สำนักงานกองทุนสนับสนุนการวิจัย
The Thailand Research Fund

รายงานวิจัยฉบับสมบูรณ์

การศึกษาจีโนมิคส์ วิตามินดีและการนวดแผนไทยเพื่อเสริมสร้างสุขภาพกระดูกและความผิดปกติที่เกี่ยวข้อง

โดย ศาสตราจารย์บุญส่ง องค์พิพัฒนกุล
ศาสตราจารย์ปิยะมิตร ศรีธรา
ศาสตราจารย์วิชัย เอกพลากร
รองศาสตราจารย์ประไพศรี ศิริจักรวาล
ผู้ช่วยศาสตราจารย์สมศรี เจริญเกียรติกุล
ดร. ละออ ชัยลือกิจ
รองศาสตราจารย์วัลยา จงเจริญประเสริฐ
แพทย์หญิงหทัยกาญจน์ นิมิตพงษ์
นางสาวสุนีย์ แซ่ตั้ง
นางสาวสุวรรณี ชั้นประเสริฐโยธิน

วันที่เสร็จโครงการ 31 กรกฎาคม 2555

สัญญาเลขที่ RTA5280001

รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาปัจจัยเสี่ยงทางพันธุกรรม ลักษณะทางเมตาลอลิก และเศรษฐศาสตร์สาธารณสุขของโรคเบาหวานชนิดที่ 2 ในคนไทย

ผู้วิจัย

ศ. บุญส่ง องค์พิพัฒนกุล	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
ศ. ปิยะมิตร ศรีธรา	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
ศ. วิชัย เอกพลากร	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
รศ. ประไพศรี ศิริจักรวาล	สถาบันวิจัยโภชนาการ มหาวิทยาลัยมหิดล
ผศ. สมศรี เจริญเกียรติกุล	สถาบันวิจัยโภชนาการ มหาวิทยาลัยมหิดล
ดร. ละออ ชัยลือกิจ	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
รศ. วัลยา จงเจริญประเสริฐ	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
พญ. หทัยกาญจน์ นิมิตพงษ์	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
น.ส. สุนีย์ แซ่ตั้ง	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล
น.ส. สุวรรณี ชั้นประเสริฐโยธิน	คณะแพทยศาสตร์ โรงพยาบาลรามธิบดี มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

CONTENT

Summary

Osteoporosis is a common health problem worldwide. Osteoporosis increases susceptibility to fractures and incurs enormous health and economic burdens. Although preventive measures are conceptually crucial, the efficacy of such effort is less than adequate and case-finding approach to reduce osteoporotic fractures has been more advocated. Innovative preventive measures for osteoporosis are therefore in need. Our previous study has demonstrated the beneficial effect of Thai traditional massage in terms of bone turnover. Further studies to explore utilization of Thai traditional massage as a measure for bone mass maintenance is warranted. Using limited genome-wide screening, our group has also previously demonstrated genetic variations associated with responsiveness to oral calcium in terms of parathyroid hormone suppression and nutrigenomics is likely an approach, which can enhance the effectiveness of calcium in osteoporosis prevention. We therefore propose to extend the study in order to achieve more coverage of the genome and attempt to identify genetic variations more directly associated with responsiveness in bone mass after calcium supplementation.

It is well established that vitamin D influences calcium metabolism and bone health. There are increasing evidences suggesting vitamin D deficiency even in tropical countries including Thailand, which may be partly related to urban lifestyle. Besides its effect on calcium and bone metabolism, vitamin D also possesses biological functions unrelated to calcium and bone and may partly explain the increasing prevalence of certain diseases associated with urbanization. The extent of the problem of inadequate vitamin D status in Thailand is currently unknown. Direct studies to investigate the long-term health effect of vitamin D inadequacy are also scarce. The information is crucial in devising simple strategies to ameliorate the problem of vitamin D inadequacy and adverse health outcomes associated with the disorder. Over the 3-year period of the study, a number of studies and findings have been generated as followed:

