Our study showed that the PBM was attained at age 18 in women in Tokyo, Japan. It also suggested the importance of intervention in these women by age 18 by using total hip BMD as an index for such intervention, as well as the importance of weight control in these individuals.

In conclusion, in light of the findings obtained on the skeletal parameters, the impact of duration of exposure to estrogen after menarche, bone and calcium metabolism, our study reveals that BMD peaks in late adolescence, which appears to be supported by the fact that the rapid decrease in BAP and NTX ceases by that age. Moreover, our results show that BMD in the total hip begins to decrease at age 18 after peaking, whereas lumbar spine BMD increases by about 5% during the 20s after age 18. We therefore consider it necessary to intervene by age 18 using total hip BMD as an index in order to increase PBM. Our results suggest an optimal timing for intervention for bone mass as well as an index for such intervention.

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ORIGINAL ARTICLE

Influence of maternal genetic and lifestyle factors on bone mineral density in adolescent daughters: a cohort study in 387 Japanese daughter-mother pairs

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Abstract We conducted a cross-sectional study in a cohort of Japanese adolescent schoolgirls (12–18 years of age) and their mothers (387 pairs). Age, lumbar bone mineral density (BMD), birth and menarche-related status, height, body weight and lifestyles were surveyed in the participants. The values of BMD, height and body weight were converted to standard deviation (SD) by age. There were 49 (12.7%) pre-menarche and 338 (87.3%) post-menarche daughters. BMD-SD, height-SD, vitamin D intake and vitamin K intake were significantly correlated between the pre-menarche daughters and mothers ($P < 0.05$), while BMD-SD, birth weight, age at menarche and all lifestyle-related factors were significantly correlated between the post-menarche daughters and mothers ($P < 0.05$). BMD-SD in the pre-menarche daughters was affected by BMD-SD in mothers ($R^2 = 0.069$, $P = 0.033$) and their own height-SD ($R^2 = 0.199$, $P = 0.001$) (model $R^2 = 0.340$), independently. BMD-SD in the post-menarche daughters was affected by BMD-SD in mothers ($R^2 = 0.073$, $P < 0.001$) as well as by their own age at menarche ($R^2 = 0.020$, $P = 0.001$), height-SD ($R^2 = 0.022$, $P < 0.001$), body weight-SD ($R^2 = 0.081$, $P < 0.001$) and intensity of exercise ($R^2 = 0.015$, $P = 0.045$) (model $R^2 = 0.372$), independently. The results suggest that BMD is strongly correlated between daughters and mothers and that a greater age at menarche leads to lower peak bone mass. It was also suggested that maintaining high-intensity physical activity and adequate body weight is important in achieving maximum BMD as factors amenable to intervention in post-menarche daughters.

Keywords Bone mineral density · Lifestyle · Nutrient intake · Exercise · Heritability

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Introduction

According to the National Institutes of Health (NIH) report, osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture [1]. As fractures occur with progressing osteoporosis, the quality of life (QOL) and the activity of daily living (ADL) of affected individuals are markedly compromised, and this makes prevention of the disease compellingly important [2–4]. Bone strength, which affects bone fractures, consists of two factors, bone mineral density (BMD) and bone quality. Of the two contributing factors, BMD accounts for 70% of bone strength [1]. Therefore, after a diagnosis of osteoporosis has been established, increasing BMD with medications is deemed necessary to reduce subsequent bone fractures. As BMD is known to decrease markedly from peri-menopause, acquisition of higher BMD before peri-menopause is of critical importance [5–8].

BMD is reported to increase in two periods, first from the 1st to 4th years and second from the 12th to 17th years, then making a spurt in adolescence [9, 10]. Moreover, results of intervention with nutrient intake and physical activity in youth were summarized in a review [11], indicating that intake of calcium, of all nutrients [12–15], and enforcement of high-impact exercise, of all physical activities, are important to acquisition of higher BMD [16–18]. Furthermore, the synergistic effect of nutrient intake and exercise

on BMD is reported [19]. On the other hand, BMD and lifestyle factors are influenced by hereditary and interfamilial homology [20, 21]. Therefore, it is important to take hereditary factors into account when managing lifestyle factors to ensure higher BMD acquisition in youth.

The purpose of this cross-sectional study was to clarify how maternal genetic or lifestyle factors might interact to influence adolescent lumber BMD in Japanese daughter–mother pairs.

Methods

Subjects

This study was carried out from July to September 2006 in Tokyo, Japan. The participants were schoolgirls aged 12–18 years old attending girls' junior and senior high schools, and their mothers (387 pairs). Participants were excluded if they had systemic or metabolic disorders or if they were receiving medications with known effects on bone metabolism. The study protocol was approved by the Ethics Committee of Tokyo Women's Medical University, and consent was obtained from all candidate subjects who agreed to participate.

Assessment of skeletal indices

Lumbar 2–4 BMD was measured in the participants by QDR-4500 (Hologic, Waltham, MA). The inter-assay variance of BMD measurement was 0.5 ± 0.5% (mean ± SD). Height and body weight were also measured, and blood samples were collected from the participants to measure their serum calcium and phosphorus levels.

Assessment of birth- and menarche-related status

Gestational age, birth weight, presence of menarche and ages at menarche and menopause were assessed in the participants by interviewing.

Assessment of lifestyle factors

As for dietary habits, nutrient intake was assessed by using the self-administered Diet History Questionnaire (DHQ) developed by Sasaki et al. [22, 23]. The daily intake of calories and all nutrients and the number of breakfasts skipped per week were calculated from the DHQ. To establish a convenient, interview-based method involving interviewing, current physical activities (yes or no), kinds of exercises, their frequency and duration per month, and their level of intensity (1, light; 2, moderate; 3, vigorous) were assessed. For participants with multiple exercises, the highest level of intensity was assigned.

Statistical analysis

In the descriptive analysis of participant characteristics, numerical data were expressed as mean ± standard deviation (SD). The daughters were divided into the pre-menarche group and post-menarche group for comparison. Given that BMD, height and body weight might be influenced by the time of exposure to female hormones, their measured values were converted to SD by age in the participants. First of all, the BMD-SD, height-SD, body weight-SD, birth-related data, age at menarche and lifestyle factors between the daughters and mothers were examined for correlation by using Spearman's rank correlation coefficient. Then, the BMD-SD in the daughters was examined for correlation with the height-SD, body weight-SD, birth-related data, age at menarche and lifestyle factors in both the daughters and mothers by using Spearman's rank correlation coefficient. Variables showing significant correlation with the BMD-SD of the daughters were selected as the candidate factors with the exclusion criterion being a P-value less than 0.05. Stepwise multivariate regression analysis was used to estimate independent contributing factors to the BMD-SD of the daughters. Finally, ANOVA test was used to clarify the effect of intervention on the BMD-SD of the daughters. A value of $P < 0.05$ was regarded as statistically significant. All statistical analyses were performed with the JMP version 5.1.2 (SAS Inst, Inc., Cary, NC).

Results

Correlation between the daughters and mothers

Measurement results for all participants are shown in Table 1. The mean age of the daughters was 14.6 ± 1.8 years (12–18 years) with the mean age in the pre-menarche group and post-menarche group being 12.8 ± 1.0 years ($n = 49$) and 14.8 ± 1.7 years ($n = 338$), respectively. The mean age of the mothers was 46.1 ± 4.0 years (36–56 years). There were significant differences between the pre- and post-menarche groups in BMD, height, body weight, frequency of exercise and maximum intensity of exercise ($P < 0.05$). There was no significant difference in birth weight between the daughters and mothers ($P = 0.711$); however, the mean gestational age and age at menarche in the daughters were significantly shorter and earlier than in their mothers ($P < 0.001$). No abnormal serum calcium or phosphorus levels were noted in any participants.

