration and bottled and tested for pH and pyrogenicity at the Cantonal Pharmacy of the University Hospital Zurich. After an overnight fast, participants presented at University Hospital Zurich, where we administered a dose of (mean ± SE) 870 ± 11 pmol of $^{41}$Ca by intravenous injection into a forearm vein, and then provided the subjects with a breakfast low in calcium and without caffeine. We monitored urinary $^{41}$Ca:$^{42}$Ca isotope ratios in 24 h urine collections from this point onward. We instructed the subjects to maintain their customary dietary habits and level of physical activity during the study.

**Urine sampling.** During the 6 mo labeling period between August/September 2009 and March/April 2010, each subject collected 24 h

1 This study was supported by DSM Nutritional Products Ltd. and the ETH Zurich, Switzerland.
3Supplemental Tables 1 and 2, Supplemental Figures 1–3, Supplemental Methods, and Supplemental Results are available from the “Online Supporting Material” link in the online posting of this article and from the same link in the online table of contents at http://jn.nutrition.org.
4To whom correspondence should be addressed. E-mail: michael.zimmermann@ethz.ch.
5Abbreviations used: BALP, bone alkaline phosphatase; BMD, bone mineral density; $C_{\text{in}}$, concentration at half-maximal effect; $E_{\text{max}}$, maximal effect; FSH, follicle-stimulating hormone; $k_{\text{dil}}$, distribution rate from the central compartment to the fast-exchanging compartment; LH, luteinizing hormone; OF, objective function; PTH, parathyroid hormone; SENECA, Survey Europe on Nutrition in the Elderly: a Concerted Action; 25(OH)D, 25-hydroxyvitamin D.
urine samples on days 7, 15, 28, 43, 71, 127, 165, 186, and 200 after administration of the isotope. During the intervention period, from March/April 2010 to November/December 2010, each subject collected 24 h urine samples biweekly on days 215, 227, 240, 255, 271, 283, 299, 311, 330, 341, 355, 368, 380, 396, 409, 423, 438, and 451. During vacations or other unavoidable absences, subjects shifted collection days (+±3 d) in consultation with the study coordinator. The subjects collected the 24 h urine samples by discarding the first morning urine and adding the morning urine of the consecutive day into preweighed polyethylene bottles containing 10 mL of 1 mol/L hydrochloric acid. Samples were either kept at 5°C if analyzed within 24 h or stored as 500 mL subsamples in acid-washed polyethylene containers at −20°C until analysis. The subjects collected fasting second morning spot urine samples every 4 wk during the intervention period in order to assess urinary deoxypyridinoline. These spot urine samples were stored at 4°C protected from light and analyzed on the day of collection.

Estimation of dietary calcium intake and vitamin D synthesis from sunlight exposure. We estimated calcium and vitamin D intake with an FFQ used in previous studies (4), expanded with selected food items containing 0.5–300 μg vitamin D per 100 g. Questions targeted the frequency and quantity of consumption over the previous 3 mo. We used EBIpro software (created by Dr. Jürgen Erhardt, University Hohenheim, Stuttgart, www.ebipro.de) to analyze the questionnaires. We estimated the amount of outdoor activities and sun exposure by using a combined questionnaire based on the Freiburger Questionnaire on Physical Activity (13) and the UV exposure questionnaire of the Institute of Health and Biomedical Innovation of Australia (16). From this, with the use of the validated FastRT web UV simulation tool of the Norwegian Institute for Air Research (17), the mean daily biosynthesis of vitamin D during the previous month, with an emphasis on the previous week, was calculated, considering exposed skin surface and sunlight application, skin type, and the UV index at the subject’s location (18). The results of this simulation tool are comparable to those generated by more complex models (19). The subjects completed the questionnaire before the first 3 mo supplementation period (in March; 524 ± 225 IU vitamin D per day) and after the first supplementation period (in June; 1318 ± 804 IU vitamin D per day), and our estimates with the use of this method showed a high correlation with measured serum 25(OH)D concentrations (Pearson’s r = 0.84, P < 0.01) when the 2 time points were pooled (n = 50) and adjusted for supplemental vitamin D intake.

