Osteoporos Int (2015) 26:2597–2605
DOI 10.1007/s00198-015-3184-0

ORIGINAL ARTICLE

Factors affecting changes in the serum levels of 25-hydroxyvitamin D: a 3-year follow-up of the ROAD study

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Received: 2 September 2014 / Accepted: 19 May 2015 / Published online: 19 June 2015

Abstract
Summary In this 3-year population-based cohort study, among 1346 subjects, the mean annual change in the serum 25-hydroxyvitamin D levels was 7.6 %/year, which tended to increase during the 3-year period. Multivariate regression analysis indicated that the L2-4 bone mineral density and total daily energy intake were significant independent associated factors.
Introduction The aim of this study was to clarify the change rate of the serum levels of 25-hydroxyvitamin D (25D) and the associated factors in a general Japanese population during a 3-year period.
Methods The baseline survey of Research on Osteoarthritis/osteoporosis Against Disability study (ROAD), a large-scale population-based cohort study, was performed between 2005 and 2007, and a follow-up survey was repeated 3 years later. Among 1690 participants at baseline, the change rate of the serum 25D levels were assessed in 1346 individuals (79.6 %; 458 men and 888 women) who completed measurements of 25D at both the baseline and follow-up examinations. The change rate was calculated, and the factors associated with the changes in the 25D levels were determined using multivariate regression analysis after adjustment for age, gender, body mass index, participated month, and regional differences at baseline.
Results The mean (standard deviation) change rate of the 25D levels in all subjects was 7.6 (13.3) %/year (men, 8.2 [12.4] %/year; women, 7.3 [13.7] %/year). Multivariate regression analysis indicated that higher bone mineral density at lumbar spine L2-4 (p=0.05) and total daily energy intake (p=0.04) were significantly associated with the change rate of the 25D levels.
Conclusions The serum levels of 25D tended to increase over the 3-year period, and higher lumbar bone mineral density and daily energy intake were found to be associated with increases in the 25D levels over time.

Keywords 25-Hydroxyvitamin D · Bone mineral density · Nutrition · Population-based cohort study · Risk factors

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Introduction

Vitamin D (VD) is known to influence bone quality and is important for maintaining bone density [1, 2]. A number of studies have reported an association between inadequate VD intake and osteoporosis [3–6], and VD deficiency has moreover been reported as detrimental to numerous other conditions, including falls, fractures, type 2 diabetes, cardiovascular disease, certain cancers, autoimmune diseases, infections, and mortality [6]. Accordingly, preventing VD inadequacy is

considered highly beneficial for the prevention of morbidity; however, there are currently few reports clarifying how the VD levels change over time and what factors may affect the changes in the VD levels.

We have previously performed a population-based cohort survey using the Research on Osteoarthritis/Osteoporosis Against Disability (ROAD) study cohorts, in which we first clarified the characteristics of subjects with VD deficiency and insufficiency, as defined by serum 25-hydroxyvitamin D (25D) levels <10 ng/mL and ≥10<30 ng/mL, respectively, using the baseline data of ROAD [7]. In this previous study, we found that VD deficiency was significantly associated with gender, residing region, serum intact parathyroid hormone (iPTH) levels, urinary β-isomerized C-terminal cross-linking telopeptide of type I collagen (β-CTX) levels, smoking habits, regular walking, calcium intake, and hyperparathyroidism. Next, using both the baseline and 3-year follow-up information of the ROAD study, we reported that the serum 25D levels at baseline could predict the occurrence of osteoporosis at the femoral neck within 3 years, but not the occurrence of knee osteoarthritis or lumbar spondylosis [8]. However, we have not yet evaluated the change rate of 25D and factors associated with the changes in the 25D levels over time.

With this in mind, in the present study, the changes from the baseline 25D levels in subjects with measurement taken both at the baseline and 3-year follow-up examinations of the ROAD study were evaluated to clarify the change rate of 25D. In addition, the factors affecting the changes in the 25D levels were assessed.

Methods

Study participants

The present study was performed using the ROAD study cohort established in 2005. The ROAD study is a national, prospective study of osteoarthritis that consists of population-based cohorts from several communities in Japan. Details of the cohort profile have been reported elsewhere [9, 10]. In brief, between 2005 and 2007, a baseline database was created that included clinical and genetic information of 3040 individuals (1061 men, 1979 women; mean age [standard deviation, SD], 70.3 [11.0] years). The subjects were recruited from resident registration listings in three communities with different characteristics: 1350 subjects from an urban region in Itabashi, Tokyo; 864 subjects from a mountainous region in Hidakagawa, Wakayama; and 826 subjects from a coastal region in Taiji, Wakayama.