All parameters examined for correlation between the daughters and mothers are shown in Table 2. BMD-SD showed a significant correlation between the daughters and mothers in both the pre- and post-menarche groups

Table 1 Characteristics of the study subjects

Variable	Daughters		Mothers ($n = 387$)
	Pre-menarche ($n = 49$)	Post-menarche ($n = 338$)	
BMD (g/cm^2)	0.81 ± 0.08	0.94 ± 0.12	1.02 ± 0.13
Birth weight (g)[a]	3,013.5 ± 422.8	3,055.9 ± 431.7	3,037.8 ± 415.9
Gestational age (weeks)[b]	38.8 ± 2.0	39.1 ± 1.9	39.7 ± 1.7
Age at menarche (years)[c]	–	11.9 ± 1.2	12.5 ± 1.2
Height (cm)	152.4 ± 7.1	157.1 ± 5.4	158.1 ± 4.7
Body weight (kg)	39.7 ± 5.4	49.0 ± 6.9	52.8 ± 7.5
BMI (kg/m^2)	17.0 ± 1.4	19.8 ± 2.4	21.1 ± 3.0
Energy intake (kcal/day)	1,965.4 ± 466.0	2,025.2 ± 570.1	1,952.4 ± 478.6
Calcium intake (mg/day)	573.7 ± 238.6	596.6 ± 268.7	581.4 ± 210
Vitamin D intake (μg/day)	7.0 ± 4.0	7.1 ± 4.4	7.5 ± 3.9
Vitamin K intake (μg/day)	278.7 ± 148.8	274.5 ± 145.2	324.4 ± 161.3
Frequency of exercise (days/month)	11.6 ± 10.1	8.4 ± 9.8	6.6 ± 9.2
Total duration of exercise (h/month)	17.3 ± 22.3	12.3 ± 18.0	6.8 ± 11.8
Maximum intensity level of exercise[d]	1.80 ± 1.31	1.32 ± 1.28	0.99 ± 1.05

Data are expressed as mean value ± standard deviation

BMI body mass index

[a] Pre-menarche, $n = 48$; post-menarche, $n = 298$; mothers, $n = 312$

[b] Pre-menarche, $n = 47$; post-menarche, $n = 277$; mothers, $n = 284$

[c] Post-menarche, $n = 338$; mothers, $n = 383$

[d] Intensity level of exercise: 1 = light; 2 = moderate; 3 = vigorous

Table 2 Correlation coefficients for daughters versus mothers in Spearman's rank test

Variable	Pre-menarche daughters–mothers ($n = 49$)		Post-menarche daughters–mothers ($n = 338$)	
	R	*P*	*R*	*P*
BMD-SD	0.284	0.048	0.301	<0.001
Birth weight (g)	0.029	0.863	0.286	<0.001
Gestational age (weeks)	0.166	0.356	0.100	0.149
Age at menarche (years)	–	–	0.298	<0.001
Height-SD	0.458	0.001	0.498	<0.001
Body weight-SD	0.114	0.435	0.240	<0.001
BMI-SD	−0.050	0.731	0.259	<0.001
Energy intake (kcal/day)	0.489	<0.001	0.323	<0.001
Calcium intake (mg/day)	0.261	0.071	0.387	<0.001
Vitamin D intake (μg/day)	0.686	<0.001	0.458	<0.001
Vitamin K intake (μg/day)	0.577	<0.001	0.407	<0.001
Frequency of exercise (days/month)	0.131	0.371	0.143	0.009
Total duration of exercise (h/month)	−0.015	0.918	0.163	0.003
Maximum intensity level of exercise[a]	0.041	0.779	0.139	0.011

SD standard deviation values calculated for each age category in this study

[a] Intensity level of exercise: 1 = light; 2 = moderate; 3 = vigorous

($P = 0.048$, $P < 0.001$). Height-SD, total energy intake, vitamin D intake and vitamin K intake in the pre-menarche daughters were each significantly correlated with those in their mothers ($P < 0.001$), while all factors examined, except gestational age, were significantly correlated between the post-menarche daughters and their mothers ($P < 0.05$).

Correlation between the BMD-SD and other factors in the daughters

Single linear regression analyses were performed to determine correlation between the BMD-SD of the daughters and the other factors examined (Table 3). Height-SD and body weigh-SD were significantly correlated with BMD-SD in the pre-menarche daughters ($P < 0.001$), while BMD-SD was significantly correlated with age at menarche, height-SD, body weight-SD, frequency, duration and intensity of exercise in the post-menarche group ($P < 0.05$). BMD-SD was not correlated with birth weight, gestational age and nutrient intake in the dietary assessment.

Multivariate analysis

Of the factors evaluated, BMD-SD, height-SD and body weight-SD in the mothers were chosen as candidate explanatory factors for BMD-SD in the pre-menarche daughters, as

Table 3 Correlation coefficients for bone mineral density in daughters and other variables in Spearman's rank test

Variable	Pre-menarche daughters–mothers ($n = 49$)				Post-menarche daughters–mothers ($n = 338$)			
	Daughters		Mothers		Daughters		Mothers	
	R	P	R	P	R	P	R	P
Birth weight (g)	0.278	0.056	0.222	0.174	0.065	0.261	−0.059	0.332
Gestational age (weeks)	0.136	0.363	−0.025	0.888	−0.011	0.850	−0.026	0.688
Age at menarche (years)	–	–	−0.143	0.327	−0.195	0.000	−0.101	0.066
Height-SD	0.561	<0.001	−0.029	0.841	0.323	<0.001	0.163	0.003
Body weight-SD	0.544	<0.001	0.103	0.480	0.433	<0.001	0.186	0.001
BMI-SD	0.214	0.141	0.047	0.746	0.292	<0.001	0.117	0.031
Energy intake (kcal/day)	0.080	0.584	0.051	0.727	−0.078	0.155	0.099	0.071
Calcium intake (mg/day)	−0.026	0.862	−0.122	0.403	0.058	0.286	0.078	0.153
Vitamin D intake (μg/day)	0.175	0.229	0.007	0.960	−0.011	0.842	0.075	0.168
Vitamin K intake (μg/day)	0.059	0.687	−0.069	0.637	0.007	0.899	0.037	0.504
Frequency of exercise (days/month)	0.021	0.889	−0.037	0.802	0.133	0.015	0.025	0.649
Total duration of exercise (h/month)	0.144	0.323	−0.110	0.453	0.146	0.007	0.016	0.774
Maximum intensity level of exercise[a]	0.166	0.255	−0.101	0.490	0.191	0.000	0.053	0.333

SD standard deviation values calculated for each age category in this study

[a] Intensity level of exercise: 1 = light; 2 = moderate; 3 = vigorous

Table 4 Multivariate regression analysis of bone mineral density in daughters and selected background parameters

Variable	Parameter estimate	P	R^2	Model R^2
(1) Pre-menarche daughters				
Height-SD in daughters, 1 SD↑	0.287	0.001	0.199	0.340
BMD-SD in mothers, 1 SD↑	0.244	0.033	0.069	
(2) Post-menarche daughters				
Age at menarche in daughters, 1 year↑	−0.121	0.001	0.020	0.372
Height-SD in daughters, 1 SD↑	0.177	<0.001	0.022	
Body weight-SD in daughters, 1 SD↑	0.351	<0.001	0.081	
Maximum intensity level of exercise in daughters				
Moderate/light	0.283	0.045	0.015	
Vigorous/moderate	0.123			
BMD-SD in mothers, 1 SD↑	0.294	<0.001	0.073	

SD standard deviation values calculated for each age category in this study

well as BMD-SD, age at menarche, height-SD, body weight-SD, frequency, duration and intensity of exercise in the mothers as candidate explanatory factors for BMD-SD in the post-menarche daughters, for analysis by using stepwise multivariate regression analysis. BMD-SD in the pre-menarche daughters was affected independently by BMD-SD ($R^2 = 0.069$, $P = 0.033$) in the mothers, height-SD ($R^2 = 0.199$, $P = 0.001$) in the daughters (model $R^2 = 0.340$, Table 4 (1). BMD-SD in the post-menarche daughters was influenced by BMD-SD ($R^2 = 0.073$, $P < 0.001$) in the mothers, age at menarche ($R^2 = 0.020$, $P = 0.001$), height-SD ($R^2 = 0.022$, $P < 0.001$), body weight-SD ($R^2 = 0.081$, $P < 0.001$) and intensity of exercise ($R^2 = 0.015$, $P < 0.045$) in the daughters (model $R^2 = 0.372$, Table 4 (2)).

Interaction between body weight-SD or intensity of exercise and BMD-SD in the post-menarche daughters

The mean BMD-SD values for the four categories of body weight in quartile analysis are presented in Fig. 1. The mean BMD-SD values for the third and fourth quartiles were positive, with the BMD-SD shown to be highest in the fourth quartile. Furthermore, the participant categories stratified by maximum intensity of exercise and their mean BMD-SD values are shown in Fig. 2. The mean BMD-SD values in the groups reporting level 2 intensity (moderate) and level 3 intensity (vigorous) were positive, with the BMD-SD shown to be highest in those reporting level 3 intensity.