Blood sampling. At screening, we collected a venous blood sample (20 mL) from a forearm vein for measurement of the screening parameters. At 4 wk intervals during the intervention period, we collected blood samples (5 mL) from a forearm vein to assess serum 25(OH)D concentrations. At 4 wk intervals during the intervention period, we collected blood samples (5 mL) from a forearm vein to assess serum 25(OH)D concentrations. Every 4 wk during the intervention period, we collected fasting second morning spot urine samples in order to assess urinary deoxypyridinoline. These spot urine samples were stored at 4°C protected from light and analyzed on the day of collection.

Urine sample preparation for calcium isotope ratio analysis. We carried out all laboratory procedures according to the guidelines of trace element analysis to minimize the risk of sample contamination. Only chemicals of analytical grade quality and ultra-pure water were used, and all acids were additionally purified by sub-boiling distillation. Samples were processed under constant separation blank monitoring. We separated calcium species from urine by oxalate precipitation, following established procedures as described previously (3, 4) with some modifications: the ion-exchange chromatography step was replaced by precipitation with the use of hydrofluoric acid (20, 21). Pellets were dried and homogeneously mixed with elemental silver powder (99.9%, spherical, 635 mesh, Alfa Aesar) for isotope ratio analysis.

Measurement of 41Ca:40Ca ratios. Because of the long half-life of 41Ca (112 = 104,000 y), decay counting would require 40Ca doses several orders of magnitude higher than used in this study. The unparalleled sensitivity of accelerator MS allowed us to directly determine the 41Ca:40Ca isotopic composition in samples with ratios ranging from 10−8 to 10−11. Samples were analyzed on the TANDY low-energy accelerator MS system at the Laboratory of Ion Beam Physics, ETH Zurich, which combines high-efficiency and a simplified measurement setup, as described previously (22, 23). Samples were analyzed relative to standard samples of known 41Ca:40Ca isotopic composition (on the order of 10−11 to 10−9) that were calibrated against IRMM-3701/5 (24). Samples showing uncertainties exceeding 10% in the first measurement were prepared a second time and measurements were repeated. Only measurements with uncertainties <10% were considered valid; the average uncertainty of those measurements was 3.4%. Chemical duplicate analysis performed on a subsample (20 samples) showed an average relative difference of 4.7% between replicates.

Other biochemical measurements. We measured serum LH, FSH, and PTH by electrochemiluminescence immunooassay with the use of the E170 autoanalyzer (Roche Diagnostics) (25). The CVs of the methods used were 2% for LH, 2.4% for FSH, and 3.6% for PTH. Serum 25(OH)D was determined by radio immunooassay (DiaSorin; 8.4% CV) with the use of controls provided by the manufacturer (26). We measured urinary deoxypyridinoline in spot urine samples by chemiluminescence ELISA with the use of the Immulite Autoanalyzer (Siemens Diagnostic Healthcare; 7.1% CV). Urinary creatinine required for normalization of deoxypyridinoline was determined by the Jaffé method (rate-blanked with compensation; 2.5% CV) (27, 28). We measured plasma albumin and calcium as safety markers by colorimetric assays from Roche Diagnostics with the use of the Modular autoanalyzer (1.7% and 0.9% CV, respectively) (29, 30). We performed all analyses described in this section at the Institute of Clinical Chemistry of the University Hospital Zurich. For all methods, external quality controls were carried out at regular intervals by INSTAND.

Pharmacokinetic data analysis. We analyzed the data with the use of the software package NONMEM version VI 2.0, running with NM-TRAN. We applied subroutines ADVAN 1–6 and the first-order conditional estimation method with INTERACTION. We determined the appropriate structural model by fitting the pharmacokinetic data to 1-, 2-, and 3-compartment models with zero-order or first-order absorption, as described previously (3).