In the present study, 1690 subjects (596 men, 1094 women; mean age, 65.2 [12.0] years; men, 66.3 [11.7] years; women, 64.7 [12.1] years) from the mountainous and coastal regions who participated in the ROAD study were analyzed. Data from those in the urban regions were not included, as baseline 25D measurements were not performed in that cohort. On the other hand, bone mineral density (BMD) measurements and blood and urinary examinations were performed in the participants from the mountainous and coastal regions.

All participants provided written informed consent, and the study was conducted with the approval of the ethics committees of the University of Tokyo (No. 1264 and No. 1326) and the University of Wakayama Medical University (No. 373).

Baseline assessments

i. Interviewer-administered questionnaire

The participants completed an interviewer-administered questionnaire that consisted of questions related to lifestyle, including occupation, smoking habits, alcohol consumption, family history, medical history, physical activity, reproductive history, and health-related quality of life.

ii. Dietary assessment

A brief diet history questionnaire (BDHQ) was administered to assess the diet of the participants. The BDHQ was modified from a comprehensive, 16-page validated self-administered diet history questionnaire [11]. The BDHQ is a four-page structured questionnaire that includes questions about the frequency of consumption of 80 principal foods. The food serving size was defined as a normal portion according to the standard weight and volume of servings commonly consumed by the general Japanese population. A total of 141 variables, including dietary energy and nutrient intake, were calculated using an ad hoc computer algorithm for the BDHQ. Detailed explanations accompanied each questionnaire. Well-trained interviewers clarified any unclear sections of the questionnaire, which was completed by the participants at their leisure.

iii. Anthropometric measurements and medical history

Anthropometric measurements such as height and weight were measured in all participants, and body mass index (BMI) was calculated as weight (kg)/height (m^2). In addition, the grip strength of both hands was measured. Experienced orthopedic surgeons collected medical information about pain, swelling, and the range of motion of the knee.

iv. Blood and urinary examinations

Samples were collected between the end of October and the middle of January. All blood and urine samples were collected between 9 a.m. and 3 p.m. Blood samples were centrifuged to obtain sera. Sera and urine samples were immediately placed on dry ice and transferred to a deep freezer within 24 h. Samples were stored at −80 °C until assayed.

The serum 25D levels were measured using a radioimmunoassay with a ^{125}I-labeled tracer (DiaSorin, Stillwater,

MN, USA) [12]. The iPTH levels were measured using an electrochemiluminescence immunoassay (Roche Diagnostics GmbH, Menheim, Germany). Serum procollagen type I N-terminal propeptide (PINP), a marker of bone formation, was measured using a radioimmunoassay (Orion Diagnostics, Espoo, Finland). Urinary levels of β-CTX, a marker of bone resorption, were determined using enzyme-linked immunosorbent assay (Fujirebio, Inc., Tokyo, Japan). Urinary β-CTX values were standardized to the urinary creatinine concentrations.

v. Bone mineral density examination

The lumbar spine and proximal femur BMD values were determined using dual-energy X-ray absorptiometry (Hologic Discovery; Hologic, Waltham, MA, USA).

Three-year follow-up

Between 2008 and 2010, the 1690 participants with baseline 25D measurements were invited again to participate in the 3-year follow-up of the ROAD survey, which repeated the baseline examinations.

The follow-up samples were collected between October and January, in the same way as for the baseline study. As a general rule in the ROAD study, those who attended the baseline visit in, for example, October, subsequently attended the follow-up in October as well. However, some participants who attended the baseline visit in October might have attended the follow-up in a later month, owing to personal reasons.

Similar to for the baseline examination, all blood and urine samples at the follow-up were collected between 9 a.m. and 3 p.m. The blood samples were centrifuged to obtain sera. Sera and urine samples were immediately placed on dry ice and transferred to a deep freezer within 24 h, and the samples were stored at −80 °C until assayed.

Changes in the vitamin D levels

The changes in the serum levels (ng/mL) of 25D, and annual change rate of the 25D levels (%/year) over the 3-year period were calculated as follows:

Change in 25D levels (ng/mL)=25D levels measured in the second survey − 25D levels measured in the baseline survey.

Annual change rate of the 25D levels (%/year)=([(25D levels measured in the second survey − 25D levels measured in baseline survey)/25D levels measured in baseline survey]/3)×100.