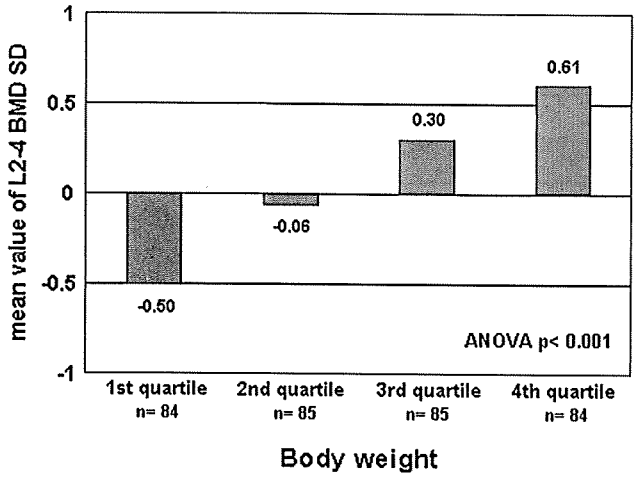

Fig. 1 Quartiles for body weight and mean lumber bone mineral density in post-menarche daughters. Mean lumber bone mineral density was significantly higher in the third and fourth quartiles than in the first and second quartiles (ANOVA, $P < 0.001$)

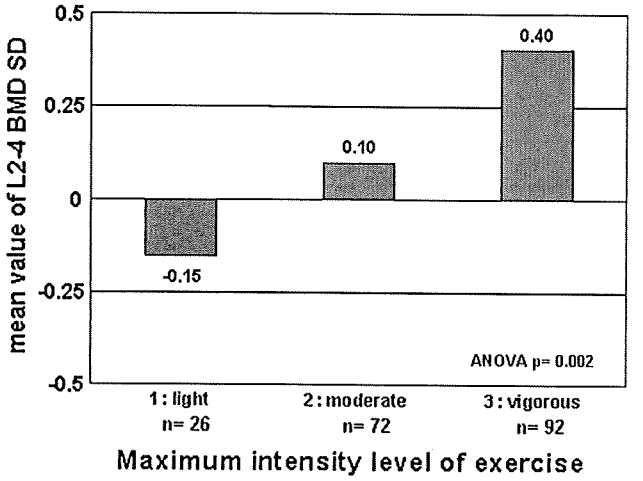

Fig. 2 Maximum intensity level of exercise and mean lumber bone mineral density in post-menarche daughters. Lumbar bone mineral density was significantly higher in those reporting intensity level 2 and 3 exercise than in those reporting intensity level 1 exercise (ANOVA, $P = 0.002$)

Discussion

To avoid steep decreases in BMD after menopause and the associated increased risk of fractures, it is assumed to be effective to achieve high peak bone mass [24]. Both heritable congenital and acquired factors are reported to affect peak bone mass [9, 10], and it is deemed useful to determine how to control these two factors. While the heritability rate for BMD has been reported in previous studies variously as ranging between 40 and 60%, at least there is no doubt that heritability plays a large role in determining BMD [25, 26]. While there are a number of reports showing a significantly positive correlation in bone mass between mothers and their children [27–29], Bounds et al. [30] reported that such positive correlation was only slightly significant in their study, further showing that both the BMC and BMD of the total hip in the mothers were not correlated with those in their children in a multivariate model, where some other factors, including height, body weight, age and sex, were taken into account. However, in another study, some other factors, including height and body weight, were also shown to be genetic constituents [31]. On the other hand, BMD gain is known to peak during puberty, and intervention through nutrient intake and physical activity during this period is likely to further increase this BMD gain [19–21]. In this study, we investigated if there might be any correlation in BMD, birth- and menarche-related data, and lifestyles between adolescent daughters and their mothers to identify factors influencing the BMD in the daughters as well as their individual influence.

BMD-SD values in both the pre- and post-menarche daughters were significantly correlated with those of their mothers, and the maternal influence on both periods were almost comparable with the R^2 value for the pre-menarche and post-menarche groups shown to be 0.069 and 0.073, respectively (Table 4). In a cross-sectional study in early adolescent daughters and their pre-menopausal mothers ($n = 72$) in which femoral neck and lumbar vertebra BMD were examined for correlation, the heritability rate for femoral neck and lumbar vertebra BMD was shown to be 0.56 and 0.70, respectively [21]. As the heritability rate is calculated as $R \times 2$, the heritability rate for lumbar vertebra BMD in this study using the multiple regression value, the $R \times 2$ was estimated as 0.53 and 0.54 in the pre- and post-menarche daughters, respectively, thus showing a similar maternal influence on both groups, in agreement with previous reports.

Although there was a significant correlation in mean age at menarche between the daughters and their mothers, but menarche occurred significantly earlier in the daughters than in their mothers, suggesting that acquired environmental factors also possibly affected age at menarche in the daughters, while congenital factors were assumed to have had a greater role. We showed that age of menarche independently affected BMD in the daughters, with the highest value of BMD shown in those in whom menarche occurred earliest at the age of 10, given that menarche normally occurs during 10–15 years of age (ANOVA, $P < 0.05$; data not shown). These results suggest that greater age at menarche may be a risk factor for low peak bone mass. There is no discrepancy between this hypothesis and the study results reported above, as this finding has to do with the duration of exposure to estrogen.

Height correlated between the daughters and their mothers without regard to presence of menarche, and

represented an independent explanatory factor for BMD. On the other hand, body weight correlated only between the post-menarche daughters and their mothers. Even though height significantly correlated with body weight, this correlation was found to be weak at 14 years of age or older (data not shown). As the growth of height was not noted beyond the age of 14 years, height was thought to correlate with BMD only before menarche.

In regard to the lifestyle factors examined, of all nutrients, only intakes of vitamin D and vitamin K correlated between the pre-menarche daughters and their mothers, but exercise habit did not. Moreover, none of these nutritional factors were correlated with BMD-SD. On the other hand, all dietary and exercise habits correlated between the post-menarche daughters and their mothers. However, exercise habit was the only factor that correlated with BMD-SD, indicating that exercise habit more strongly affected BMD than dietary habit. Calcium intake did not significantly affect BMD-SD. The daughters had a mean calcium intake of 593.7 mg/day, a lower value than that reported in other studies showing the usefulness of calcium intake [12–15]. As per the report of Kelly et al. [32] demonstrating that the availability of calcium may play a permissive role in allowing the skeleton to respond to both genetic and other environmental influences, such as physical activity, calcium may have affected our subjects to a lesser degree, given that physical activity was thought to be higher in them. Additionally, since both dietary and exercise habits correlated with BMD, it is possible that exercise habit may have affected BMD in the subjects to a greater degree, given their dietary intake, which was thought to be more or less adequate.

In this study, it was shown that intensity of exercise independently correlated with BMD-SD. Many studies have reported the influence of physical activity on BMD in young adults, especially that of high-impact exercises [16–18], which appears to be an established consensus. We aimed in this study to establish a simple questionnaire-based assessment tool. This tool was shown to be highly useful at home and school, as it helped to demonstrate that the influence of exercise habit on BMD-SD was most conspicuous in those who reported level 3 (vigorous) exercise, suggesting that vigorous exercise may lead to higher peak bone mass.

Study findings suggest that future peak bone mass in daughters may be predicted on the basis of BMD measurements in their mothers. Additionally, a greater age at menarche was thought to lead to shorter exposure to estrogen, thus representing a risk for decreased peak bone mass. Furthermore, it was suggested that controlling body weight against normal weight values may be a useful measure in intervening through lifestyle modification aimed at increasing peak bone mass and that maintaining high-intensity exercise may be very important in achieving maximum peak bone mass.

In conclusion, in this study a strong correlation was confirmed between daughters and their mothers in their BMD and lifestyle factors. And especially, it is suggested that the BMD in the post-menarche daughters may be affected by maternal BMD, age at menarche, body weight and physical activity. Of these factors, body weight and exercise are amenable to intervention. Therefore, controlling adequate weight and maintaining high-intensity exercise may help to achieve higher peak bone mass.