The basic principle behind the use of 41Ca to monitor changes in bone calcium metabolism is shown in Figure 1. After administration of the isotope, urinary excretion of the tracer is followed during the labeling period. This labeling curve serves as an index from which perturbations from interventions can be assessed once steady-state kinetics for 41Ca are established, i.e., when all of the 41Ca that is recovered...
in the urine was previously incorporated into the bone. In a first step, we used serum 25(OH)D concentration data, vitamin D intake through diet and supplementation, and vitamin D synthesis from UV exposure to establish a model describing serum 25(OH)D pharmacokinetics.

In a second step, we combined 41Ca data from the labeling period of our subjects with data from our previous study in which a comparable group of older Swiss women (who were not supplemented with increasing doses of vitamin D) was followed for an extended period of time after labeling (3). In the previous study, the compartmental modeling approach was able to predict the run of the labeling curve and average offsets were on the order of 10% during a theoretical intervention period when compared with measured values. However, the variation in the 41Ca:40Ca isotope ratios in the urine of the subjects in the current study during the labeling period was unexpectedly higher than in the previous study, even though we labeled with an intravenous dose. This may have been due to small changes in calcium metabolism in the individual subjects that have occurred over the labeling period because of changes in diet, lifestyle, or other factors. Because of this high variation, we were uncertain whether we could resolve a shift in the urinary 41Ca:40Ca isotope ratio from the vitamin D supplementation during the intervention period with only the predicted run of the labeling curve from the present study. Therefore, in order to improve our ability to resolve this shift, we decided to introduce as a control group the 22 subjects from the previous study.

We felt justified in combining the data, because the previous cohort was comparable to the present cohort, in that it also consisted of apparently healthy women from northern Switzerland (mean age 66.7 ± 4.9 y, n = 22) who were at least 5 y postmenopausal and had no known systemic disease or were taking any medication or dietary supplements that could potentially influence calcium homeostasis or bone status, including calcium, vitamin D, bisphosphonates, estrogens and diuretics. The previous cohort had a mean BMI of 26.1 ± 3.3 kg/m², mean calcium intake assessed by FFQ was 803 ± 257 mg calcium per day, and mean BMD was −1.48 ± 0.72 (T-score in femur neck) at the beginning of the study (3). Compared with using only the data from the labeling period of the current study, this combined dataset allowed us to develop a model that more accurately predicted 41Ca concentrations for our study cohort during the intervention period in the absence of vitamin D supplementation.

In a third step, we incorporated the serum 25(OH)D model into the 41Ca model, fixed the pharmacokinetic parameters for 41Ca, and then allowed the individual predicted increases in serum 25(OH)D concentration from supplement administration to influence one or several 41Ca transfer rates during the intervention period. We modeled the pharmacodynamic effect of the serum 25(OH)D concentration on calcium pharmacokinetics by using a maximal effect (E_max) model, in which the concentration at half-maximal effect (C_50) corresponds to the increase in serum 25(OH)D concentrations leading to the half-maximal effect. A more detailed description of the method is available in the Supplemental Methods.

Statistical analysis. We did additional statistical analysis with the use of IBM SPSS Statistics 20. All data were checked for normal distribution and the skewed data were log-transformed before analysis. We reported descriptive statistics as arithmetic means (95% CIs) for normally distributed data and as geometric means (95% CIs) for non-normally distributed data that were normally distributed after log-transformation. We examined the changes in the different markers of bone metabolism and serum 25(OH)D concentrations over time by using repeated measures ANOVA with post hoc Bonferroni tests. Significance was set at P < 0.05.

This study was carried out in accordance with the ethical standards presented in the Helsinki Declaration. The Ethics Committee of the Canton of Zurich approved the study and written informed consent was obtained from all participants. The study was registered at clinicaltrials.gov as NCT01053481.

