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Effects of vitamin D supplementation on bone turnover markers and other bone-related substances in subjects with vitamin D deficiency

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ABSTRACT

In observational studies, vitamin D deficiency is a risk factor for low bone density and future fractures, whereas a causal relation has been difficult to show in randomized controlled trials (RCTs). Similarly, vitamin D deficiency has been associated with increased bone turnover, but RCTs with vitamin D have not shown conclusive effects. This could be due to inclusion of vitamin D sufficient subjects and low vitamin D doses. In the present study 399 subjects with mean baseline serum 25-hydroxyvitamin D (25(OH)D) 34.0 nmol/L completed a four months intervention with vitamin D_3 20,000 IU per week versus placebo. Mean serum 25(OH)D increased to 89.0 nmol/L in the vitamin D group and decreased slightly in the placebo group. A small, but significant, decrease in the bone formation marker procollagen of type 1 amino-terminal propeptide (P1NP) was seen in the vitamin D group as compared to the placebo group (mean delta P1NP -1.2 pg/mL and 1.5 ng/mL, respectively, $P < 0.01$). No significant effects were seen on serum carboxyl-terminal telopeptide of type 1 collagen (CTX-1), Dickkopf-1, sclerostin, tumor necrosis factor-alpha, osteoprotegerin, receptor activator of nuclear factor κB ligand, or leptin. Subgroup analyses on subjects with low baseline serum 25(OH)D did not yield additional, significant results. In subjects with high baseline serum parathyroid hormone (PTH) > 6.5 pmol/L and post-intervention decrease in PTH, the decrease in P1NP was more pronounced, they also exhibited significantly reduced serum CTX-1 and increased serum sclerostin. In conclusion, supplementation with vitamin D appears to suppress bone turnover, possibly mediated by PTH reduction. Our findings need to be confirmed in even larger cohorts with vitamin D insufficient subjects.

1. Introduction

Vitamin D facilitates intestinal calcium absorption and thereby provide calcium necessary for bone mineralization [1]. Prolonged and severe vitamin D deficiency leads to rickets in children and osteomalacia in adults, and it is important with vitamin D supplements to prevent this in those at risk [2]. Consequently, recommendations regarding vitamin D intake and what can be considered a sufficient serum level of 25-hydroxyvitamin D (25(OH)D), which is a marker of vitamin D status, are based upon bone health [3]. The vitamin D receptor (VDR), as well as enzymes necessary for hydroxylation of vitamin D to its active form 1,25-dihydroxyvitamin D, are present in tissues throughout the body, and therefore vitamin D may possibly also have extra-skeletal effects [4]. In observational studies, vitamin D deficiency is associated with a number of diseases and an increased mortality risk, but positive effects of vitamin D have been hard to demonstrate in

Abbreviations: BMI, Body mass index; BMD, Bone mineral density; BTMs, Bone turnover markers; CTX-1, Carboxyl-terminal telopeptide of type 1 collagen; DKK1, Dickkopf-1; DXA, Dual-energy x-ray absorptiometry; FGF23, Fibroblast growth factor 23; OPG, Osteoprotegerin; PTH, Parathyroid hormone; RCTs, Randomized controlled trials; RANKL, Receptor activator of nuclear factor κB ligand; P1NP, Total procollagen of type 1 amino-terminal propeptide; TNF-α, Tumor necrosis factor-alpha; VDR, Vitamin D receptor; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D

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randomized controlled trials (RCTs) [5].

There is a positive cross-sectional association between bone mineral density (BMD) and serum levels of 25(OH)D [6,7]. However, most interventional studies have failed to show effects of vitamin D supplementation on BMD and fracture prevention [8]. This may, as has been the case for vitamin D RCTs in general, be attributed to adequate vitamin D status in the study subjects [9]. Thus, in a study of institutionalized patients with apparently severe vitamin D deficiency, significant benefits were demonstrated [10].

The skeleton undergoes a constant remodeling with a delicate balance between bone resorption and bone formation, which is orchestrated by the osteocytes. The activity of this process can be monitored by serum levels of bone turnover markers (BTMs). Carboxyl-terminal telopeptide of type 1 collagen (CTX-1) and procollagen of type 1 amino-terminal propeptide (P1NP), which are markers of bone resorption and bone formation, respectively, are useful for evaluating both anti-resorptive and anabolic osteoporosis treatment [11]. Whether these markers are suitable in assessing the skeletal effects of vitamin D supplementation is not settled, and so far, studies on the effect of vitamin D supplementation on BTMs have been diverging [12–14].