1. <u>Vitamin D deficiency in Thais</u> Vitamin D deficiency is common in western countries due mainly to the reduced ultraviolet radiation in sunlight in these geographical regions. In the present study, we found that vitamin D deficiency is not uncommon in Thais particularly those residing in urban areas. The tendency to avoid sun exposure as well as increased urbanization is likely to be the underlying causal factors. It is well established that vitamin D is essential for calcium and bone metabolism. However, it has been increasingly recognized that vitamin D also possesses myriad non-skeletal biological functions. Observational studies have demonstrated the association of vitamin D status with a number of chronic disorders besides

osteoporosis, including cardiovascular diseases, certain cancers, and diabetes mellitus. In the present study, we also demonstrated the association between poor vitamin D status and diabetes as well as thyroid dysfunction in Thais. However, findings of significant associations do not prove causality and large-scale clinical trials to determine casualty are costly. Currently we are addressing the issue by utilizing the Mendelian randomization approach using a single nucleotide polymorphism in the vitamin D binding protein gene as the instrument. The causal role of vitamin D in non-skeletal disorders will have large impact if proved beneficial, even only for a few disorders,

2. Nutrigenomics of calcium supplementation Osteoporosis and osteoporotic fractures are important health problems. Both anti-resorptive and bone-forming agents are widely used for preventing fractures in patients with osteoporosis. However, these drugs are costly and not accessible for a sizable proportion of patients who then need to depend on calcium alone for the treatment of osteoporosis. In the present study, we have identified a number of single nucleotide polymorphisms that are associated with skeletal response to calcium including those in the genes encoding malic enzyme 1 and bone morphogenetic protein 6. Such findings should be useful in targeting calcium supplementation to those who are more likely to respond favorably.

3. Thai traditional massage and bone health The effect of massage therapy on bone metabolism in adults has only scarcely been explored. In the present study, we examined the effect of Thai traditional massage on biochemical markers of bone turnover. Thai traditional massage results in an increase in bone formation as assessed by serum P1NP, particularly in postmenopausal women who are older and have a smaller body build. Thai traditional massage may be an option to promote bone health in this group to individuals.

4. Others New understandings of factors potentially associated with bone mass were explored in this project. Recently, it has been found that uric acid is associated with bone mass in the elderly, which may be due to its antioxidant property. We have identified in the present study the relationship between uric acid and bone mass in both younger males and females. Study to examine the causality of uric acid in this regard is being planned. Moreover, using the Mendelian randomization approach we showed that adiposity is causally related to bone mass which is suggestive of the physiological role of adipose tissue in controlling bone mass.

Keywords: vitamin D, Thai traditional massage, osteoporosis, calcium supplementation

REGIONAL VARIATION AND DETERMINANTS OF VITAMIN D STATUS IN SUNSHINE-ABUNDANT THAILAND

Abstract

Background: Vitamin D insufficiency is highly prevalent. Most of the studies concerning vitamin D status were generated from countries situated at temperate latitudes. It is less clear what the extent of vitamin D insufficiency is in countries situated in the tropics and how geographical regions within country would affect vitamin D status. In the present study, we investigated vitamin D status in Thais according to geographical regions and other risk factors.

Methods: Subjects consisted of 2,641 adults, aged 15 – 98 years, randomly selected from the Thai 4th National Health Examination Survey (2008-9) cohort. Serum 25 hydroxyvitamin D were measured by liquid chromatography/tandem mass spectrometry. Data were expressed as mean ± SE.