Results

Baseline subject characteristics at enrollment before labeling with 41Ca. The age of the subjects was 64 ± 6.0 y, with a range of 52–75 y, and all were at least 5 y postmenopause. BMD measured at the spine and hip were 0.951 ± 0.127 g/cm² and 0.833 ± 0.089 g/cm², respectively, and

### Table 1

Changes in bone-related clinical chemistry variables in 24 postmenopausal women who, in addition to seasonally increasing vitamin D biosynthesis, received oral supplementation with cholecalciferol

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Month 3</th>
<th>Month 6</th>
<th>Month 9</th>
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<tbody>
<tr>
<td>Serum 25(OH)D, µg/L</td>
<td>28.8 (25.1, 32.5)</td>
<td>16.2 (13.5, 18.8)</td>
<td>29.8 (27.2, 32.4)</td>
<td>36.9 (34.2, 39.7)</td>
</tr>
<tr>
<td>Plasma parathyroid hormone, ng/L</td>
<td>52.1 (44.0, 58.4)</td>
<td>44.6 (40.5, 48.7)</td>
<td>44.2 (40.8, 47.7)</td>
<td>41.6 (37.0, 46.2)</td>
</tr>
<tr>
<td>Urinary deoxypyridinoline, nmol/mmol creatinine</td>
<td>8.5 (7.8, 9.3)</td>
<td>8.1 (7.2, 9.0)</td>
<td>8.4 (7.5, 9.3)</td>
<td>8.7 (7.6, 9.8)</td>
</tr>
</tbody>
</table>

1 Values are arithmetic means (95% CIs) or geometric means [95% CIs]. Supplementation dosages were 10 µg/d during months 1–3 (spring), 25 µg/d during months 4–6 (summer), and 50 µg/d during months 7–9 (fall). Labeled means in a row without a common letter differ, P < 0.05 (repeated-measures ANOVA with post hoc Bonferroni test, P < 0.05). BALP, bone alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D.

### Table 2

Measured and predicted serum 25(OH)D concentrations before and after oral vitamin D supplementation along with seasonally increasing biosynthesis in 24 postmenopausal women

<table>
<thead>
<tr>
<th></th>
<th>Serum 25(OH)D at screening</th>
<th>Serum 25(OH)D at baseline, (nadir, early spring)</th>
<th>Serum 25(OH)D after oral vitamin D supplementation²</th>
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<tr>
<td></td>
<td>Predicted, µg/L</td>
<td>Predicted, µg/L</td>
<td>After 3 mo</td>
</tr>
<tr>
<td></td>
<td>28.2 (25.3, 31.1)</td>
<td>20.9 (19.1, 22.6)</td>
<td>26.9 (25.1, 28.7)</td>
</tr>
</tbody>
</table>

1 Values are arithmetic means (95% CIs). The screening was carried out in August 2009, whereas baseline measurements were done in March 2010. 25(OH)D, 25-hydroxyvitamin D.
2 10, 20, and 50 µg/d, respectively, for 3 mo each.
3 Labeled means in this row without a common letter differ, P < 0.05 (repeated measures ANOVA with post hoc Bonferroni test, P < 0.05).
4 Predicted values were calculated with the use of the selected 1-compartment vitamin D model.
T-scores were $-0.791 \pm 1.13$ (total spine, L1-L4) and $-0.791 \pm 0.528$ (total hip). In August/September 2009, late Swiss summer, 12.5% of women had a serum 25(OH)D concentration $<20 \mu g/L$. From the FFQ, we estimated calcium intake to be 1.35 (1.15, 1.55) g/d because of high consumption of dairy products. Of the 25 subjects enrolled, 24 completed the study. We excluded one subject because she consumed multivitamin supplements during the intervention.

**Changes in serum or plasma concentrations of serum 25(OH)D, PTH, and other biomarkers of bone metabolism.** Compliance with supplementation during the study was high, and ranged between 99% and 100% for the 3 intervention periods (10 $\mu$g/d: 99%, range 92–100%, 25 $\mu$g/d: 99%, range 92–100% and 50 $\mu$g/d: 100%, range 96–100%). At the beginning of the intervention in March 2010 (end of winter), 17 of 24 women had a serum 25(OH)D concentration $<20 \mu g/L$. Serum 25(OH)D concentrations significantly increased during the intervention period ($p < 0.001$) (Table 1); after 3 mo of supplemental 10 $\mu$g/d of cholecalciferol, along with seasonally increasing UV exposure, none of the women had a serum 25(OH)D concentration $<20 \mu g/L$. Plasma PTH concentrations decreased over the entire intervention period ($p = 0.02$), and serum BALP concentrations significantly decreased only between baseline and 6 mo ($p = 0.02$). There were no significant changes in urinary deoxyxypyrindinoline concentrations during the study (Table 1).