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ORIGINAL ARTICLE

The impact of lifestyle factors on serum 25-hydroxyvitamin D levels: a cross-sectional study in Japanese women aged 19–25 years

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Abstract Insufficient levels of serum 25-hydroxyvitamin D [25(OH)D] lead to low bone mineral density (BMD) by increasing serum levels of intact parathyroid hormone (PTH), and are associated with a high mortality rate. Therefore, the 25(OH)D level is used as an indicator of frailty in older persons. To obtain higher serum 25(OH)D levels, management of lifestyle habits and nutrient intake is important beginning in a person's younger years. This study evaluated the degree of association between serum 25(OH)D concentrations and lifestyle factors in young Japanese women. A cohort study was conducted from December 2003, and the survey was finished by February 2004. The subjects were 274 Japanese women aged 19–25 years old. The parameters evaluated in these subjects included: (1) serum concentrations of 25(OH)D, intact PTH, calcium, and phosphorus; (2) BMD in the lumbar spine and hip; and (3) lifestyle factors (nutrient intake, physical activity, and duration of sunlight exposure). The serum 25(OH)D level was negatively associated with the intact PTH level (Spearman; $r = -0.17$, $P = 0.006$). The BMD was significantly higher in the high 25(OH)D and low intact PTH group than the other group ($P < 0.05$). The serum 25(OH)D level was significantly correlated with daily intake of dietary vitamin D ($r = 0.20$, $P = 0.001$), the mean number of steps taken per day ($r = 0.16$, $P = 0.010$) and the mean time spent in sedentary activity ($r = -0.14$, $P = 0.018$) among the lifestyle factors evaluated. Multiple regression analysis showed the degree of association between lifestyle factors and serum 25(OH)D to be small ($R^2 = 0.084$). Daily intake of dietary vitamin D and daily walking may be useful for increasing the serum 25(OH)D level in young Japanese women.

Keywords 25-Hydroxyvitamin D · Nutrient intake · Physical activity · Japanese women

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Introduction

Vitamin D in the body is primarily produced in basal epidermis by ultraviolet radiation, and it is also supplied from intake of foods [1, 2]. Vitamin D is then transported to the liver where it is metabolized to 25(OH)D. 25(OH)D is converted in the kidneys to active 1,25 dihydroxyvitamin D [1,25$(OH)_2$D], and exerts its effects as a bone metabolic hormone with intact parathyroid hormone (PTH) [3].

It is well established that serum 25(OH)D concentration is the best clinical indicator of vitamin D status. A lower level of serum 25(OH)D leads to bone fractures [4, 5] and serum 25(OH)D concentration is reported to fall [6] in the elderly, with the vitamin D deficiency being common in the

elderly in the range of 30–90% in the Western population [4, 7]. The serum 25(OH)D concentration is related to lifestyle factors such as vitamin D intake and sunlight exposure [8, 9], and the circulating 25(OH)D level serves as an indicator of vitamin D sufficiency [10].

In a previous study in a Japanese population, 4.6% of the subjects had low serum 25(OH)D levels in the peri-/postmenopausal period [11], but women in their twenties had significantly lower serum 25(OH)D concentrations than those in their thirties and older [12], and 40.3% of the subjects had vitamin D insufficiency as college students [13]. Additionally, low serum concentrations of 25(OH)D and high serum concentrations of intact PTH were found to predispose young individuals to low bone mineral density (BMD) [13].

It is reported that the primary dietary sources of vitamin D in food are fish and eggs in Japanese [14], but there is no report on the relationship between 25(OH)D and nutrient intake or other lifestyle factors in Japanese women.

The aim of this study was to clarify the relationship between 25(OH)D concentrations and lifestyle factors, such as nutrient intake, physical activity and duration of exposure to sunshine, in young Japanese women.

Subjects and methods

Study participants

The present Kawada-cho Peak Bone Mass Study is a cohort study in young Japanese women in Tokyo, Japan [15]. The participants consisted of healthy female volunteers who were students at the School of Nursing (college-degree four-year course) and the Nursing Vocational School (non-college-degree three-year course) of Tokyo Women's Medical University, Tokyo, Japan. We obtained written consent from 348 candidate study subjects who agreed to participate voluntarily. Participants were excluded if they had systemic or metabolic disorders or medications with known effects on bone metabolism and had abnormalities in hormonal regulation or nutritional habits, including menstrual disturbance and eating disorders. Of these, 274 women finally participated in the study. The study protocol was approved by the Ethics Committee of Tokyo Women's Medical University School of Medicine.

Study design

The baseline survey was carried out from December 2003 to February 2004. Each participant completed a questionnaire about background information including age, weight, birth weight, age at menarche, and current menstrual status, along with the questionnaires described below.

Laboratory assessments

All blood samples were taken when the participants gathered to receive the questionnaires, and to undergo blood chemistry tests for serum calcium, phosphorus, and albumin. Serum 25-hydroxyvitamin D concentrations were determined by the Nichols Advantage Chemiluminescence protein-binding assay (CLPBA) method [16]. Intact PTH was measured as a marker for vitamin D insufficiency by using a two-site immunoradiometric assay (Nichols Institute Diagnostics).

Bone mineral density measurements

BMD at the lumbar spine (L2–L4) was measured by dual-X ray absorptiometry (DXA) using the QDR-4500 absorptiometer (Hologic Inc, Bedford, MA). The manufacturer's lumbar spine phantom was scanned daily for quality control and to correct for instrument drift. As previously reported, coefficient of variation in our measurements was <0.7% for the day-to-day quality control scans. BMD was reported as grams per square centimeter.

Lifestyle factors

Assessment of food and nutrient intakes

Dietary habits during the past month were assessed with a validated, self-administered Diet History Questionnaire (DHQ) [17, 18], which was completed by each participant at home and was checked by ≥2 dietitians. The DHQ is a 16-page structured questionnaire that consists of the following 7 sections: general dietary behavior, major cooking methods, frequency of consumption of 6 alcoholic beverages as well as their portion sizes, semi-quantitative frequency of intake of 121 selected foods and nonalcoholic beverage items, dietary supplements, frequency of consumption of 19 staple foods (rice, bread, noodles, and other wheat foods) and *miso* (fermented soybean paste) soup as well as their amounts, and open-ended food items consumed regularly (≥1 time/week) not listed in the DHQ. The food and beverage items and portion sizes in the DHQ were derived primarily from data in the National Nutrition Survey of Japan and several recipe books on Japanese dishes [17]. Dietary intake of 147 food and beverage items, energy, fat, total carbohydrate, alcohol, and dietary fiber were calculated by using an ad hoc computer algorithm developed for the DHQ, which was based on the Standard Tables of Food Composition in Japan [18].

Information on dietary supplements and data from the open-ended questionnaire items were not used for calculation of dietary intake. Detailed descriptions of the methods used for calculating dietary intake and the validity

of the DHQ were published elsewhere [17, 19]. Spearman's correlation coefficients between the DHQ and the 3rd estimated dietary records were 0.48, 0.48, 0.55, and 0.48, respectively, for energy, protein, fat, and carbohydrate in 47 women [17]. In addition, Pearson's correlation coefficients between the DHQ and the 16th semi-weighed dietary records were 0.32, 0.30, 0.52, 0.46, 0.43, 0.30, and 0.40, respectively, for energy, protein, fat, carbohydrate, calcium, phosphorus, and vitamin D in 92 women, with the Spearman's correlation coefficients being 0.39, 0.65, and 0.32, respectively, for fish, meats, and eggs (unpublished observation, S. Sasaki, 2006). For analysis of intake levels, we used energy-adjusted values, i.e., percentage of energy accounted for by protein, fat, and carbohydrate, and amount per 1000 kcal of energy for other nutrients and foods.

Assessment of physical activity

JALSPAQ Information about the subjects' participation in exercises, household activities, walking and cycling for transportation, as well as their occupational type were assessed with a self-administered Japan Arteriosclerosis Longitudinal Study Physical Activity Questionnaire (JALSPAQ). JALSPAQ is a 2-page structured questionnaire that consists of the following five activities and four additional questions: sleep, work related activities, traveling to and from places (walking and cycling), housework (cooking, laundry, cleaning, caring for one's children and elderly), exercise and non-exercise leisure time activities.

Data on leisure time activities were collected from free-response items. Questions included: (1) exercise duration per session; (2) frequency of sessions per month; and (3) intensity of sessions. Activities were coded with the Compendium of Physical Activity [20, 21], which reflects the type and MET intensity of each activity.