Statistical analyses

All statistical analyses were performed using STATA statistical software (STATA Corp., College Station, TX, USA). Differences in proportions were compared using the chi-square test. The significance of differences between continuous variables was evaluated using analysis of variance for comparisons among multiple groups or Scheffe's least significant difference test for pairs of groups. All p values and 95 % confidence intervals are two-sided. A value of $p<0.05$ was considered statistically significant.

Multivariate regression analysis was used to test the association of the related factors with the changes in the 25D levels. In the analysis, we used the values of the annual change rate of serum 25D levels (%/year) as the objective variable and selected potential associated factors as explanatory variables, after adjusting for age (+1 year), gender (0, men; 1, women), BMI (+1 kg/m^2), participated month (0, October, November, December; 1, January), and regional differences (0, mountainous area; 1, coastal area) at the baseline survey, as previously described [7, 8].

As for the potential associated factors used as explanatory factors, we assessed the following factors with a significant or marginal ($p<0.1$) association with VD status in the simple linear analysis, which were assessed for the association with VD deficiency herein and in the previous reports of the ROAD study [7, 8]: smoking (0, never, ever; 1, current), alcohol consumption (0, never, ever; 1, current), regular walking outside (0, <5 times/week; 1, ≥5 times/week), regular exercising outdoors (e.g., football, tennis, baseball, and golf) after the most recent graduation (0, no; 1, yes), serum levels of iPTH (+1 pg/mL), baseline BMD values at the lumbar spine (+1 g/cm^2) or femoral neck (+1 g/cm^2), serum levels of PINP (+1 standard deviation, μg/L), urinary levels of β-CTX (+1 standard deviation, μg/mmol creatinine), and daily amounts of total energy (+100 kcal/day), calcium (+100 mg/day), and VD (+10 μg/day) intake, as calculated based on the BDHQ questionnaire.

Results

Eligible participants

Of the 1690 participants with 25D measurements at baseline, 251 (14.9 %) did not participate in the second survey. The reasons for these 251 dropouts were as follows: 40 individuals died, 97 were ill, 16 moved away, 8 were absent, 51 declined to participate in a second visit, and 39 stated "other" reasons. Furthermore, 55 individuals (3.3 %) did not complete the second survey, and the values of serum 25D were not obtained in 11 individuals at the follow-up because of insufficient serum samples for the measurement. In addition, 14 subjects were diagnosed as suspicion of hyperparathyroidism based on elevated serum iPTH >100 pg/mL at baseline, and 13 subjects prescribed oral VD at baseline and during the 3 years between the baseline and second surveys were excluded. As a result, in

the present study, 1346 subjects were analyzed (79.6 %; 458 men and 888 women; mean age, 63.8 [11.8] years).

Table 1 shows the baseline characteristics of the 1346 subjects classified by gender. The mean age (SD) of the subjects in the present study was 63.8 (11.8) years and the mean BMI (SD) was 23.1 (3.3) kg/m^2. More than half of all individuals lived in the coastal area, and approximately 30 % of all men were smokers, while the corresponding proportion of smokers in women was very small (3.5 %). More than half of all subjects had a habit of walking outside, including to, or during, work, and 15 % exercised regularly outdoors. The daily intakes of total energy (kcal), calcium (mg), and VD (μg) are also shown in Table 1, and these were all significantly higher in men than in women. Moreover, the prevalence of