**Compartmental analysis of vitamin D pharmacokinetics.** Detailed results of the model-finding process are described in the Supplemental Results. Because they did not improve the data fit, more complex models were discarded and a one-compartment serum 25(OH)D model was used to incorporate into the calcium model (Supplemental Table 1 and Supplemental Figure 1). Predicted serum 25(OH)D concentrations with the use of the model next to the measured values for time points before and after each intervention period are shown in Table 2.

**Model for calcium distribution and excretion.** We combined data from our previous $^{41}$Ca study (3) with the data of the present study to refit our previous model (3) and predict calcium distribution, excretion, and plasma $^{41}$Ca concentrations after the labeling period in the absence of vitamin D supplementation. The new 3-compartment model, schematically represented in Figure 2, described both the old (3) and new (from the present study) $^{41}$Ca data sets well. More details can be found in the Supplemental Results and in Supplemental Table 2.

**Use of the 3-compartment calcium model to predict site of action and effect of increased serum 25(OH)D concentrations through supplementation.** With the use of NONMEM population pharmacokinetic analysis, we tested different models for the effect of varying serum 25(OH)D concentrations on $^{41}$Ca (the effect of serum 25(OH)D concentration was incorporated into 1 or 2 $^{41}$Ca transfer rates) and to determine the most likely site of action of the increased serum 25(OH)D concentration. Objective function (OF) values and parameter estimates of the $E_{\text{max}}$ and $C_{50}$ for the different models are compared in Table 3. We obtained the best predictions and the lowest OF when serum 25(OH)D concentration was allowed to influence distribution rate from the central compartment to the fast-exchanging compartment ($k_{12}$). These results suggest that the effect of an increase in serum 25(OH)D concentration is an increase in the transfer rate of calcium from the first to the second compartment; the predicted markers are shown in Table 3. A likelihood profile for the $C_{50}$ resulted in a 95% CI of 1.08–4.80 $\mu g/L$.

**Principal Figure 2** Schematic representation of the 3 compartment model to describe $^{41}$Ca kinetics. $C_i$ represents the central compartment to which the $^{41}$Ca dose was administered. The tracer is then transferred to a fast-exchanging compartment ($C_2$) and thereafter to a slow-exchanging compartment ($C_3$) that are probably located in the bone. $k_{10}$, elimination rate; $k_{12}$, distribution rate from the central compartment to the fast-exchanging compartment; $k_{21}$, distribution rate from the fast-exchanging compartment to the slow-exchanging compartment; $k_{32}$, distribution rate from the slow-exchanging compartment to the fast-exchanging compartment.

For 3 representative subjects, the actual measurements (squares) and individual predictions for $^{41}$Ca concentrations in plasma (calculated from $^{41}$Ca:$^{40}$Ca isotope ratios in urine) in pmol/L are compared in Figure 3. Fine lines represent individual predictions without taking vitamin D into account, whereas thick lines show individual predictions with the use of a model in which vitamin D from supplementation is allowed to have an effect on $k_{12}$ (Table 3, rows 1 and 3). The remaining individual values are shown in Supplemental Figure 2. Taken together, the mean shift in $^{41}$Ca:$^{40}$Ca isotope ratio between the predicted (no supplementation) and the measured values for all subjects was 21%, 22%, and 10% after 3, 6, and 9 mo of supplementation, respectively.