Summary estimates of physical activity energy expenditure were calculated in terms of standard metabolic equivalents (METs) as MET-hours/day. MET values were obtained by multiplying the hours spent on each of the categories evaluated and the products summed to give kilocalories per kilogram per day. Total energy expenditure was estimated as the sum of energy expended in the 5 activity categories. The validity of the JALSPAQ was assessed using a sample of 271 volunteers. The correlation between the 24-h physical activity reported and that reported on the JALSPAQ was 0.36 in men and 0.38 in women. The correlation between the values registered by the uni-axial accelerometer and those reported on the JALSPAQ was 0.38 in men and 0.38 in women.

Accelerometer Lifecorder EX, a uniaxial accelerometer (Suzuken Co. Ltd, Nagoya, Japan), measures acceleration in the vertical direction. The accelerometer was designed to detect movements of the body trunk by being attached to the waist, and to record the number of steps taken and the intensity of physical activity registered on a unique scale of 1–9 at 4-s intervals. Detailed descriptions of the algorithm used for calculating TEE and the validity of the Lifecorder have been published elsewhere [22].

Assessment of exposure to sunlight

The estimated duration of exposure to sunlight was calculated based on the following information obtained from the JALSPAQ: time spent on traveling to and from places (i.e., to work, for shopping) and outdoor leisure time activities considered to involve exposure to sunlight.

Statistical analysis

Continuous variables were expressed as a mean and SD to describe the status of the participants. To evaluate the relationship between serum 25(OH)D, intact PTH concentrations and the lumbar spine (L2–L4) BMD, Wilcoxon's rank sum test was used. The participants were then divided into four groups by median values for serum 25(OH)D and intact PTH concentrations. All continuous variables of interest (background information, physical activity, and nutrient intake) were analyzed for correlation with serum 25(OH)D concentrations, using Spearman's rank correlation coefficient and stepwise multiple regression analysis. All statistical analyses were performed by using the JMP (Japanese version 5.1.2, SAS Institute, Cary, NC).

Results

The participant characteristics are presented in Table 1. The serum calcium concentration was significantly positively correlated with the serum 25(OH)D concentration (Spearman; $r = 0.23$, $P < 0.001$), and was also inversely correlated with the serum intact PTH concentration (Spearman; $r = -0.21$, $P = 0.001$,). The serum 25(OH)D and intact PTH concentrations were significantly inversely correlated (Spearman; $r = -0.17$, $P = 0.006$, Fig. 1). Other background characteristics (age, birth weight, age at menarche, and BMI) were not correlated with 25(OH)D or intact PTH. Seventy-six participants were found to comprise the group showing the high 25(OH)D ($\geq$median of 18.0 ng/mL) and low intact PTH ($<$median of 40.3 pg/mL) concentrations, and were considered to combine the most appropriate conditions. The lumbar spine BMD was significantly higher in the high 25(OH)D and low intact PTH group ($n = 76$, mean $\pm$ SD $= 1.02 \pm 0.10$ g/cm^2) than the other group ($n = 198$, 0.99 ± 0.11 g/cm^2: Wilcoxon,

Table 1 Basic characteristics of the participants[a]

Item	Value	Range
Age (y)	20.6 ± 1.4	19–25
Birth weight (g)	3143.2 ± 446.5	1800–4800
Age at menarche (y)	12.0 ± 1.3	9–17
BMI (kg/m^2)	21.2 ± 2.7	15.2–31.2
BMD (g/cm^2)		
Lumbar spine (L2–L4)	1.00 ± 0.11	0.74–1.30
Total proximal femur	0.90 ± 0.10	0.63–1.24
Intact parathyroid hormone (pg/mL)	40.5 ± 11.6	15.0–71.1
25(OH)D (ng/mL)	18.7 ± 4.8	5.8–32.3

[a] All values are mean ± SD; range in parentheses. $n = 274$ except for birth weight ($n = 261$) and age at menarche ($n = 272$)

BMD bone mineral density, *25(OH)D* 25-hydroxyvitamin D

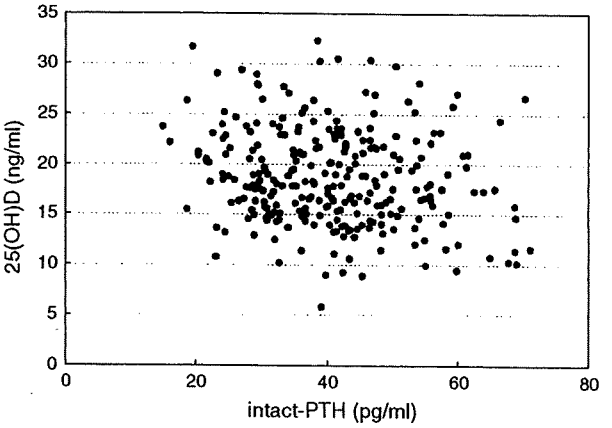

Fig. 1 The relationship between the serum intact PTH and 25(OH)D concentrations. The serum intact PTH and 25(OH)D concentrations were significantly inversely correlated (Spearman; $r = -0.17$, $P = 0.006$)

$P = 0.038$). The same results observed in the hip BMD (0.92 ± 0.10 vs. 0.89 ± 0.10 g/cm^2; $P = 0.049$). Age, BMI, serum phosphorus, serum bone metabolic markers, birth information and age at menarche were not significantly different between the two groups ($P > 0.05$).

Analysis of the values for lifestyle factors is shown in Table 2. To exclude the influence of intake volume, nutritional intake values were stratified by total consumption calories. The mean daily energy expenditure as calculated from the JALSPAQ questionnaire was 1786.8 ± 300.7 kcal, and was found to be consistent with the accelerometer values.

Of the nutrients examined, vitamin D (including supplements) and fish showed a significant correlation with the

Table 2 Daily nutrient intake and physical activity

Item	Value	Range
Energy, and selected nutrient and food intakes assessed by the DHQ		
Energy (kcal/day)	1863.4 ± 629.3	685.9–6134.3
Proteins (% of energy)	13.4 ± 2.4	4.3–21.7
Fat (% of energy)	28.9 ± 6.0	12.8–46.5
Carbohydrates (% of energy)	54.5 ± 6.9	15.0–80.2
Calcium (mg/1000 kcal)	424.1 ± 207.9	76.9–1508.1
Vitamin D (μg/1000 kcal)	9.9 ± 6.7	0.4–46.4
Fish (g/1000 kcal)	46.0 ± 36.7	0–279.3
Egg (g/1000 kcal)	20.0 ± 15.6	0–111.2
Physical activity		
As assessed by the JALSPAQ		
Total energy expenditure (METs-h/day)	33.3 ± 2.6	29.1–48.3
Sleep (h)	6.4 ± 1.2	4.0–12.0
School curriculum (h)	5.1 ± 1.3	0–10.7
Traveling to and from places (h)	1.2 ± 0.8	0.2–4.5
Housework (h)	0.8 ± 0.7	0–3.6
Exercise (h)	0.1 ± 0.2	0–1.5
Leisure (h)	0.4 ± 0.8	0–4.9
Sedentary activity (h)	10.0 ± 2.1	3.1–17.3
As assessed by the accelerometer[a]		
Total energy expenditure (kcal/day)	1820.4 ± 171.0	1364.0–2300.0
Energy expenditure for exercise (kcal/day)	222.3 ± 75.8	56.0–497.0
Steps (steps/day)	8839.5 ± 2638.8	2273–18022
Exposure to sunlight[b] (h/day)	1.2 ± 0.8	0.17–4.5

All values are mean ± SD; $n = 274$ except for accelerometer ($n = 267$), daily time allocation ($n = 273$)

DHQ the Diet History Questionnaire, *JALSPAQ* the Physical Activity Questionnaire by the Japan Arteriosclerosis Longitudinal Study

[a] Lifecorder EX, a uniaxial accelerometry sensor by Suzuken Co., Ltd

[b] Duration of exposure to sunlight was calculated from the questionnaire responses: amount of time spent on traveling to and from places (i.e., to work, for shopping), outdoor leisure time activities considered as involving exposure to sunlight

serum 25(OH)D concentration ($P < 0.05$; Table 3). The mean intake of vitamin D was significantly correlated with the intake of fish (Spearman; $r = 0.74$, $P < 0.001$).

Of the items examined for physical activity, the mean daily energy expenditure on exercise and the number of steps taken per day as calculated based on the accelerometer were significantly associated with the serum 25(OH)D concentration. Analysis of the daily time allocation showed that the mean time spent in sedentary activity was negatively correlated with the serum 25(OH)D concentration.