Table 1 Background characteristics of the study subjects at baseline

	Total (n=1346)	Men (n=458)	Women (N=888)	p (men vs. women)
Mean values (SD) of 25D (ng/mL) and changes in the 25D levels (ng/mL)				
Baseline 25D levels	23.4 (6.5)	25.9 (6.4)	22.2 (6.2)	<0.0001***
Measurement of 25D in the second survey	27.7 (8.5)	31.4 (9.8)	25.8 (7.1)	<0.0001***
Changes in the 25D levels between baseline and the second survey	4.3 (7.3)	5.5 (8.5)	3.6 (6.5)	<0.0001***
Annual change rate (%/year) of 25D between baseline and the second survey	7.6 (13.3)	8.2 (12.4)	7.3 (13.7)	0.26
Mean values (SD) of selected characteristics				
Age (years)	63.8 (11.8)	64.9 (11.6)	63.2 (11.8)	0.0120*
Height (cm)	155.7 (9.0)	164.0 (7.0)	151.4 (6.6)	<0.0001***
Weight (kg)	56.1 (10.7)	52.5 (8.6)	63.1 (10.7)	<0.0001***
BMI (kg/m^2)	23.1 (3.3)	23.4 (3.2)	22.9 (3.4)	0.0068**
Prevalence of selected characteristics (%)				
Residing in the coastal area	53.6	51.8	54.6	0.32
Current smoking habit (regularly, ≥1 time/month)	12.4	29.4	3.5	<0.001***
Current alcohol consumption (regularly, ≥1 time/month)	40.8	68.5	26.6	<0.001***
Regularly walking outside (≥5 times/week, including for work)	56.5	62.1	53.6	0.004**
Regularly exercising outdoors (e.g., football, tennis, baseball, and golf) after the most recent school graduation	15.3	36.0	4.6	<0.001***
Mean values (SD) of selected measurements				
Serum levels of iPTH (pg/mL)	38.9 (14.7)	37.5 (13.8)	39.6 (15.1)	0.0155*
BMD (L2-4) (g/cm^2)	0.94 (0.20)	1.06 (0.20)	0.88 (0.18)	<0.0001***
BMD (femoral neck) (g/cm^2)	0.66 (0.16)	0.62 (0.12)	0.74 (0.14)	<0.0001***
Serum levels of PINP (μg/L)	57.9 (26.8)	46.9 (20.4)	65.5 (28.0)	<0.0001***
Urinary levels of β-CTX (μg/mmol Cr)	185.7 (126.8)	124.9 (73.0)	217.0 (136.9)	<0.0001***
Mean values (SD) of the total amounts of selected nutritional intakes (/day)				
Total energy (kcal)	1947 (584)	2326 (656)	1751 (428)	<0.0001***
Calcium (mg)	558 (230)	582 (259)	546 (215)	0.0069**
Vitamin D (μg)	20.4 (12.3)	22.6 (13.9)	19.3 (11.3)	<0.0001***
Prevalence of medication of osteoporosis prescribed by doctors (%)				
Calcium	0.4	0.2	0.5	0.51
Bisphosphonate	1.8	0.2	2.6	0.002**
SERMs	1.0	0.0	1.6	0.007**
Calcitonin	0.5	0.0	0.7	0.08
Prevalence of hyperparathyroidism (%)	6.1	5.2	6.5	0.35
Prevalence of osteoporosis (%) according to the WHO criteria				
Osteoporosis (L2-4)	13.6	2.4	19.4	<0.001***
Osteoporosis (femoral neck)	13.0	3.1	18.1	<0.001***

SD standard deviation, *25D* 25-hydroxyvitamin D, *BMI* body mass index, *iPTH* intact parathyroid hormone, *BMD* bone mineral density, *L2-4* lumbar spine L2-L4, *PINP* procollagen type I N-terminal propeptide, *β-CTX* β-isomerized C-terminal telopeptide cross-links of type I collagen, *Cr* creatinine, *SERMs* selective estrogen receptor modulators, *WHO* World Health Organization

*p<0.05; **p<0.01; ***p<0.001

osteoporosis measured in both the lumbar spine of L2-4 and femoral neck was significantly higher in women than in men, as were the mean values of iPTH. However, the proportion of medication use for osteoporosis was very small, even in women (Table 1).

Changes in the VD levels in subjects who participated in both the baseline and second surveys

In the total 1346 subjects, the mean change in the serum 25D level (SD) was 4.3 (7.3) ng/mL. The corresponding changes in men and women were 5.5 (8.5) and 3.6 (6.5) ng/mL, respectively, and the mean annual change rates in the 25D levels in the total subjects, men, and women were 7.6 (13.3), 8.2 (12.4), and 7.3 (13.7) %/year, respectively. There was no significant gender difference in the annual change rate of serum 25D (Table 1).

The mean annual change rates in the 25D levels (SD) in subjects in their 30s or younger, 40s, 50s, 60s, 70s, and 80s or older were 1.3 (7.4), 5.1 (12.2), 7.4 (17.5), 8.8 (12.6), 7.7 (11.1), and 8.2 (11.0) %/year, respectively, indicating that the serum levels of 25D increased in all age groups over time. Further, the 25D levels were significantly increased in subjects in their 60s compared to those in their 30s or younger (p=0.048).