In the present study, we included subjects with serum 25(OH)D levels below 42 nmol/L at screening, gave a weekly vitamin D_3 dose of 20,000 IU, and evaluated effects on CTX-1 and P1NP, and also on several factors involved in bone metabolism.

2. Methods

2.1. Subjects and study design

The main endpoint of the study was change in cardiovascular risk factors, and the design of the study and these results have previously been reported in detail [15]. The study was performed in Tromsø, northern Norway (69 degrees north), and the subjects were recruited from the population-based Tromsø study [16], which was performed for the seventh time in 2015–2016. In the Tromsø study all citizens ≥40 years (n = 32,591) were invited, 21,083 attended, and serum 25(OH)D was successfully measured in 20,922. Among these, 1489 subjects with serum values < 42 nmol/L and with age < 80 years were invited to participate. Six hundred and thirty-nine responded and were screened by phone regarding medical history, use of vitamin D supplements, solarium on a regular basis, planned holiday(s) in tropical areas during the study period, and for women < 50 years, use of acceptable contraception. A total of 455 subjects passed this initial telephone screening and met for the first visit at the Clinical Research Unit at the University Hospital of North Norway where an informed consent form was signed, clinical examinations performed, and fasting blood samples drawn. These examinations did not reveal any contraindication for participation in 422 subjects who then attended the next visit within 2–5 days. At this second visit, the study drugs (vitamin D_3 (cholecalciferol) capsules (20,000 IU (500 μg)) Dekristol, Mibe, Jena, Germany) or identical looking placebo capsules containing arachis oil (Ayanda GmbH & CoKG, Falkenhagen, Germany) were dispensed. Five capsules were given as a loading dose followed by one capsule each week. Measurement of BMD was performed at this visit in the last 336 subjects included.

The randomization was stratified according to gender, vitamin D status in the Tromsø study (above/below 25 nmol/L), smoking status and BMI above/below 27 kg/m^2. All nurses, doctors, other study personnel and study participants were blinded throughout the study. The subjects were asked not to take any vitamin D supplements (including cod liver oil) or use solarium during the intervention period.

Four months later the third and fourth visits were performed, identical to the first and the second. Compliance was calculated as the ratio between capsules used (capsules supplied minus capsules returned) and number of weeks between second and fourth visit.

2.2. Measurements

Serum calcium, parathyroid hormone (PTH) and 25(OH)D were analyzed as previously described [17]. The serum 25(OH)D assay was an in-house liquid chromatography–tandem mass spectrometry method that detects both 25(OH)D_3 and 25(OH)D_2 and the sum of these are presented as 25(OH)D in the results. CTX-1 and P1NP were measured by electrochemiluminescence immunoassays with a Cobas e601 kit (Roche Diagnostics, NJ, USA), at the Hormone Laboratory, Oslo University Hospital, Norway. Dickkopf-1 (DKK1), leptin, osteoprotegerin (OPG), sclerostin, and tumor necrosis factor-alpha (TNF-α) were analyzed using multianalyte profiling Milliplex MAP assay, and receptor activator of nuclear factor κB ligand (RANKL) by a single analyte assay (Millipore Corporation, Billerica, MA, USA). According to the manufacturer the serum RANKL detection limit was 5.0 pg/mL, but the minimum actual readout from the instrument was 0.1 pg/mL. However, around 80% of the samples had values below detection range, and therefore the median values for RANKL in the cohort was 0.0 pg/mL.

BMD was measured by dual-energy x-ray absorptiometry (DXA) (GE Lunar Prodigy, Lunar Corporation, Madison, WI, USA) at the hip and lumbar spine. For hip the total hip (mean of left and right, or one side if not both could be measured) was used in the analyses. For the lumbar spine L1 (which had valid measurement in almost every subject) was used as for the other vertebrae several measurements were non-valid or of less quality.

2.3. Statistical analyses

Normal distribution was evaluated with skewness, kurtosis and visual inspection of histograms and found normal for all parameters except CTX-1, leptin, OPG and sclerostin that attained normal distribution after log transformation and were used as such when being dependent variables in regression analyses. RANKL was not normally distributed and could not be log-transformed and therefore analyzed with non-parametric statistics. All delta values (value at end of study minus value at baseline) except delta RANKL were normally distributed. Comparisons between groups at baseline were performed with the Student's *t*-test or the Mann-Whitney *U* test. Comparisons between the intervention and placebo groups at the end of the study were performed with a general linear model with value at end of study as the dependent variable, gender, and randomization status as fixed factors, and age, BMI, and baseline value as covariates [18]. Interaction between gender and randomization status was tested in the same model and not found significant. Correlations were evaluated with Spearman's rho. The distributions of variables across categories of serum 25(OH)D) were evaluated with linear regression or the Kruskal-Wallis test.