Results: Subjects residing in Bangkok, the capital city of Thailand, had lower 25(OH)D levels than other parts of the country (Bangkok, central, northern, northeastern and southern regions: 64.8 ± 0.7, 79.5 ± 1.1, 81.7 ± 1.2, 82.2 ± 0.8 and 78.3 ± 1.3 nmol/L, respectively ($p < 0.001$) respectively; $p < 0.001$). Within each region, except for the northeastern part of the country, subjects living inside municipal areas had lower circulating 25(OH)D (central, 77.0 ± 20.9 nmol/L vs 85.0 ± 22.1 nmol/L, $p < 0.001$; north 79.3 ± 22.1 nmol/L vs 86.8 ± 21.8 nmol/L, $p < 0.001$; northeast 84.1 ± 23.3 nmol/L vs 87.3 ± 20.9 nmol/L, $p = 0.001$; south, 76.6 ± 20.5 nmol/L vs 85.2 ± 24.7 nmol/L, $p < 0.001$). Overall, the prevalence of vitamin D insufficiency was 64.6 %, 46.7 %, and 33.5 % in Bangkok, municipal areas except Bangkok, and outside municipal area in other parts of the country, respectively. In addition, the prevalence of vitamin D insufficiency according to geographical regions was 43.1%, 39.1%, 34.2% and 43.8% in the central, north, northeast and south, respectively. After controlling for covariates in multiple linear regression analysis, the results showed that low serum 25(OH)D levels were associated with being female, younger age, living in urban and Bangkok

Conclusions: Vitamin D insufficiency is common and varies across geographical regions in Thailand.

Background

Vitamin D is produced endogenously when the skin is exposed to sunlight, or obtained exogenously from nutrients or supplements. The major role of vitamin D is to maintain calcium homeostasis and bone health. In addition, recent research has revealed that vitamin D may play an important role in a variety of non-skeletal health activities, such as modulation of neuromuscular and immune function, reduction of inflammation, and regulation of cell proliferation, differentiation and apoptosis. Vitamin D insufficiency is highly prevalent and is now recognized as a worldwide health problem [1-3]. It has been observed to varying degrees in many different countries, regardless of geographical location [4]. However, most of the studies concerning vitamin D status have focused on countries situated at temperate latitudes. It is less clear what the extent of vitamin D insufficiency is in countries situated in the tropics, and how geographical regions within a country could affect vitamin D status.

Thailand, a Southeast Asian country, is located at latitudes between 5°30′ N and 20°30′ N. Vitamin D intake among Thais is generally low because few natural vitamin D-rich food sources are found in Thailand, and foods are not fortified with vitamin D. Up to now, there has been a lack of reliable epidemiological data concerning vitamin D status in Thais. Therefore, the purpose of this study is to investigate vitamin D status in Thais according to geographical region by assessing levels of serum 25-hydroxyvitamin D (which is the major metabolite and represents the stored form of vitamin D) by a reference method, liquid chromatography/tandem mass spectrometry (LC-MS/MS).

Subjects and methods

Population

This study used data from the Thai 4th National Health Examination Survey (NHESIV) conducted between August 2008 to March 2009 by the National Health Examination Survey Office, Health System Research Institute. Subjects aged 15–98 years were randomly selected from 21 provinces in four geographical regions of Thailand as well as the capital city, Bangkok (Fig1) using stratified, multistage probability sampling of the population aged ≥15 years with a sample size of 21,960 individuals. The skin color of Thais are categorized into skin types 4 (Brown)-5(Dark Brown) by the Fitzpatrick Classification Scale, both of which possess the high capability to produce melanin. Demographic data such as age, sex, and religion were included. Body weight and height were measured using standard procedure. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Fasting blood samples were obtained and transferred to a freeze at a central laboratory in Ramathibodi Hospital, a university hospital Bangkok, where they were kept at -80 Cº. The present study used a subsample of the NHES-IV serum samples to measure serum levels of 25-hydroxyvitamin D (25(OH)D). The subsamples were randomly selected according to age group (15-29, 30-44, 45-59, 60-69, 70-79, and ≥80 years), sex, urban/rural and region. In each stratum, 25 individuals were randomly selected using statistical software. A total of 2,700 were sampled of which 2,641 serum samples were available. The study was approved by the ethics committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Serum 25-hydroxyvitamin D (25(OH)D) measurement

Serum 25(OH)D2 and 25(OH)D3 were analyzed by LC-MS/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) using a MassChrom® 25-OH-Vitamin D3/D2 diagnostics kit (Chromsystems, Munich, Germany). The summation of serum 25(OH)D2 and 25(OH)D3 was used to reflect vitamin D status. The inter-assay and intra-assay coefficients of variation of total serum 25(OH)D level were 6.3% and 5.0%, respectively.