Figure 1 shows the distribution of the annual change rates in the 25D levels (SD) classified by gender. In men, the mean annual change rates in the 25D levels in participants in their 30s or younger, 40s, 50s, 60s, 70s, and 80s or older were 2.2 (6.9), 2.8 (7.8), 7.5 (16.8), 10.5 (12.0), 8.7 (10.7), and 6.4 (8.1) %/year, respectively, and there was a significant difference between men in their 30s and younger and those in their 60s (p=0.036). The corresponding values in women were 1.0 (7.6), 6.2 (13.5), 7.4 (17.8), 8.0 (12.9), 7.0 (11.4), and 9.1 (12.2) %/year, respectively, and there was no significant difference between any particular age groups (Fig. 1).

Factors associated with changes in the serum 25D levels

Table 2 shows the results of the multivariate regression analysis assessing the associations with the annual change rate in the 25D levels. The analysis was adjusted for age (+1 year), gender (0, men; 1, women), BMI (+1 kg/m^2), participated month (0, October, November, December; 1, January), and regional differences (0, mountainous area; 1, coastal area) at baseline, as previously described [8]. The results of the analysis indicated that the BMD values measured at lumbar spine L2-4 and the total daily energy intake were significantly associated with the change rate of the 25D levels (Table 2). In other words, the higher the BMD of the lumbar spine and the higher the total daily energy intake, the higher the increase in the serum 25D levels.

To test the independence of the associations between BMD at the lumbar spine and total energy of daily food with the change in the 25D levels, the multivariate model was adjusted for both these variables; as a result, both variables remained significantly associated with the changes in the 25D levels, revealing that these variables were mutually and independently associated with the changes in the 25D levels (Table 2).

Discussion

In this 3-year follow-up of a large cohort of subjects, the serum levels of 25D at baseline and at a second survey were measured, and we estimated the change rate of 25D levels and the factors contributing to this change rate. This study showed that the mean change rate of the levels of serum 25D tended to

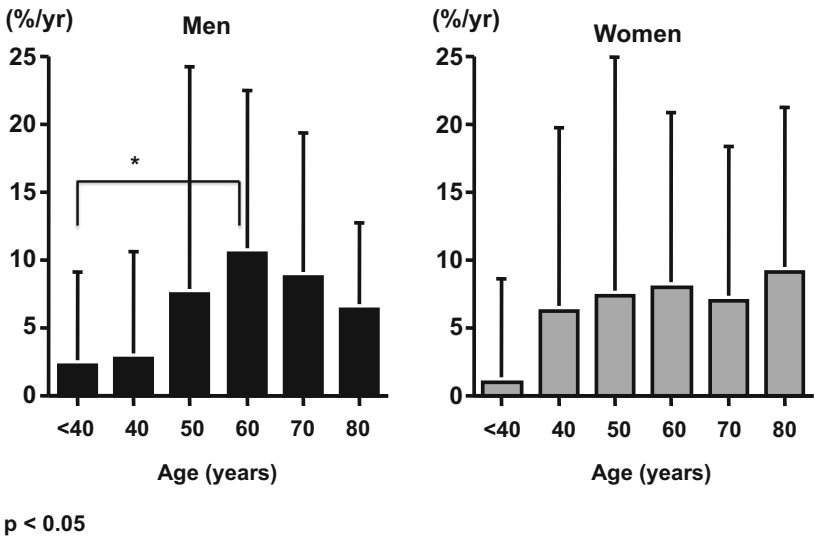

Fig. 1 Age-gender distribution (%) of the annual change rate of 25D

Table 2 Partial regression coefficients (beta) of potential factors associated with the annual change rate of serum 25D levels (%/year)

Explanatory variables collected at baseline	Reference	Multivariate regression analysis 1[a]			Multivariate regression analysis 2[b]		
		Beta	R^2	p	Beta	R^2	p
Lifestyle factors							
Smoking	0: ex or never smoker; 1: current smoker	0.04	0.143	0.18			
Alcohol consumption	0: ex or never drinker; 1: current drinker	0.12	0.142	0.46			
Regularly walking outside	0: no; 1: yes	0.02	0.135	0.46			
Regularly exercising outdoors (e.g. football, tennis, baseball, golf, etc.) after the most recent school graduation	0: no; 1: yes	−0.04	0.143	0.15			
Mean values of selected measurements							
Serum levels of iPTH (pg/mL)	+1 pg/mL	−0.01	0.141	0.62			
BMD (L2-4) (g/cm2)	+1 g/cm^2	0.06	0.144	0.05*	0.06	0.144	0.050*
BMD (femoral neck) (g/cm2)	+1 g/cm^2	−0.004	0.140	0.91			
Serum levels of PINP (μg/L)	+1 SD	0.009	0.140	0.73			
Urinary levels of β-CTX (μg/mmol Cr)	+1 SD	0.002	0.142	0.94			
Nutritional factors							
Total energy from daily food (kcal/day)	+100 kcal	0.06	0.145	0.039*	0.06	0.145	0.039*
Calcium from daily food (mg/day)	+100 mg	0.03	0.143	0.198			
Vitamin D from daily food (μg/day)	+1 μg	−0.01	0.142	0.578			