$P < 0.05$ (two-tailed) was considered statistically significant. Data are presented as mean ± SD or as median (5th, 95th percentile). All statistical analyses were performed using IBM SPSS version 25 software.

2.4. Power calculation

For the main end-point of the study, cardiovascular risk factors (systolic blood pressure, serum LDL-cholesterol, insulin resistance (HOMA)), a total number of 450 subjects were needed to attain a power of 0.8 and $P < 0.05$ [15]. A specific power calculation of effects on the BTMs was not performed.

2.5. Ethics

The study was approved by the Regional Committee for Medical Research Ethics (REK NORD 2013/1464) and by the Norwegian Medicines Agency (2013–003514-40). The study is registered at ClinicalTrials.gov NCT02750293. All subjects gave their written informed consent.

Table 1
Characteristics of the subjects at baseline in relation to gender and serum 25(OH)D level.

	All subjects (n = 406)	Males (n = 212)	Females (n = 194)	Serum 25(OH)D < 25 nmol/L (n = 92)	Serum 25(OH)D 25–49 nmol/L (n = 266)	Serum 25(OH)D > 49 nmol/L (n = 48)
Males/females	212/194			51/41	131/135	30/18
Current smokers/non-smokers	86/320	47/165	39/155	21/71	58/208	7/41
Age (years)	51.9 ± 8.7	52.0 ± 9.0	51.6 ± 8.3	49.6 ± 8.0	52.5 ± 8.8	52.6 ± 8.6
BMI (kg/m^2)	27.8 ± 4.9	28.1 ± 4.6	27.4 ± 5.3	27.9 ± 4.5	27.9 ± 5.1	26.8 ± 4.6
Serum calcium (mmol/L)	2.27 ± 0.07	2.26 ± 0.007	2,26 ± 0.08	2.27 ± 0.07	2.27 ± 0.08	2.28 ± 0.06
Serum PTH (pmol/L)	6.7 ± 2.0	6.6 ± 1.9	6.9 ± 2.2	7.3 ± 2.4	6.6 ± 1.9	6.2 ± 1.6*
Serum 25(OH)D (nmol/L)	34.0 ± 12.9	33.9 ± 13.2	34.1 ± 12.5	19.4 ± 3.9	34.4 ± 6.2	59.7 ± 9.4*
Serum PINP (pg/mL)	44.8 ± 15.1	44.5 ± 13.7	45.2 ± 16.6	46.6 ± 15.2	44.7 ± 15.3	42.5 ± 13.6
Serum CTX (pg/mL)	0.34 (0.18, 0.62)	0.36 (0.19, 0.67)	0.35 (0.16, 0.59)	0.37 (0.17, 0.73)	0.35 (0.17, 0.65)	0.33 (0.21, 0.59)
Serum OPG (pg/mL)	306 (192, 479)	306 (188, 498)	306 (208, 460)	292 (1981, 485)	308 (194, 479)	310 (221, 477)
Serum RANKL (pg/mL)	0.0 (0.0, 46.8)	0.0 (0.0, 55.8)	0.0 (0.0, 24.9)	0.0 (0.0, 38.7)	0.0 (0.0, 52.3)	0.0 (0.0, 33.7)
Serum TNF-α (pg/mL)	2.40 ± 0.81	2.58 ± 0.82***	2.20 ± 0.76	2.41 ± 0.81	2.36 ± 0.79	2.61 ± 0.9
Serum sclerostin (pg/mL)	1806 (1030, 3140)	2044 (1126, 3286)***	1642 (998, 2764)	1872 (1034, 3005)	1791 (1052, 3169)	1957 (980, 2936)
Serum DKK1 (pg/mL)	1456 ± 396	1461 ± 402	1451 ± 392	1458 ± 338	1461 ± 421	1429 ± 364
Serum Leptin (pg/mL)	11,081 (1725, 53,375)	7488 (1212, 30,290)***	20,094 (3051, 68,867)	11,949 (1802, 51,042)	11,029 (1764, 54,815)	10,343 (752, 44,940)
BMD total hip (g/cm^2)****	0.993 ± 0.133	1.032 ± 0.118***	0.950 ± 0.136	1.008 ± 0.131	0.987 ± 0.136	0.995 ± 0.113
BMD L1 (g/cm^2)****	1.067 ± 0.156	1.084 ± 0.159**	1.048 ± 0152	1.082 ± 0.138	1.059 ± 0.166	1.081 ± 0.138