The estimated duration of exposure to sunlight as calculated from the time spent on outdoor activities showed no significant association with the serum 25(OH)D concentration.

The vitamin D intake, the steps taken per day and the time spent in sedentary activity were chosen for stepwise multiple regression analysis, with the 25(OH)D concentration as the outcome variable ($P < 0.05$). As a result, each of these factors was found to significantly impact the 25(OH)D concentration (Table 4), while the r values were small.

Table 3 Correlation coefficients (r) for serum 25(OH)D levels versus lifestyle factors

Variable	r	P
Selected nutrient and food intakes assessed by the DHQ		
Calcium (mg/1000 kcal)	0.11	0.077
Vitamin D (μg/1000 kcal)	0.20	0.001
Fish (mg/kcal)	0.18	0.002
Egg (g/1000 kcal)	0.07	0.249
Physical activity		
As assessed by the JALSPAQ		
Total energy expenditure (METs-h/day)	0.08	0.164
Sedentary activity (h)	−0.14	0.018
As assessed by the accelerometer[a]		
Total energy expenditure (kcal/day)	0.07	0.265
Energy expenditure for exercise (kcal/day)	0.15	0.016
Steps (steps/day)	0.16	0.009
Exposure to sunlight[a] (h/day)	0.04	0.487

Spearman's rank correlation coefficient

DHQ Diet History Questionnaire, *JALSPAQ* the Physical Activity Questionnaire by the Japan Arteriosclerosis Longitudinal Study

[a] Duration of exposure to sunlight was calculated from the questionnaire responses: amount of time spent on traveling to and from places (i.e., to work, for shopping), outdoor leisure time activities considered to involve exposure to sunlight

Table 4 Lifestyle factors showing significant correlation to serum 25(OH)D

Variable	Parameter estimate	Standard estimate	P	R^2	Model R^2
Vitamin D (μg/1000 kcal)	0.258	3.724	0.001	0.037	0.084
Steps (number/day)	0.000	2.147	0.010	0.024	
Sedentary activity (h)	−0.287	−2.039	0.038	0.015	

Stepwise multiple regression analysis

Discussion

Vitamin D and PTH have an important role in controlling the plasma calcium concentration. Any fall in the ionized calcium concentration is detected by the calcium receptor of the parathyroid gland, followed by the secretion of PTH by the parathyroid gland. PTH then activates vitamin D production, which in turn promotes calcium absorption from the intestines, increases bone resorption by the osteoclasts and compensates for the plasma calcium concentration which is accompanied by the reduction of calcium accumulated in the bone [23].

Insufficient intake of vitamin D is known to cause untoward conditions, such as secondary hyperparathyroidism and decreased BMD [3], and vitamin D deficiency is known to be a significant risk factor for osteoporosis and secondary hyperparathyroidism. Vitamin D, as it results from both cutaneous production and from dietary intake, reflects the conditions of daily living. Around 80–90% of (the precursor of) vitamin D is absorbed through the intestines or produced at the skin through exposure to sunlight, becoming a biologically active hormone after hydration [24]. It is thus recommended that hands, face and arms, or arms and legs, be exposed to sunlight for a period equal to 25% of the time required to cause a light pinkness to the skin [25]. Vitamin D intake varies from country to country [14]. The standard value recommended for intake of dietary vitamin D is 5 μg for 15–18-year-olds in Japan.

Serum 25(OH)D concentration is the best clinical indicator of the vitamin D concentration in blood. The serum 25(OH)D concentration is lower in the elderly [26, 27], lower in women than in men [27] and lower in winter than in the other seasons [1, 25]. Low concentrations of 25(OH)D, defined as below 25 nmol/L, lead to an increase in the serum PTH concentration and to increased bone resorption [2]. Insufficiency of 25(OH)D in youth is associated with low BMD of the forearm [28] and hampers acquisition of maximum peak bone mass at the lumbar spine [29]. In addition, it is reported in a study evaluating BMD of the calcaneus that low levels of 25(OH)D may adversely affect bone strength [12].

In this study, we measured serum 25(OH)D levels using Nichols Advantage CLPBA. It detects serum 25(OH)D2 with much less sensitivity than serum 25(OH)D3. In Japan, vitamin D2 preparations are not prescribed for patients and vitamin D2 supplements are less used. Furthermore, we had

reported that the ratio of 25(OH)D2 to total serum 25(OH)D in Japanese was extremely small [30]. Therefore, there is no doubt that the 25(OH)D2 levels as measured on the Nichols Advantage did not affect our study results.

We investigated the association between serum 25(OH)D, intact PTH levels and BMD. The serum 25(OH)D concentration is negatively correlated with intact PTH. The low intact PTH and high 25(OH)D group showed higher serum calcium concentrations and BMD than the other group. Background data including age, BMI, serum parameters and birth information were not significantly different between the two groups. High 25(OH)D levels were assumed to control the intact PTH level, and to contribute toward an increase in calcium absorption and, consequently, in BMD.

Analysis of the lifestyle factors showed that exposure to sunlight had no impact on serum 25(OH)D. Previous study reports indicated positive correlation between sunlight exposure and serum 25(OH)D [24, 25]. But this study indicated no correlation between them. We estimated the reasons for this discrepancy as follows. First, the amount of vitamin D synthesis by sunlight reaches the upper limit of normal in Tokyo, at 35° north latitude [26]. Furthermore, Hollis et al. reported that an adequate UVB exposure level (18–20 mJ/cm^2) in sunlight to induce pre-vitamin D on the epithelium is not generally reached during winter in the northern United States above latitude 40° [10]. Second, the measurement of sunlight exposure time may have some methodological problems. However, our results showing no association between the estimated time of exposure to sunlight and the serum 25(OH)D level did not contradict the positive correlation between sunlight exposure and serum 25(OH)D. Landin-Wilhelmsen et al. have reported that physical activities are often associated with being outdoors, and active individuals should therefore have a better chance of having sun exposure [8]. On the contrary, our study showed that there was no significant correlation between sunlight exposure and serum 25(OH)D levels. We might speculate that our participants may have applied some ultraviolet protection cosmetics when they exercised, though we did not check on it. That's likely the reason why only physical activities correlated with 25(OH)D.

Dietary intake of vitamin D (including supplements) and fish had an impact on serum 25(OH)D (Table 3). The participants consumed 56.9 ± 45.4 g of fish per day, which was found to be significantly correlated with vitamin D. The steps taken per day or energy expenditure on exercise had a positive impact, while the time spent in sedentary activity (watching TV, playing computer games) had a negative impact on serum 25(OH)D, suggesting that physical activity acted in an additive manner with vitamin D intake in Japanese young women. Although there have been reports showing correlation between physical activity and serum 25(OH)D [8, 9], the present study was too small to draw any conclusion in this regard. Calcium is the most abundant of minerals available in the human body, of which 99% is found in bone with the rest in blood and muscle. Vitamin D participates in the contraction of muscle and is known to maintain myodynamia by transporting calcium from bone to muscle when it is calcium-deficient. Moreover, Kwon et al. reported that concomitant low serum albumin and vitamin D levels are associated with decreased muscle strength and balancing capability in elderly people [21].

The present study had several limitations. First, this cohort study was confined in geographical coverage to Tokyo only. Therefore, the distribution of the research parameter sunlight exposure could have been narrow. Second, participants were only students or nurses by occupation, possibly suggesting a similar lifestyle pattern among the participants. And third, since sunlight exposure was estimated from the JALSPAQ, the use of ultraviolet protection cosmetics was not able to be ruled out.

However, this is the first report investigating the association between the impact of lifestyle factors and serum 25(OH)D levels in Japanese young women which appears to partially explain the correlation between the steps taken per day and the serum 25(OH)D level. Further research is needed to verify the reported correlation between physical activity and serum 25(OH)D.

In conclusion, the serum 25(OH)D concentration was positively affected by dietary vitamin D or fish intake and the mean steps taken per day or energy expenditure on exercise, and was negatively affected by the time spent in sedentary activity. These findings may suggest that lifestyle modification at an early age may contribute to preventing osteoporosis or frailty in later years.