25D 25-hydroxyvitamin D, *iPTH* intact parathyroid hormone, *BMD* bone mineral density, *iPTH* intact parathyroid hormone, *PINP* procollagen type I N-terminal propeptide, *β*-CTX, β-isomerized C-terminal telopeptide cross-links of type I collagen, *Cr* creatinine

$^{+}p<0.1$;$^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

[a] Multivariate regression analysis was performed after adjustment for age (+1 year), gender (0: men; 1: women), body mass index (+1 kg/m2), participated month (0, October, November, December; 1, January), and regional differences (0: mountainous area; 1: coastal area) at the baseline survey

[b] Multivariate regression analysis was performed after adjustment for BMD (L2-4) and total daily energy intake mutually, in addition to age (+1 year), gender (0: men; 1: women), body mass index (+1 kg/m2), participated month (0, October, November, December; 1, January), and regional differences (0: mountainous area; 1: coastal area) at the baseline survey

increase during the 3-year period. There was no gender difference, and subjects in their 60s showed the largest increases in the change rate. Moreover, we found that the BMD values at the lumbar spine and the total daily energy intake at baseline were significantly associated with increases in the serum 25D levels.

The prevalence of VD inadequacy in postmenopausal women in Japan is well known to be very high [13, 14], and our previous report furthermore revealed a very high prevalence of VD insufficiency and a low prevalence of VD deficiency in Japanese men and women [7]. However, to our knowledge, this is the first report on the change rate of the serum levels of 25D measured in a general Japanese population.

As mentioned, in the present study, the mean changes in the serum 25D level tended to increase during the 3-year period in both men and women. It is possible that this result might be due to the intervention effect of the repeated surveys. We have performed individual consultations with all participants in order to explain the individual findings and inform them regarding the results of the survey. In such consultations, we provided the subjects with information on the association between osteoporosis and lifestyle factors, including nutrition, such as the importance of the total energy, calcium, and vitamin D intake from their daily food. Such consultations after each survey might have helped educate the participants, and as a result, it may have influenced the course of the 25D levels of the subjects. However, this procedure could not be avoided, and we believe that this is in fact a favorable effect, allowing for improved health outcomes of the study cohort, rather than a limitation of the present study.

Of note, at the translation of the changes of the measurements, there is one particular issue that should be considered, namely the regression to the mean (RTM) phenomenon. RTM is a statistical phenomenon that can cause natural variation. It happens when unusually large or small measurements tend to be followed by measurements that are closer to the mean [15]. To reduce the effects of this phenomenon, Barnett et al. recommended using a suitable study design, such as random allocation of subjects to the comparison groups and multiple measurements. Unfortunately, we could not change our study from a cohort study into a randomized controlled trial, or repeat the measurements of 25D at each visit, owing to budget limitations. Nonetheless, to reduce the RTM in the data

analysis, the authors also recommended adjusting each subject's follow-up measurements according to their baseline measurement, i.e., analysis of covariance [15]. Therefore, we here used the rate of the changes in the 25D levels as an objective variable and adjusted the baseline 25D levels in advance, which might have helped reducing the RTM phenomenon. By contrast, Sonderman et al. evaluated the reproducibility of 25D measurements using a prospective cohort study of 225 participants and indicated that the 25D serum measurements provided reasonably representative measures [16].