Data are shown as mean ± SD or median (5th, 95th percentile).
* P < 0.001, linear trend across groups.
** P < 0.05.
*** P < 0.001 vs females (Student's *t*-test or Mann-Whitney *U* test).
**** 336 subjects (181 males, 155 females); 83, 218, and 35 subjects in the serum < 25 nmol/L, 25–49 nmol/L, and > 49 nmol/L groups, respectively.

3. Results

3.1. Baseline

Altogether, 406 subjects not using anti-resorptive medication had successful measurements of the BTMs. Their characteristics in relation to gender and serum 25(OH)D are shown in Table 1. Males had significantly lower leptin and higher TNF-α, sclerostin and BMD than females. There was a significant negative association between serum 25(OH)D and PTH (Tables 1 and 2, Fig. 1). Except for a correlation with OPG, no significant relations between 25(OH)D and the BTMs or bone-related substances were seen (Table 2). There were several correlations between the BTMs and the bone-related substances, age, BMI and BMD. There was no significant correlation between 25(OH)D and BMD (Table 2).

3.2. Intervention study

In total, 399 subjects completed the intervention with successful BTM measurements, 202 in the vitamin D and 197 in the placebo group. These two groups did not differ significantly at baseline (Table 3). At the end of the intervention, serum 25(OH)D had increased from a mean level of 32.8 nmol/L to 88.9 nmol/L in the vitamin D group, whereas a decline from 35.1 nmol/L to 30.6 nmol/L occurred in the placebo group. Compared with the placebo group, there was a significant decrease in serum PTH, an increase in serum calcium, and a small but significant (P < 0.01) decrease in serum P1NP. Changes in CTX-1 or the bone-related substances did not differ significantly between the two groups, and there were no effects on BMD (Table 3). No serious study-drug related side effects were recorded. The compliance rate was between 84 and 100% in 14% of the subjects, and the rest had a compliance rate of 100%. Two subjects developed hypercalcemia (both had serum calcium = 2.57 mmol/L); one female had primary hyperparathyroidism, and one male had normal serum calcium upon retesting.

Table 2
Spearman's rho coefficient between age, BMI, serum calcium, PTH and 25(OH)D and bone turnover markers at baseline in 406 subjects.

	Age	BMI	Serum calcium	Serum PTH	Serum 25(OH)D
Age (years)		−0.083	0.071	0.132**	0.128**
BMI (kg/m^2)	−0.083		−0.005	0.192**	−0.028
Serum calcium (mmol/L)	0.071	−0.005		−0.120*	0.061
Serum PTH (pmol/L)	0.132**	0.192**	−0.120*		−0.171**
Serum 25(OH)D (nmol/L)	0.128**	−0.028	0.061	−0.171**	
Serum PINP (pg/mL)	0.045	−0.109*	0.098*	0.058	−0.047
Serum CTX (pg/mL)	0.128**	−0.205**	0.117*	0.096	−0.040
Serum OPG (pg/mL)	0.429**	−0.041	0.052	−0.040	0.098*
Serum RANKL (pg/mL)	−0.102*	0.135**	−0.009	−0.043	−0.100*
Serum TNF-α (pg/mL)	0.055	0.176**	0.070	0.003	0.011
Serum sclerostin (pg/mL)	0.322**	0.097	−0.001	−0.061	−0.013
Serum DKK1 (pg/mL)	−0.112*	0.200**	0.076	−0.005	−0.037
Serum Leptin (pg/mL)	−0.064	0.642**	−0.096	0.237**	−0.001
BMD total hip (g/cm^2)***	−0.271**	0.439**	−0.012	−0.047	- 0.001
BMD L1 (g/cm^2)***	−0.273**	0.253**	−0.120*	−0.029	0.013

* P < 0.050.
** P < 0.01 Spearman's rho.
*** n = 336.