The effect of increases in serum 25(OH)D concentrations from supplementation in the optimal $E_{\text{max}}$ model (from Table 3) and the effect on the calcium transfer rate $k_{12}$ are shown in Figure 4. With a $C_{50}$ of 2.33 $\mu g/L$ and an $E_{\text{max}}$ of 31.7%, mean $\pm$ SE increases in serum 25(OH)D concentrations after 3 mo of supplementation with 10 $\mu$g/d (during spring), 25 $\mu$g/d (during summer), and 50 $\mu$g/d cholecalciferol (during fall) were 3.0 $\pm$ 0.2, 8.35 $\pm$ 0.3, and 12.1 $\pm$ 0.4 $\mu g/L$, respectively.

**Table 3** Selection of a site of action for increased serum 25(OH)D through supplementation in the calcium 3-compartment model by population pharmacokinetics with the use of a serum 25(OH)D 1-compartment model as described$^1$

<table>
<thead>
<tr>
<th>Effect of vitamin D supplementation</th>
<th>$\triangle$OF</th>
<th>$C_{50}$, $\mu g/L$</th>
<th>$E_{\text{max}}$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effect</td>
<td>0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>On $k_{10}$</td>
<td>—3.52</td>
<td>0.633</td>
<td>10.3</td>
</tr>
<tr>
<td>On $k_{12}$</td>
<td>—5.48</td>
<td>2.33</td>
<td>31.7</td>
</tr>
<tr>
<td>On $k_{21}$</td>
<td>—5.39</td>
<td>1.22</td>
<td>&lt;9.72</td>
</tr>
<tr>
<td>On $k_{23}$</td>
<td>—5.28</td>
<td>0.272</td>
<td>13.9</td>
</tr>
<tr>
<td>On $k_{32}$</td>
<td>—5.07</td>
<td>0.0524</td>
<td>&lt;8.35</td>
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$^1$ $C_{50}$ concentration at half-maximal effect; $E_{\text{max}}$ maximal effect; $k_{10}$, elimination rate; $k_{12}$, distribution rate from the central compartment to the fast-exchanging compartment; $k_{21}$, distribution rate from the fast-exchanging compartment to the slow-exchanging compartment; $k_{32}$, distribution rate from the slow-exchanging compartment to the fast-exchanging compartment; OF, objective function; 25(OH)D, 25-hydroxyvitamin D.

$^2$ Elimination rate.

$^3$ Distribution rate. $k_{12} = \theta_i \times e^{11} \times [1 + E_{\text{max}} \times C_{50}/C_{50} + C_{50}]$, where $C_{50}$ is the predicted concentration from the supplement in the vitamin D compartment, $\theta_i$ is the estimated fixed-effects parameter, and $e^{11}$ is the estimated random effect parameter by subject ($i = 1–24$).
from the blood or extracellular space to the fast-exchanging compartment. These data suggest that the main cause of reduced cutaneous biosynthesis may be the lower end of the range of serum 25(OH)D concentration was achieved by supplementing the subjects with cholecalciferol daily for 3 mo during summer. However, our data also suggest that to achieve 90% of the expected E\textsubscript{max} on bone calcium flux, serum 25(OH)D concentrations would have to be increased to ~47 \mu g/L. Our findings are generally consistent with the study by Heaney et al. (35), which concluded that the lower end of the range of serum 25(OH)D concentrations that reflect normal vitamin D status is ~35 \mu g/L.

The optimal daily intake of vitamin D and serum 25(OH)D concentrations for maximum benefits on bone health has been widely debated, and our findings provide important new evidence on this relation. Our findings suggest that, in healthy postmenopausal women with adequate calcium intake and sufficient physical activity, a serum 25(OH)D concentration of ~37 \mu g/L achieves an effect on bone calcium flux that is >80% of the expected E\textsubscript{max}. In our study, this serum 25(OH)D concentration was achieved by supplementing the subjects with 25 \mu g cholecalciferol daily for 3 mo during summer. However, the changes in the 41Ca:40Ca ratio during the intervention indicate a positive effect from vitamin D supplementation on bone calcium flux, and this was consistent with the change in the blood-based biomarkers of bone turnover during the 9 mo study: PTH and BALP significantly decreased early in the intervention, consistent with reduced bone turnover (36–38). However, the decrease in PTH and BALP in the final 3 mo of the intervention was not significant (P = 0.32 for PTH and P = 1.00 for BALP, respectively, between month 6 and month 9; compare Table 1), and based on our calcium model, there was only an