In this study, in the multivariate regression analysis, the BMD values at the lumbar spine, and the total daily energy intake measured at baseline were found to be associated with an increased change rate of the serum 25D levels, both mutually and independently from one another. As stated above, many studies have reported an association between inadequate VD intake and osteoporosis [3–6]. Cauley et al. reported that, in their observational study of a US cohort consisting of 1532 middle-aged women, the serum 25D levels were inversely associated with non-traumatic fractures, whereas menopause-related changes in the lumbar spine and femoral neck BMD values were not significantly associated with the serum 25D level [17]. In another cohort consisting of 1470 postmenopausal Japanese women, the incidence of proximal femur and long bone fractures tended to decrease as the serum 25D levels increased [18]. Regarding the relationship between 25D and the occurrence of osteoporosis, using the identical ROAD cohort as in the present study, we have previously shown that the serum 25D levels at baseline were significantly associated with subsequent osteoporosis occurrence, especially at the femoral neck [8]. These findings suggest that higher levels of 25D at baseline favorably impact future bone health. However, there is currently no report regarding the association between baseline BMD and changes in the 25D levels, as assessed in the present study.

Herein, we found that the baseline BMD values at lumbar spine L2-4 were significantly associated with increased 25D levels. Together with the results of our previous report in which we showed that the 25D levels at baseline influence the subsequent occurrence of osteoporosis [8], we conclude that the BMD values and 25D levels mutually influence one another over time. However, we did not examine the mechanisms of this mutual association in detail. Moreover, we also cannot explain why, in the present study, this association was observed only at the lumbar spine of L2-4 and not at the femoral neck. Further analysis is required to confirm this observed association.

Furthermore, among the nutritional factors examined, higher daily energy intake at baseline was found to significantly accelerate the increase in the serum levels of 25D. Adequate nutritional intake, especially adequate intake of calcium and VD, is recommended to maintain bone health [6, 19]. In addition, several other nutrients have been reported to improve bone health. Nieves reviewed several reports of nutrients and osteoporosis and concluded that the skeletal benefits of flavonoids, carotenoids, omega-3 fatty acids, and vitamins A, C, E, and K were limited to observational data or a few clinical trials [20]. Further, the author reported that potassium bicarbonate may improve calcium homeostasis, that high homocysteine levels may relate to the fracture risk, and that magnesium supplementation is likely only required in those with low magnesium levels [21]. Of note, regarding the associations between nutrients, BMD, and fractures, Nieves noted that the nutrients do not act in isolation [21], and it is hence important to evaluate the effects of different combinations of nutrients using analyses of dietary patterns. Accordingly, our results showed that the total daily energy intake, rather than daily VD intake, positively influenced the change rate of the 25D levels. Total energy can be considered a comprehensive factor containing most above mentioned nutrients, and we hence conclude that to prevent serum 25D decreases, it is important to improve energy-poor diets.

There are several limitations to this study. First, although the ROAD study includes a large number of participants, the participants in the present study (from the mountainous and coastal regions only) may not be completely representative of the general population. To address this issue, we compared the anthropometric measurements, smoking frequency, and alcohol consumption between the present study participants and the general Japanese population. The values for the general population were obtained from the report on the 2005 National Health and Nutrition Survey conducted by the Ministry of Health, Labour and Welfare, Japan [22], when our baseline ROAD started. The mean BMI values of men aged 30 or younger, 40, 50, 60, 70–74, 75–79, and 80 years or older, as reported in the National Health and Nutrition Survey, were 23.29 (3.68), 23.99 (3.27), 23.74 (3.07), 23.75(2.94), 23.68 (3.18), 23.31 (3.04), and 22.27 (2.64) kg/m^2, respectively, and those of women were 21.37 (3.81), 22.44 (3.49), 23.06 (3.37), 23.54 (3.66), 23.16 (3.42), 23.42 (3.53), and 22.50 (3.97) kg/m^2, respectively. In the present study, the corresponding mean BMI values were 23.51 (3.44), 24.70 (4.32), 23.60 (2.96), 23.86 (3.19), 23.11 (2.82), 22.24 (2.82), and 23.13 (2.74) kg/m^2, respectively, for men, and 21.14 (2.67), 21.82 (3.73), 23.03 (3.24), 23.25 (3.15), 23.65 (3.55), 22.44 (3.82), and 22.23 (3.21), respectively, for women. No significant differences were identified between our participants and the total Japanese population, except that male and female participants aged 75–79 years in the present study had significantly smaller BMI than the overall Japanese population ($p<0.05$). This difference should be taken into consideration when evaluating the potential risk factors in subjects aged 75–79 years. In addition, the proportion of current smokers and drinkers (those who regularly smoke or drink more than one drink/month) in the general Japanese population was compared with