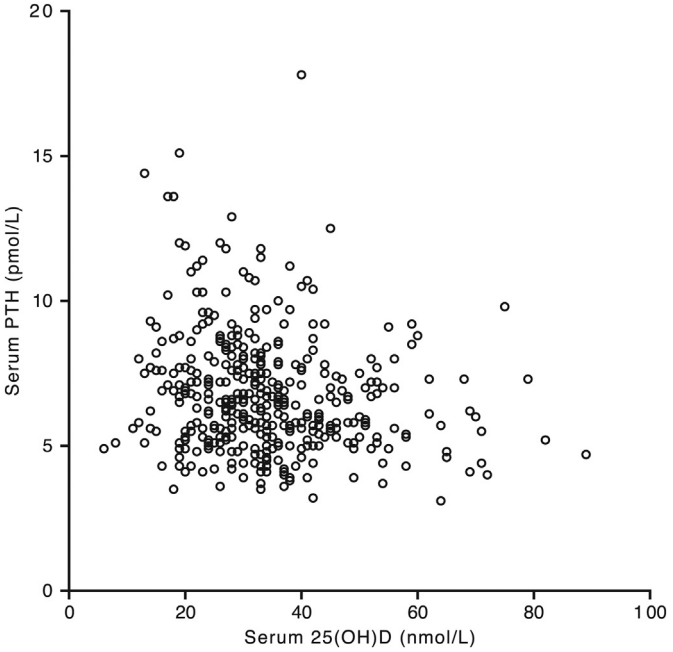

Fig. 1. Serum PTH in relation to serum 25(OH)D in the 406 subjects at baseline.

Nine subjects in the vitamin D group had serum 25(OH)D > 125 nmol/L at the end of the study. None of them developed hypercalcemia, and their mean serum calcium was 2.31 mmol/L.

When the two groups were pooled at the end of the study, there was a significant increase in serum calcium and decrease in serum PTH across categories of serum 25(OH)D (in steps of 25 nmol/L),whereas no significant changes occurred the BTMs or bone-related substances (Supplementary Table 1). In the same cohort, there was in particular a negative correlation between delta serum 25(OH)D and delta PTH, a negative correlation between delta serum 25(OH)D and delta P1NP, and positive correlations between delta PTH and delta P1NP and delta CTX-1. There was a positive association between delta BMI and delta leptin (Supplementary Table 2).

3.3. Subgroup analyses

To examine potential effects in subgroups based on baseline serum 25(OH)D and serum 25(OH)D response to treatment, subjects with baseline 25(OH)D < 40 nmol/L and final serum 25(OH)D in the vitamin D group > 70 nmol/L (n = 126) and < 40 nmol/L in the placebo group (n = 121) were analyzed separately. However, similar results were found as when all subjects were included, with a mean decrease in P1NP of 1.47 pg/ml in the vitamin D group, versus an increase in the placebo group of 1.06 pg/ml (P < 0.05, linear regression with gender, age, BMI and baseline value as covariates). Changing the above cut-off did not reveal other positive effects, even when lowering the baseline cut-off to < 25 nmol/L, (n = 90) (data not shown). When analyzing separately subjects with baseline serum 25(OH)D > 40 nmol/L, there was still a significant difference in delta P1NP between the groups with a decrease in the vitamin D group of 0.68 pg/ml, and an increase in the placebo group of 3.14 pg/ml (P < 0.01).

To evaluate if vitamin D effects could be related to serum PTH levels and responses, subjects with serum PTH > 6.5 pmol/L at baseline were selected from the intervention group if serum PTH had declined ≥1 pmol/L (n = 42) and from the placebo group if no decrease in serum PTH had occurred (n = 59). In addition to a significant decrease of P1NP in the vitamin D group, a significant decline in serum CTX-1 and an increase in serum sclerostin were seen as compared to the placebo group (Table 4).

4. Discussion

In this RCT, including 399 subjects with low 25(OH)D levels, those given 20,000 IU vitamin D weekly for four months reached a mean serum 25(OH)D level of 89 nmol/L, reflected in a decline in serum PTH and increase in serum calcium. In spite of a substantial rise in 25(OH)D, there was only a small, but significant reduction in serum P1NP.

No additional effects on BTMs or bone-related substances were seen in 90 subjects who had baseline 25(OH)D < 25 nmol/L and with the anticipated response to the intervention. However, in another subgroup with baseline serum PTH > 6.5 pmol/L and with the expected PTH response to supplementation, the reduction in P1NP was more pronounced, and a significant reduction in CTX-1 occurred. Moreover, this subgroup displayed a significant increase in serum sclerostin. This

Table 3
Baseline and end of study values in the 399 subjects who completed the four months intervention.