Discussion

Our supplementation regimen, in combination with increasing sunlight exposure, was effective in gradually increasing serum 25(OH)D concentrations from insufficiency to clear sufficiency in the participating women. At the beginning of the intervention, at the end of winter, these women were insufficient in vitamin D, with a mean (95% CI) serum 25(OH)D concentration of 16.2 (13.5, 18.8) \mu g/L (31). However, compared with other older populations in Europe, the insufficiency was only mild, with this value being at the upper end of the range for serum 25(OH)D concentrations of 8.4–18.4 \mu g/L found in 80–85-y-old women in 12 European countries in the Survey Europe on Nutrition in the Elderly: a Concerted Action (SENECA) study (6). After 3 mo of receiving 10 \mu g/d of cholecalciferol during spring, none of the women had a serum 25(OH)D concentration <20 \mu g/L; after 3 mo of receiving 50 \mu g/d during fall, none of the women had a serum 25(OH)D concentration <30 \mu g/L. The 30 \mu g/L threshold has been recommended as the desirable serum 25(OH)D concentration for fall and fracture reduction by several expert organizations (8, 31, 32).

We used nonlinear mixed-effects modeling to estimate the impact of improving vitamin D status on measured variables. After discarding more complex models, a 1-compartment serum 25(OH)D model was chosen to predict serum 25(OH)D status over the course of the intervention. Comparing the measured and predicted serum 25(OH)D concentrations in the subjects at baseline and after each of the 3 interventions showed a good overall fit (Table 2). The largest difference, with higher predicted than measured concentrations, was seen at baseline, i.e., in early spring. This suggests the model underestimated the decrease in serum 25(OH)D concentrations during the winter months because of reduced cutaneous biosynthesis.

We used population pharmacokinetic analysis to determine the most likely site of action of serum 25(OH)D concentrations within the 3-compartment model for calcium distribution and excretion. Based on the lowest OF and the highest maximal effect achieved, the most likely site of action for vitamin D was found to be in the transfer from compartment 1 to compartment 2, i.e., the central compartment to the fast-exchanging–calcium compartment. Although compartments can, but do not have to, correspond to anatomic spaces, these data suggest that the main effect of serum 25(OH)D concentration was on transfer of calcium from the blood or extracellular space to the fast-exchanging compartment, likely the bone surface. It has been long established that a functional membrane consisting of osteoblasts and osteocytes with a labile calcium pool separates plasma from mineralized bone, likely the abovementioned fast exchanging compartment (33, 34). This is consistent with one of the recognized sites of action of 1,25-dihydroxyvitamin D to maintain plasma calcium concentrations, i.e., to stimulate bone uptake and release of calcium (7, 10).

The mean dose-response of serum 25(OH)D concentrations in postmenopausal women (n = 24) taking increasing doses of oral vitamin D supplementation, and the corresponding effect on k\textsubscript{12} in the 3-compartment model for calcium. With the reported C\textsubscript{50} of 2.33 \mu g/L and E\textsubscript{max} of 31.7%, the mean ± SE increase in serum 25(OH)D concentration of 3.0 ± 0.2 \mu g/L after 3 mo of 10 \mu g/d of vitamin D caused an average change in k\textsubscript{12} of 18.0%. Mean ± SE increases in 25(OH)D concentration after 3 mo supplementation with 25 and 50 \mu g/d were 9.9 ± 0.5 and 20.9 ± 1.0 \mu g/L, and caused average effects of 25.7% and 28.5% on k\textsubscript{12}, respectively. C\textsubscript{50}, concentration at half-maximal effect; E\textsubscript{max}, maximal effect; k\textsubscript{12}, distribution rate from the central compartment to the fast-exchanging compartment. 25(OH)D, 25-hydroxyvitamin D.
additional 2.8% decrease in mean calcium flux during this later period. The minimal changes in these variables during the final 3 mo of the intervention support our conclusion that a serum 25(OH)D concentration of 35–40 µg/L is adequate for most postmenopausal women with high calcium intake, and higher serum 25(OH)D concentrations are likely to provide minimal additional benefits on bone calcium flux. Since we carried out this study, new sensitive biomarkers of bone turnover have become routinely available [e.g., C-terminal telopeptide in serum or N-terminal telopeptide in urine (39)] and had we used these, we may have been able to detect further changes in bone turnover over the final 3 mo of the intervention.