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Variations in Circulating Osteoprotegerin and Soluble RANKL during Diurnal and Menstrual Cycles in Young Women

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Key Words
Bone turnover markers · BAP · NTX · Osteoprotegerin · RANKL

Abstract
Background/Aims: Physiological bone turnover shows diurnal variations and changes within the menstrual cycle. The aim of this study was to assess the variability of osteoprotegerin (OPG) and soluble RANKL (sRANKL) serum levels during diurnal and menstrual cycles. ***Method:*** Blood was collected from 15 young women at 6-hour intervals between 08.00 and 20.00 h during the follicular phase. Moreover, to compare the follicular and luteal phases, blood was also collected at 14.00 h during the luteal phase. Serum bone-specific alkaline phosphatase (BAP), N-telopeptide of type I collagen (NTX), OPG and free sRANKL were measured. ***Results:*** No diurnal variations in BAP, OPG, sRANKL and sRANKL/OPG ratio were detected. NTX was significantly higher in the morning than in the afternoon and at night ($p = 0.02$). There were no menstrual variations in either. ***Conclusions:*** The consistent absence of diurnal variations in circulating OPG and sRANKL levels may reflect the absence of diurnal variation in their expression in the bone microenvironment. In this case, the nocturnal rise and the fall in bone resorption in the luteal phase should be accounted for by other factors than RANKL/OPG-mediated factors. Timing of sampling is unlikely to influence the results of circulating OPG and sRANKL measurement.

Introduction

Since its discovery, the signaling system consisting of RANK ligand (RANKL), its receptor RANK and its decoy receptor osteoprotegerin (OPG) has been identified as an essential regulator of bone resorption and bone mass [1–3]. OPG, a soluble neutralizing decoy receptor, inhibits osteoclastogenesis by blocking RANKL [3]. This signaling system plays a critical role in a variety of bone and vascular diseases, including osteoporosis, arthritis, Paget's disease and various malignant diseases [4]. Measurement of circulating OPG and soluble RANKL (sRANKL) may have important clinical implications in the diagnosis and management of patients with such diseases [4]. It is therefore important to identify factors affecting the variability of these measurements.

Diurnal variations have been detected in bone turnover [5–7], which is regulated by various endocrine hormones and cytokines. However, only a few studies have addressed the issue of variations in circulating OPG [8–10], and there is only one study reporting on serum sRANKL [10].

Moreover, bone remodeling changes during the menstrual cycle. It is generally accepted that bone resorption is enhanced during the follicular phase and decreases during the luteal phase [11]. Reports concerning variations in circulating OPG [11, 12] and serum sRANKL [12] during the menstrual cycle are also few. The aim of this study was to assess the variability of OPG and sRANKL

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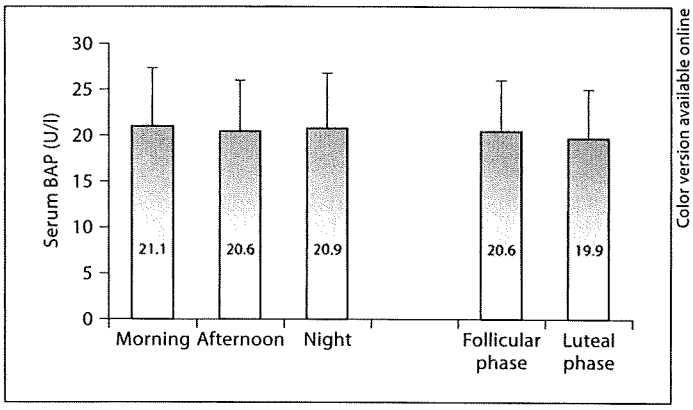

Fig. 1. Variations in serum BAP during the diurnal period (left) and the menstrual cycle (right).

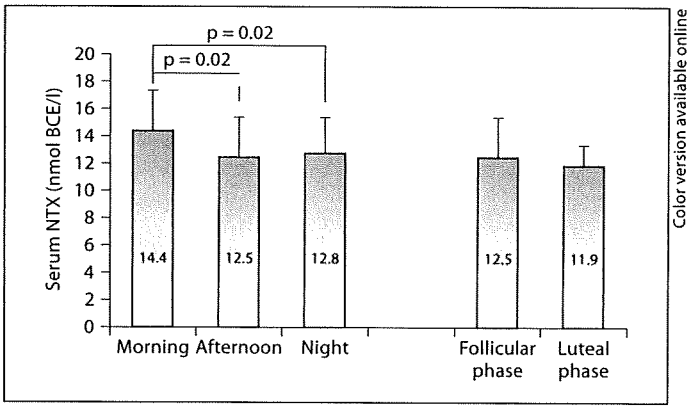

Fig. 2. Variations in serum NTX during the diurnal period (left) and the menstrual cycle (right).

serum levels during the diurnal period and within menstrual cycles in healthy young women whose bone densities were thought to be at their maxima.

Subjects, Materials and Methods

Fifteen healthy young women volunteered to participate in this study (median age, 21.5 ± 1.1 years; range, 20–24 years). The subjects were student nurses with normal menstrual cycles, nonsmokers, had no existing disease and were not receiving any bone-affecting medications. Mean age of menarche was 11.9 ± 1.8 years (range, 9–16 years); mean height, 159.0 ± 4.4 cm; mean weight, 51.9 ± 9.1 kg and mean BMI, 20.5 ± 3.0. Bone mineral density was not assessed since the subjects had no specific risk factors for osteoporosis. The local ethics research committee approved the study and all subjects gave written, informed consent.

Daily basal body temperature was measured in all subjects for 3 consecutive months. After a normal ovulation cycle was confirmed, blood samples were collected at 3 time points, i.e. morning (08:00 h), afternoon (14:00 h), and night (20:00 h) during the follicular phase. Moreover, to allow comparison between the follicular and luteal phases, blood was collected in the afternoon (14:00 h) during the luteal phase.

All subjects had their meals at approximately 07:00 h, 13:00 h, and 19:00 h. They were freely ambulant, but avoided moderate to heavy exercise throughout the 12-hour sampling period; they were not hospitalized during the sampling days and usually returned home or to school between blood collections. The collected specimens were aliquotted and frozen at –80°C after serum separation.

Measurements were made by enzyme-linked immunosorbent assay (ELISA) using the following kits: OPG (osteoprotegerin ELISA™, Immundiagnostik AG, Bensheim, Germany; intra-assay variance, 9.3%; inter-assay variance, 8.0%), sRANKL (RANKL ELISA™, Immundiagnostik AG; intra-assay variance, 9.0%; inter-assay variance, 5.0%) , bone-specific alkaline phosphatase (BAP; osteolinks™, Quidel Corporation, San Diego, Calif., USA; intra-assay variance, 4.0%; inter-assay variance, 3.4%), N-telopeptide of type I collagen (NTX; osteomark NTx serum™, Ostex International Inc., Seattle, Wash., USA; intra-assay variance, 7.8%; inter-assay variance, 13.5%). Furthermore, estradiol (E_2) was measured with ECLIA (Electrochemiluminescence immunoassay, Eclusys™, Roche Diagnostics KK, Tokyo, Japan; intra-assay variance 2.1%; inter-assay variance, 2.3%); follicle-stimulating hormone and luteinizing hormone were measured with CLIA (Chemiluminescence immunoassay, Architect FSH™, Architect LH™, Abbott Japan, Tokyo, Japan; intra-assay variance, 3.7, 4.1%; inter-assay variance, 4.8, 3.5%).

All data obtained were expressed as means ± SD, and SAS software (SAS Institute Inc., Cary, N.C., USA) was used for statistical analysis. Wilcoxon's test was used to examine variations, and correlations were assessed by Spearman's rank coefficient. A p value <0.05 was considered statistically significant.

Results

Daily basal body temperature showed that all subjects had normal ovulation cycles with the menstrual cycle being 29.4 ± 3.1 (25–35) days, the follicular phase being 15.3 ± 1.5 (13–18) days, and the luteal phase being 14.1 ± 3.1 (10–18) days. Blood was sampled at day 8.4 ± 3.9 (days 1–15) of the follicular phase and at day 23.8 ± 5.1 (days 15–35) of the luteal phase (day 8.4 ± 3.7 of the hyperthermic phase).

BAP levels did not differ between morning, afternoon, and night (fig. 1). However, serum NTX levels were significantly higher in the mornings [14.4 ± 3.0 nmol bone collagen equivalents (BCE)/l] than that in the afternoons (12.5 ± 2.8 nmol BCE/l; p = 0.02) and nights (12.8 ± 2.4 nmol BCE/l; p = 0.02) (fig. 2).