	Vitamin D group (n = 202)		Placebo group (n = 197)	
	Baseline	End of study	Baseline	End of study
Males/females	108/94		104/93	
Current smokers/non-smokers	44/158		44/153	
Age (years)	51.5 ± 8.6		52.5 ± 8.8	
BMI (kg/m^2)	27.8 ± 5.0	28.0 ± 5.0	27.7 ± 4.7	27.9 ± 4.8
Serum calcium (mmol/L)	2.27 ± 0.07	2.29 ± 0.08*	2.27 ± 0.07	2.27 ± 0.97
Serum PTH (pmol/L)	6.6 ± 2.2	5.9 ± 2.0**	6.7 ± 1.8	7.3 ± 2.1
Serum 25(OH)D (nmol/L)	32.8 ± 11.1	88.9 ± 19.4**	35.1 ± 13.4	30.6 ± 9.6
Serum PINP (pg/mL)	45.0 ± 15.4	43.8 ± 13.6*	44.1 ± 14.8	45.6 ± 15.2
Serum CTX (pg/mL)	0.34 (0.18, 0.61)	0.35 (0.18, 0.67)	0.34 (0.17, 0.62)	0.35 (0.16, 0.60)
Serum OPG (pg/mL)	307 (200, 477)	321 (211, 497)	308 (188, 498)	318 (194, 524)
Serum RANKL (pg/mL)	0 (0, 42.1)	0 (0, 72.9)	0 (0, 46.8)	0 (0, 80.0)
Serum TNF-α (pg/mL)	2.37 ± 0.79	2.63 ± 0.77	2.39 ± 0.82	2.67 ± 0.86
Serum sclerostin (pg/mL)	1815 (1063, 3054)	2159 (1321, 3553)	1818 (998, 3181)	2147 (1217, 3509)
Serum DKK1 (pg/mL)	1476 ± 380	1605 ± 420	1442 ± 411	1573 ± 454
Serum Leptin (pg/mL)	11,243 (1693, 53,375)	12,561 (1575, 59,002)	10,877 (1764, 50,797)	11,983 (2239, 52,890)
BMD total hip (g/cm^2)***	1.001 ± 0.135	1.001 ± 0.136	0.985 ± 0.136	0.987 ± 0.137
BMD L1 (g/cm^2)***	1.074 ± 0.159	1.077 ± 0.161	1.056 ± 0.153	1.056 ± 0.155

Data are shown as mean ± SD or median (5th, 95th percentile).

* P < 0.01.

** P < 0.001, versus placebo group, linear regression with age, gender, BMI and baseline value as covariates.

*** N = 336, 166 in the vitamin D group and 170 in the placebo group.

Table 4
Baseline and end of study values in subjects who at baseline had serum PTH > 6.5 pmol/L and for those in the vitamin D group with a decrease in serum PTH > 1 pmol/l and for those in the placebo group that did not have a decrease in serum PTH by the end of the study.

	Vitamin D group (n = 42)		Placebo group (n = 59)	
	Baseline	End of study	Baseline	End of study
Males/females	25/17		29/30	
Current smokers/non-smokers	5/37		9/50	
Age (years)	52.0 ± 7.9		55.1 ± 8.6	
BMI (kg/m^2)	28.8 ± 5.3	28.8 ± 5.3	28.2 ± 4.5	28.3 ± 4.5
Serum calcium (mmol/L)	2.27 ± 0.07	2.30 ± 0.07	2.28 ± 0.08	2.26 ± 0.08
Serum PTH (pmol/L)	9.1 ± 2.6*	6.6 ± 2.1***	8.0 ± 1.3	9.3 ± 1.7
Serum 25(OH)D (nmol/L)	30.4 ± 10.3*	87.4 ± 20.8***	35.1 ± 12.7	28.5 ± 8.3
Serum PINP (pg/mL)	48.6 ± 19.7	44.0 ± 13.4***	46.8 ± 15.6	49.1 ± 16.9
Serum CTX (pg/mL)	0.38 (0.21, 0.66)	0.35 (0.18, 0.69)**	0.37 (0.19, 0.74)	0.41 (0.19, 0.69)
Serum OPG (pg/mL)	287 (205, 431)	291 (190, 490)	305 (215, 512)	305 (206, 540)
Serum RANKL (pg/mL)	0.0 (0.0, 38.5)	0.0 (0.0, 83.9)	0.0 (0.0, 28.2)	4.7 (0.0, 80.0)
Serum TNF-α (pg/mL)	2.17 ± 0.77	2.57 ± 0.87	2.40 ± 0.89	2.79 ± 0.93
Serum sclerostin (pg/mL)	1734 (1059, 2995)	2200 (1398, 3482)**	1876 (998, 3180)	2087 (1398, 3515)
Serum DKK1 (pg/mL)	1405 ± 343	1620 ± 417	1419 ± 381	1556 ± 494
Serum Leptin (pg/mL)	12,875 (3303, 53,330)	12,915 (4231, 60,209)	14,871 (1851, 58,217)	14,820 (2697, 56,397)
BMD total hip (g/cm^2)****	1.031 ± 0.157	1.031 ± 0.156	0.950 ± 0.134	0.952 ± 0.132
BMD L1 (g/cm^2)****	1.078 ± 0.161	1.080 ± 0.162	1.025 ± 0.151	1.019 ± 0.149