Statistical analysis

Data were expressed as mean ± SE. Differences between two groups were assessed by Student's *t*-test. Comparisons among three or more groups were analyzed by analysis of variance followed by Scheffé's test. Stepwise multiple linear regression analysis was used to examine the independent determinants of variables. A p value less than 0.05 was considered statistically significant. All analyses were performed using Stata version 10.1 (StataCorp LP, Texas, USA) and SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA). All the data analyses were weighted to the probability of sampling to take into account for complex survey design.

Results

The Baseline characteristics of the population study were shown in Table 1. The frequency distribution of serum 25(OH)D levels was shown in Fig 2. Data on average duration of sunlight (Table 2) and temperature in Thailand were obtained from the Thai Meteorological Department, for year 2008 and 2009. The minimum and maximum temperature ranged from 6.0 °C to 42.4 °C. Mean serum 25(OH)D levels in Thais according to geographical region are shown in Table 2. Subjects residing in Bangkok had lower 25(OH)D levels than those in other parts of the country, and the mean value was also below the sufficient level (75 nmol/L); whereas subjects residing in the northeastern region had the highest mean serum 25(OH)D level. Within each region, except for the northeastern part of the country, subjects living inside municipal areas had lower circulating 25(OH)D: central, 73.5 ± 1.2 nmol/L vs 82.5 ± 1.7 nmol/L, $p < 0.001$; north 75.6 ± 1.9 nmol/L vs 83.3 ± 1.1 nmol/L, $p < 0.001$; northeast 81.3 ± 1.4 nmol/L vs 82.4 ± 0.9 nmol/L, $p = 0.001$; south, 71.9 ± 1.1 nmol/L vs 80.1 ± 1.3 nmol/L, $p < 0.001$ (Fig. 3). When only municipal areas were analyzed, subjects in Bangkok still had significant lower 25(OH)D levels than the rest of the country ($p < 0.01$) (Fig. 3). In addition to geographical region, there were significant differences in mean serum 25(OH)D levels by gender, age, living in municipal areas, BMI status and religion (Table 2 and Table 3). Moreover, lower serum 25(OH)D levels was observed in younger age (Table 3). Overall, the prevalence of vitamin D insufficiency, as defined by 25(OH)D levels less than 75 nmol/L, in Bangkok was 64.6 %; in municipal areas other than Bangkok, 55.1 %; and outside municipal areas in other parts of the country, 39.1 %. Table 4 shows the prevalence of vitamin D insufficiency at 25(OH)D < 75 nmol/L and 25(OH) < 50 nmol/L by geographical regions and gender . Individuals lived in Bangkok had the highest prevalence of vitamin D insufficiency, whereas people in the northeast had the lowest prevalence. After controlling for covariates in multiple linear regression analysis, the results showed that low serum 25(OH)D levels were associated with being female, younger age, living in urban and Bangkok (Table 5).

Discussion

Our study represents the first large-scale examination of vitamin D status in the Thai population. Despite the fact that Thailand is located near the equator, a sizable proportion of Thais have inadequate vitamin D status. When using a 25(OH)D threshold of 75 nmol/L, nearly half of Thais are vitamin D insufficient. When a lower threshold of 50 nmol/L was used, the prevalence of vitamin D insufficiency was found to be more than 10% in Bangkok, which is as high as the prevalence of diabetes in Thailand [5]. Studies examining vitamin D status in the tropics are scarce, but have mostly demonstrated similarly low vitamin D status. For example, even in the sunniest areas like Saudi Arabia, the United Arab Emirates, Australia, Turkey, India and Lebanon, a high prevalence of vitamin D insufficiency has been reported in 30 to 50% of children and adults, with 25(OH)D levels under 50 nmol/L [6-10]. On the other hand, people living near the equator who are exposed to sunlight without sun protection have robust levels of 25(OH)D, well above 75 nmol/L [11]. Taken together, this suggests that low vitamin D status is not an uncommon problem even in countries that receive abundant sunshine. Despite this, outdoor sun exposure can be limited and is likely to be the main contributing factor.