that in the present study population. The proportion of current smokers was significantly higher in the general Japanese population than in our study population in both men and women (men, 34.8 vs. 29.4 %, $p<0.05$; women, 8.8 vs. 3.5 %, $p<0.05$). Moreover, the proportion of current drinkers was significantly higher in women in the general Japanese population than in women in the present study, while there was no significant difference in men (men, 69.8 vs. 68.5 %, $p=0.62$; women, 30.8 vs. 26.6 %, $p<0.05$), suggesting that the participants of the present study lead healthier lifestyles, at least in terms of their smoking habits, as compared to the general Japanese population. This selection bias should be taken into consideration when generalizing the results obtained from the present study. Second, the measurements of 25D were only conducted two times, once at baseline and once at the 3-year follow-up, and we could not exclude the effect of incidental life changes of the participants, such as holidays or dietary changes for special anniversaries around the examination date. Although this random effect on the subjects seems difficult to avoid, the large number of participants of the present study is considered to dilute any individual variance, and the high participation rate of the follow-up study is a major strength of the present study. Third, in the present study, limited information on the association between the changes in the exposure variables, including lifestyle factors, and the changes in the serum levels of 25D, was provided. As one example of a change in a potential associated factor, we assessed the association between new prescriptions of VD during the 3-year period between the baseline and the second survey and the changes in the serum levels of 25D. Over the 3-year follow-up, 32 individuals (3 men, 29 women) were newly prescribed oral VD supplementation. Multivariate regression analysis was performed to assess the association between the annual change rate in the 25D levels and new prescription of VD supplementation (1, yes; 0, no) after adjustment for age (+1 year), gender (0, men; 1, women), BMI (+1 kg/m^2), participated month (0, October, November, December; 1, January), and regional differences (0, mountainous area; 1, coastal area) at baseline. As a result, we found that there was no significant association between the presence of new VD supplementation and the changes in the serum levels of 25D (beta=−0.17, $p=0.51$). We plan on assessing the effects of changes in other potential associated factors, such as dietary-, physical-activity-, and other lifestyle-related factors on the changes in the serum levels of 25D over this 3-year period, in order to clarify the contributing factors to the increase in 25D, as a next step of our research.

In conclusion, we here found that the serum levels of 25D tended to increase over a 3-year period in a large cohort of general Japanese inhabitants and that higher lumbar BMD and total daily energy intake are significant independent factors associated with increases in the 25D levels over time.

Acknowledgments This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology for Scientific Research to NY (B26293139, B23390172, B20390182, and Challenging Exploratory Research 24659317), TA (C20591737, B23390357), and SM (C20591774, B23390356, Challenging Exploratory Research 23659580); for Young Scientists to HO (A18689031, Challenging Exploratory Research 24659666); and for Collaborating Research with NSF to NY (Director; 08033011-00262). Moreover, this work was supported by H17-Men-eki-009 (Director, KN), H18-Choujyu-037 (Director, TN), H20-Choujyu-009 (Director, NY), H23-Chojyu-002 (Director, TA), H25-Nanchi-to (Men)-005 (Director, ST), and H25-Chojyu-007 (Director, NY) from the Ministry of Health, Labour and Welfare in Japan, and by grants from the Japan Osteoporosis Society (NY, SM, HO, and TA), and research aids from the Japanese Orthopaedic Association (JOA-Subsidized Science Project Research 2006-1 and 2010-2; Director, HK). The authors wish to thank Dr. Takako Nojiri and Mr. Kazuhiro Hatanaka of the Gobo Public Health Centre; Dr. Naoki Hirabayashi of the Kawakami Clinic, Hidakagawa Town; Mrs. Tomoko Takijiri, Mrs. Kumiko Shinou, Mrs. Rie Takiguchi, Mrs. Kyoko Maeda, Ms. Ikuyo Ueyama, Mrs. Michiko Mori, Mrs. Hisayo Sugimoto, and other members of the public office in Hidakagawa Town; Dr. Shinji Matsuda of the Shingu Public Health Centre; and Mrs. Tamako Tsutsumi, Mrs. Kanami Maeda, Mr. Shoichi Shimoichi, Mrs. Megumi Takino, Mrs. Shuko Okada, Mrs. Kazuyo Setoh, Mrs. Chise Ryouno, Mrs. Miki Shimosaki, Mrs. Chika Yamaguchi, Mrs. Yuki Shimoji, and other members of the public office in Taiji Town for their assistance in locating and scheduling participants for examinations. Finally, we also thank Ms. Kyoko Yoshimura, Mrs. Toki Sakurai, and Mrs. Saeko Sahara for their assistance with data reduction and administration.

Conflicts of interest None.

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