Data are shown as mean ± SD or median (5th, 95th percentile).
* P < 0.05; versus placebo group, Student's *t*-test.
** P < 0.05 versus placebo group, linear regression with delta value as dependent variable and age, gender, BMI and baseline value as covariates.
*** P < 0.001, versus placebo group, linear regression with end of study as dependent variable and age, gender, BMI and baseline value as covariates.
**** n = 37 in the vitamin D group, 54 in the placebo group.

indicates that in subjects with "functional" vitamin D deficiency, vitamin D supplementation leads to reduced bone turnover through suppression of PTH.

This inhibition of bone turnover is consistent with observational data where vitamin D deficiency appears to be associated with increased bone turnover [7]. We measured CTX-1 and P1NP for assessment of bone turnover as recommended by the International Osteoporosis Foundation, and these markers have been included in the majority of studies on vitamin D and BTMs [19]. Most studies have found no effect of vitamin D supplementation [12–14], but slight reductions in CTX-1 [20] and P1NP [21] have also been reported. However, none of these studies included an adequate number of subjects with vitamin D deficiency and gave sufficient vitamin D doses, as was done in our study.

As anticipated, the effect on P1NP and CTX-1 in the current study was small compared with conventional antiresorptive drugs. We observed a decline in serum P1NP of 6.0% after four months of vitamin D supplementation (compared to the placebo group), as opposed to a reduction in CTX-1 and P1NP of 40% after treatment with oral bisphosphonates [22]. Nevertheless, vitamin D administration was shown to induce an additional reduction in CTX-1 of 25% in postmenopausal, osteoporotic women with serum 25(OH)D < 50 nmol/L treated with alendronate [23]. In line with this, in subjects on antiresorptive treatment a significantly higher BMD and lower fracture rate have been observed in those with 25(OH)D above 50 nmol/L compared to those below [24]. Thus, the response to antiresorptive treatment of postmenopausal osteoporosis seems to depend on vitamin D status.

Meta-analyses on pooled data from randomized trials on vitamin D supplementation and skeletal effects, show only a small, positive effect at the femoral neck and a modest fracture reduction [25]. The role of vitamin D in preserving bone health has therefore been questioned. However, the majority of the populations included had normal baseline levels of 25(OH)D, and thus the results may not apply to individuals with low 25(OH)D levels. In contrast, Chapuy reported a 2.7% increase at proximal femur BMD and 32 and 43% lower risk, respectively, of non-vertebral and hip fractures in elderly with serum 25(OH)D levels < 50 nmol/L given calcium and vitamin D supplements [10]. In spite of these substantial skeletal effects, no change occurred in the bone formation marker osteocalcin. Similarly, Dawson-Hughes observed a 2.6% rise in spine BMD and a decline by 50% in vertebral fractures after 3 years with vitamin D and calcium supplements [26]. They observed a decline in PTH and osteocalcin, whereas bone resorption assessed by 24-h urinary N-telopeptide/creatinine ratio did not differ between groups. These studies suggest that BTMs not necessarily reflect the skeletal effects of vitamin D supplementation, and may not be the appropriate tool to assess thresholds for vitamin D sufficiency. An alternative interpretation could be that the definition of vitamin D deficiency might need to be re-appraised.

The size of the dosage and whether supplements are given daily or intermittently as in the current study, could also affect the BTMs response. Previous studies have suggested that large intermittent doses may have adverse effects with a transient increase in fracture and fall risk in spite of adequate vitamin D levels [27,28]. Rossini et al. observed an acute rise in CTX-1 and cross-linked N-telopeptide of type I collagen after a single oral dose of 600,000 IU vitamin D, whereas bone-specific alkaline phosphatase was unaffected [29]. A loading dose of 300,000 IU vitamin D has also been shown to induce supraphysiological levels of 1,25(OH)$_2$D and a rise in the osteocyte-products sclerostin and fibroblast growth factor 23 (FGF23) [30,31], resulting both in inhibition of bone formation and mineralization [32–34], and stimulation of bone resorption. Thus, these substances are proposed to mediate the adverse skeletal effects observed after a high dose with vitamin D. A rise in 1,25(OH)$_2$D and FGF23 has also been observed at a daily dosage of 2800 IU vitamin D [35], which corresponds to the weekly dose of 20,000 IU in our study.