The mean serum 25(OH)D levels in Thais seem relatively high when compared to those reported in various countries in the West [3, 12], Middle East [13] and Asia [14]. This might be caused by a higher exposure to sunshine all year round, since the latitude of Thailand is more southerly (closer to the equator) than the countries studied. Nevertheless, differences in vitamin D status were found between regions in Thailand; subjects residing in the southern parts of the country, women in particular, generally had lower serum 25(OH)D concentrations than those residing in the northern region. This finding conflicts with the belief that vitamin D status decreases with increasing latitude. However, our results are in agreement with a European study which showed a positive relationship between serum 25(OH)D and northern latitude [4]. This finding could be explained by the common use of cod liver oil and vitamin supplements in many northern European countries; while people in southern Europe typically have more skin pigmentation (with consequently less vitamin D production) and may prefer shade instead of sunshine. An explanation for this observation in Thailand might be regional differences in religion. Southern Thailand has a much higher percentage of Muslims, and the clothing style of Muslim women generally allows for greater body coverage. In addition, most northern Thai people are agricultural. Working in the fields and spending more time outdoors in the sunshine probably accounts for much of this difference.

Both lifestyle and environmental factors are important determinants of serum 25(OH)D concentration because of their relationship to ultraviolet exposure. In the present study, a difference in vitamin D status between populations in rural and urban areas was clearly demonstrated. Lower vitamin D levels in the urban populations were evident in almost all geographical regions of Thailand. Although a number of studies have investigated the vitamin D status of urban or rural residents, the disparity in vitamin D status between rural and urban populations has been investigated less often; but existing studies have generally shown lower vitamin D reserves among urban populations [15-17]. A number of factors may be causally related to lower vitamin D status associated with urbanization. Besides lifestyle factors, which may preclude adequate outdoor sun exposure, it is also likely that air pollution may have a contributory role. Tropospheric ozone is a common urban air pollutant and an efficient absorber of ultraviolet radiation [18]. The phenomenon is likely to be more marked in big cities, and may partially explain why residents of Bangkok, the largest city in Thailand, had the lowest 25(OH)D concentrations.

Lower vitamin D status has been demonstrated to be more prevalent with advancing age in most studies [19-22]; this may be caused by less sun exposure and the decreased ability of the skin to produce vitamin D [23]. In contrast, we demonstrated in the present study that vitamin D levels unexpectedly became higher with increasing age. Younger age, rather than older, was an independent risk factor for inadequate vitamin D status. The phenomenon of higher vitamin D levels with advancing age was also observed in both sexes, making it less likely that the observation was simply a chance finding. Although most studies have demonstrated lower vitamin D levels with advancing age, such findings have been predominantly generated from studies of populations residing in temperate geographical locations. There are only limited data on this issue for countries in the tropics. It was found in a study of postmenopausal women in Malaysia that vitamin D levels do not decrease with age [24]. Likewise, 25(OH)D levels remain more or less constant from age 20 to more than age 60 in Iranian men [25]. It is therefore conceivable that despite the decreased dermal synthesis of vitamin D in the elderly, the abundant sunlight may overcome this disadvantage, given that sun exposure is not limited. The elderly in Thailand, after retirement, may have more leisure time and spend more time in the sun. On the other hand, it is also likely that the increased use of sunblock by younger people may be partly accountable for their lower vitamin D status compared to the older population.

Conclusion

Vitamin D insufficiency is highly prevalent in the general adult population in Thailand. Vitamin D status is better in northern than in southern regions of the country. Low serum 25(OH)D levels were associated with being female, younger age, living in urban and Bangkok.