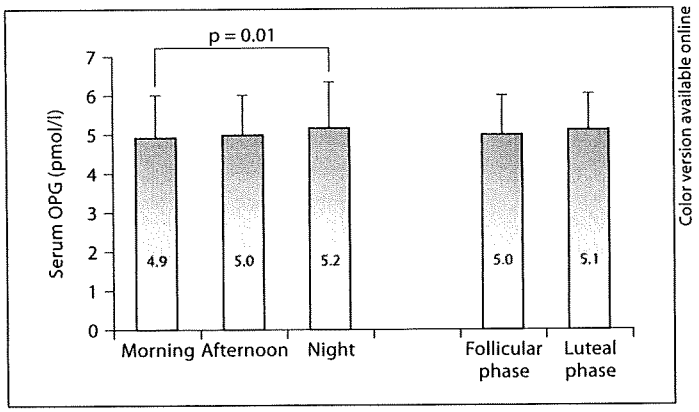

Fig. 3. Variations in serum OPG during the diurnal period (left) and the menstrual cycle (right).

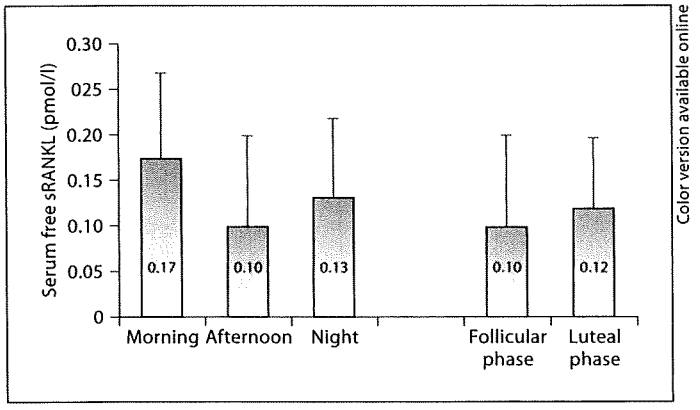

Fig. 4. Variations in serum free sRANKL during the diurnal period (left) and the menstrual cycle (right).

Table 1. Serum levels during the ovarian cycle

Variable	Follicular (n = 15)	Luteal (n = 15)	p value
Cytokines			
OPG, pmol/l	5.0 ± 0.99	5.14 ± 0.92	0.13
Free sRANKL, pmol/l (n = 8)	0.10 ± 0.09	0.12 ± 0.08	0.18
Hormones and biochemical markers of bone metabolism			
Estradiol, pg/ml	65.0 ± 46.7	105.5 ± 66.8	<0.05
FSH, mIU/ml	5.4 ± 1.6	3.0 ± 1.6	<0.05
LH, mIU/ml	5.2 ± 2.2	4.5 ± 3.9	0.26
BAP, U/l	20.6 ± 5.5	19.9 ± 5.1	0.32
NTX, nmol BCE/l	12.5 ± 2.8	11.9 ± 1.5	0.48

All data are expressed as means ± SD. FSH = Follicle-stimulating hormone; LH = luteinizing hormone.

When BAP and NTX levels were compared between the follicular and the luteal phases (20.6 ± 5.5 and 19.9 ± 5.1 U/l, 12.5 ± 2.8 and 11.9 ± 1.5 nmol BCE/l, respectively), no significant variations were noted (fig. 1, 2, table 1). In addition, BAP levels were remarkably stable intra-individually, with a between-phase variation of 7.0% and a correlation coefficient of 0.95 ($p < 0.001$). There were no significant correlations in NTX levels ($p = 0.11$).

OPG levels were significantly higher at night (5.2 ± 1.2 pmol/l) than in the morning (4.9 ± 1.1 pmol/l) ($p = 0.01$) (fig. 3) although the difference (6.1%) did not exceed the inter-assay variance (8.0%). Thus there was no indication of significant diurnal variation. Furthermore, serum levels of sRANKL tended to be higher in the mornings (0.17 ± 0.09 pmol/l) than in the afternoons (0.10 ± 0.10 pmol/l); however, the difference did not reach statistical significance ($p = 0.107$) (fig. 4).

When OPG and sRANKL levels were compared between the follicular and the luteal phases (5.0 ± 1.0 and 5.1 ± 0.9, 0.10 ± 0.09 and 0.12 ± 0.08 pmol/l, respectively), no variations were noted (fig. 3, 4, table 1). In addition, OPG levels were remarkably stable intra-individually, with a between-phase variation of 6.8% and a correlation of 0.92 ($p < 0.001$). There were no significant correlations in sRANKL levels ($p = 0.16$).

Discussion

Previous studies have reported various elements that have affected diurnal variation in OPG values [8–10]. However, in this study, we did not find significant variation in serum OPG and sRANKL levels. Studies reporting variations in circulating OPG and sRANKL during the circadian cycle are few and inconsistent. Joseph et al. [8] found significant diurnal variations with a diurnal increase as well as a nocturnal decrease in OPG in a group of 6 premenopausal women, 6 postmenopausal women and 6 elderly men. Tarquini et al. [9] found no significant diurnal variations in OPG, but found 12-hour cycle variations, with small-amplitude (<5%) peak concentrations being reached around noon and midnight in a group of 6 women and 3 men. Dovio et al. [10] found no significant diurnal variations in OPG and total sRANKL in a group of 20 women. Differences in study design (gender, age,

sampling sequence, assays and data processing) may explain these discrepancies.

In our study, comparison of the follicular and luteal phases of the menstrual cycle did not show any significant variation in both OPG and free sRANKL. Furthermore, OPG showed remarkable intra-individual stability, with no menstrual-cycle-dependent changes, thus suggesting that OPG serum levels were controlled by stable environmental factors.

In agreement with our results, Abrahamsen et al. [11] found no menstrual-cycle-dependent changes in OPG in a group of 11 premenopausal women, while Hofbauer et al. [12] found that OPG and free sRANKL serum levels remained unchanged in a group of 19 premenopausal women. Our results suggest that bone resorption may be suppressed in the luteal phase through a mechanism that does not involve significant changes in serum OPG and sRANKL.

Our study was limited to a group of young women in their follicular phase to prevent the menstrual cycle from affecting diurnal variations. Subject age was also taken into consideration to ensure maximum bone density in the subjects. However, rhythmic changes may still occur in the luteal phase in elderly women or in men. We did not address the effects of food intake, a determinant of diurnal variations in bone resorption, especially in NTX levels. Moreover, blood was collected only a few times, and not at night. Additionally, free sRANKL profiles were investigated using a currently available assay (RANKL ELISA™), but about one third of the sRANKL concentrations obtained were below the assay's reported detection limit; these data were not included in the study. Nonetheless, it is interesting to note that there was a tendency towards higher values in the morning. Finally, while free sRANKL was assessed in this study, no data are available for total sRANKL. sRANKL is present in blood both in free form and as a complex with OPG. Furthermore, sRANKL is unstable, is considered to degrade rapidly, or bind to OPG to form large stable conglomerates [13]. Therefore, potential rhythmic changes in total sRANKL levels cannot be ruled out. In murine models, RANKL is more active in membrane form than in soluble form [14, 15]. The appearance of more sensitive RANKL assays is eagerly awaited.

The results of our study could be related to the long plasma half-lives of the molecules evaluated. However, no data are available on sRANKL, and the data on OPG plasma half-life come from two animal studies. The half-life of OPG has been shown to be approximately 20 min or less than 30 min in rats [16, 17], but no human data are available. If these values are applied to humans, the absence of diurnal variation in OPG levels cannot be explained by its plasma half-life.

The absence of variation could be due to a substantially stable release of these molecules from the bone microenvironment into the bloodstream. If this is the case, the nocturnal rise in osteoclast activity may be mediated by pathways independent of RANKL/OPG. On the other hand, since circulating OPG and sRANKL are derived from several sources [18–23], all studies measuring circulating OPG and sRANKL are also qualified by the multiplicity of their extraskeletal sources and the possible discrepancies between circulating concentrations in the bone microenvironment. These limitations, however, are inherent to all studies assessing circulating concentrations of paracrine agents. Thus, rhythmic changes in gene expression and release of these products into the extracellular space may occur in cells in bone. If feasible, determining the expression of OPG and RANKL mRNA extracted from osteoblastic cells may provide more accurate estimates of variations than those from measurement of plasma concentrations [24–26].

In summary, we report that significant variations in OPG and sRANKL serum levels during diurnal and menstrual cycles in a cross-sectional study of young healthy women were not observed. These findings suggest that the sampling time is unlikely to influence measurement results.

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