Dawson Hughes et al. also reported a rise in sclerostin after three years with 800 IU vitamin D in combination with calcium [26]. Presumably, the mechanism for the rise in sclerostin is different when vitamin D is given in a high loading dose compared to a daily dose of 800 IU. In the latter case, the elevation could be attributed to a higher bone mass and a larger pool of osteocytes, as indicated by some studies [36]. Thus, in a recent study, vitamin D deficiency was associated with decreased number of viable osteocytes in human iliac crest and vitamin D was shown to promote the transition of osteoblasts to osteocytes and to play a role in regulation of osteocyte number [37]. Whether the higher sclerostin level observed in the subgroup analysis in our study is due to a vitamin D-induced increase of osteocytes and a higher bone mass remains to be seen.

P1NP and CTX-1 are indicators of bone turnover, but do not provide information on which pathways an intervention works. To elucidate this, we measured several substances involved in regulation of bone metabolism. Apart from sclerostin, no differences were seen in RANKL, OPG, TNF-α or leptin levels, which at least for TNF- α and leptin are in accordance with previous publications [38,39].

Effects of vitamin D on bone depend on the calcium status. If in calcium deficit, VDR stimulation leads to bone resorption to maintain the serum calcium level, whereas positive effects on bone metabolism prevail when the calcium supply is adequate [1]. Consequently, vitamin D supplementation is not effective or may even exaggerate bone loss if not combined with sufficient calcium intake. Unfortunately, data on calcium intake were not available in the present study. However, a previous study reported a daily calcium intake of about 500 mg in our population [40], which is insufficient according to the recommendations of The Institute of Medicine [3]. Since low serum calcium is a potent stimulus of PTH secretion, this may partly counteract the suppressive effect of vitamin D supplementation on PTH and BTMs.

Hypomagnesemia could also attenuate the effect of vitamin D supplements on BTMs by blunting the PTH response [41,42]. One could therefore speculate that subjects with increased serum PTH level who responded with a decline in PTH and BTMs after vitamin D supplementation, had vitamin D deficiency without concomitant calcium or magnesium insufficiency. In contrast, the more modest effect on BTMs in the subgroup with low vitamin D status alone could be attributed to insufficient calcium or magnesium intake.

Our study has several limitations. As mentioned above, we did not have information on calcium intake, which may be low in our population [40]. Our results may therefore not apply to populations with a higher intake of calcium. Even though all subjects at screening were insufficient according to the standard criterion (serum 25(OH)D < 50 nmol/L) [3], none displayed extreme vitamin D deficiency (serum 25(OH)D < 10 nmol/L), and most were not functionally vitamin D deficient. The subgroup analysis based on serum PTH levels and responses was not pre-specified, and even though the results were biologically plausible, should be viewed with caution. Furthermore, the effect on BTMs probably reaches its maximum when optimal level of vitamin D level is acquired, thereafter tapering off. Given the potential negative effects of high-dose vitamin D [30–34], the BTMs should therefore ideally have been measured directly after intake of the supplements and at several time points thereafter to capture the fluctuations in bone metabolism. There was no correlation between serum 25(OH)D and BMD, probably due to including mainly subjects with low serum 25(OH)D levels. And finally, the observation period was too short for evaluating effects on BMD.

On the other hand, our study has considerable strengths as we included the largest group of subjects with vitamin D deficiency so far studied regarding BTMs, and achieved serum 25(OH)D levels above 70 nmol/L in nearly all subjects. We found the expected relations between the BTMs and age, BMI, and gender, and the vitamin D supplementation caused significant increase in serum calcium and reduction in PTH, which give the study considerable internal strength.

4.1. Concluding remarks

The main lesson from our study is that vitamin D supplementation has minor effects on BTMs in subjects without extreme vitamin D deficiency. Thus, BTMs are probably not useful in monitoring skeletal response to vitamin D supplements. Intervention studies including subjects with higher baseline serum 25(OH)D than in our study, should be discouraged.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.04.002.

Conflict of interest

None.

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