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Table 1 Baseline characteristics of the population studies

Characteristics	Men (n =1,321)	Women (n = 1,320)	Total
Age (years)	39.6 $\pm$ 0.5	41.0 $\pm$ 0.4	40.3 $\pm$ 0.3
BMI (kg/m^2)	22.7 $\pm$ 0.2	24.4 $\pm$ 0.2*	23.6 $\pm$ 0.1
rural	71.5 %	70.2 %	70.8 %
urban	28.5 %	29.8 %	29.2 %
Religion: Muslim	2.5 %	3.9 %	3.2 %
non Muslim	97.5 %	96.1 %	96.8 %
Region: Bangkok	8.8 %	9.4 %	9.1 %
Central	24.4 %	24.9 %	24.6 %
North	18.3 %	18.4 %	18.3 %
Northeast	34.9 %	33.5 %	34.2 %
South	13.6 %	13.8 %	13.7 %

Values are mean $\pm$ SE or percentage

* Significantly different from the men ($p < 0.001$)

Table 2 Duration of sunshine and mean serum vitamin D levels according to geographical region and gender

Regions	Duration of sunshine (hours/day)	25(OH)D (nmol/L)		
		Men	Women	Total
Bangkok	4.7 – 9.1	69.0 ± 0.6	61.1 ± 1.3*	64.8 ± 0.7‡
Central	4.1 -8.5	86.5 ± 1.7†	73.0 ± 1.1$^{*, \dagger, \ddagger}$	79.5 ± 1.1$^{\dagger, \ddagger}$
North	3.6 – 8.1	88.5 ± 1.7†	75.1 ± 1.8$^{*, \dagger}$	81.7 ± 1.2†
Northeast	3.5 -8.0	87.8 ± 1.3†	76.7 ± 1.1$^{*, \dagger}$	82.2 ± 0.8†
South	2.0 – 8.8	87.7 ± 2.9†	69.5 ± 0.8$^{*, \dagger, \ddagger}$	78.3 ± 1.3$^{\dagger, \ddagger}$
Total	2.0 – 9.1	85.9 ± 1.1	73.0 ± 0.8*	79.3 ± 0.8

Values are range or mean ± SE

*Significantly different from men ($p < 0.001$), † significantly different from Bangkok ($p < 0.001$),‡ significantly different from Northeast ($p < 0.05$)

Table 3 Mean serum 25(OH)D levels between gender by age, municipal area, BMI and religion

Variables		25(OH)D (nmol/L)			
		Men	Women	*p*	Total
Age (years)	15 - 29	79.3 ± 1.3	69.3 ± 1.1	<0.001	74.4 ± 0.9
	30 - 44	89.1 ± 1.7*	70.7 ± 1.2 †	<0.001	79.9 ± 1.1*,
	45 - 59	86.5 ± 1.5*, †	75.3 ± 1.2*, †	<0.001	80.6 ± 1.0*, †
	60 - 69	90.7 ± 1.3*	80.2 ± 1.1*	<0.001	85.1 ± 1.0*
	70 - 79	95.0 ± 1.4*	83.8 ± 1.7*	<0.001	88.6 ± 1.2* †
	> 80	96.9 ± 1.5*	80.7 ± 1.7*	<0.001	88.2 ± 1.4* †
Municipal area	rural	88.9 ± 1.1	75.8 ± 0.8	<0.001	82.3 ± 0.6
	urban	78.4 ± 1.6‡	66.6 ± 1.1‡	<0.001	72.3 ± 1.3‡
BMI (kg/m2)	≥ 25	84.1 ± 1.9	73.7 ± 1.1	<0.001	77.5 ± 1.1
	< 25	86.5 ± 1.1	72.6 ± 0.9	<0.001	80.3 ± 0.8§
Religion	Muslim	81.9 ± 5.2	61.1 ± 4.2	= 0.001	69.0 ± 4.4
	non Muslim	86.0 ± 1.0¶	73.5 ± 0.7¶	<0.001	79.7 ± 0.7¶

*Significantly different from age 15 -29 years ($p < 0.001$), †significantly different from age > 80 years, ‡significantly different from rural ($p < 0.001$), § significantly different from BMI ≥ 25 ($p < 0.001$), ¶ significantly different from Muslim ($p < 0.05$)