This study has several strengths. The extremely high precision of the 41Ca method can detect even small variations in bone calcium flux that may not be captured by measures of BMD or by blood biomarkers, and studies can be done with small sample sizes. Once subjects are uniformly labeled, this method can discern subtle effects on bone calcium flux from lifestyle and diet modifications in interventions lasting for only several months. The collection of urine to measure the calcium ratios is noninvasive and feasible even in frail older adults, although there are challenges in obtaining complete 24 h urine collections. Only one subject dropped out during the study, and compliance with supplementation was high.

There are several limitations to our study. A characteristic of our subjects that may limit generalizability of the results is their high baseline dietary calcium intake. The SENECA study found low calcium intake, between 0.3 and 0.6 g/d, in 71–76 y old women in 10 countries in Europe (40). In contrast, our Swiss subjects consuming a diet high in dairy products had a calcium intake of ~1.3 g/d. Because a high calcium intake may reduce the requirement for vitamin D (13), our subjects may have had a lower vitamin D requirement to optimize calcium bone flux compared with subjects with lower dietary calcium intake. A second limitation to our study is the imprecision of tools available for estimating sunlight exposure and resulting biosynthesis of vitamin D. Thus, we are only able to make inferences about the effects of the supplements on vitamin D status. Also, although we did explain to our subjects that they should not vary their usual levels of physical activity during the study, we did not measure possible changes in lean mass in the subjects during the study or monitor possible changes in their physical activity; these may have been important covariates.

Another limitation of the study was that, instead of using only the predicted run of the labeling curve from the present study as a comparator, we introduced as a control group the 22 subjects from our previous study, who were not statistically different in age, body weight, femoral neck BMD, and dietary calcium intake at baseline (3). This was necessary because there was unexpectedly high variation in the plasma 41Ca concentrations in our subjects during the labeling period, and thus it was uncertain whether we could resolve a shift in the urinary 41Ca:40Ca isotope ratio during the intervention period with the use of only the predicted run of the labeling curve. We chose to give the 41Ca label intravenously, rather than orally as previously (3), because the intravenous dose eliminates potential between-subject variation in oral absorption of the label, and we expected this to result in less variation in plasma 41Ca concentrations during the study, but this was not the case; there was more variation in the present study (Supplemental Figure 3).

In the previous study, the compartmental modeling approach was able to predict that the run of the labeling curve and average offsets were on the order of ±10% during a theoretical intervention period compared with measured values (3). Finally, we examined only the effect of varying serum 25(OH)D concentrations on bone calcium flux, but it is possible that other important physiologic functions of vitamin D (41–43) may be optimized by vitamin D intake and serum 25(OH)D concentrations that differ from those in this study.

In conclusion, using 41Ca to label the skeleton, we demonstrated a positive effect from increasing serum 25(OH)D concentrations on bone calcium flux in postmenopausal women. Our findings suggest that a serum 25(OH)D concentration of ~37 µg/L achieves >80% of the expected maximal effect, whereas increasing serum 25(OH)D concentration to ~47 µg/L has minimal additional benefit. Therefore, a target serum 25(OH)D concentration of ~40 µg/L could be recommended to minimize bone calcium flux and potentially improve bone health in postmenopausal women. Further studies with the use of 41Ca labeling should examine whether this serum 25(OH)D concentration is also effective in postmenopausal women with lower calcium intake, differing diets, and varying levels of physical activity.

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