Table 4 Prevalence of vitamin D insufficiency by geographical region and gender

Regions	Age, yrs (range)	Serum 25(OH)D levels					
		< 75 nmol/L			< 50 nmol/L		
		Men	Women	Total	Men	Women	Total
Bangkok	15 - 93	66.7 %	75.5 %	64.6 %	10.8 %	24.2 %	14.3 %
Central	15 - 91	36.2 %	59.2 %	43.1 %	2.1 %	11.4 %	6.5 %
North	15 - 98	27.9 %	50.8 %	39.1 %	0.9 %	6.5 %	4.3 %
Northeast	15 - 91	25.1 %	51.0 %	34.2 %	0.1 %	3.7 %	2.8 %
South	15 - 92	29.4 %	65.8 %	43.8 %	1.5 %	12.9 %	6.3 %
Total	15 - 98	32.6 %	57.3 %	45.2 %	1.9 %	9.3 %	5.7 %

Table 5 Independent variables for serum 25(OH)D levels by multiple regression analysis

Independent variables	Regression coefficient	SE	*p*
gender	12.97	0.957	< 0.001
Age	0.28	0.019	< 0.001
Urban	-6.47	1.04	< 0.001
BMI	-0.08	0.094	0.422
Muslim	-0.82	2.358	0.733
Region*:			
Bangkok	-12.01	1.519	<0.001
Central	-1.73	1.543	0.279
North	-0.69	1.575	0.664
South	-3.32	1.648	0.060

*reference group: Northeast

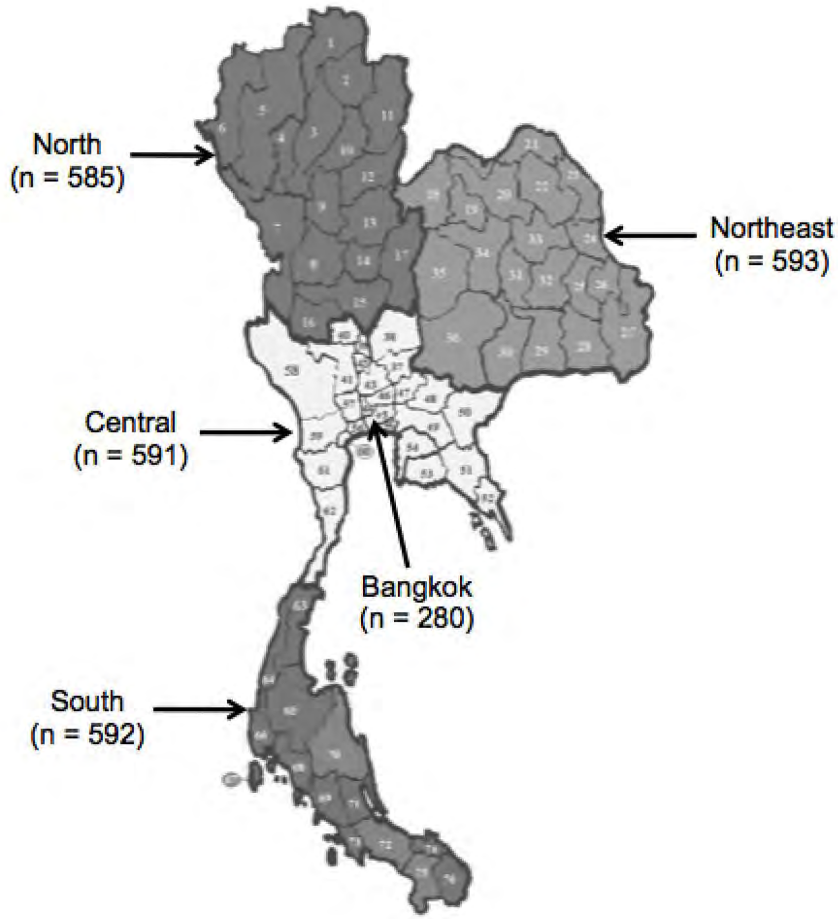

Fig. 1 Geographical regions of the study.

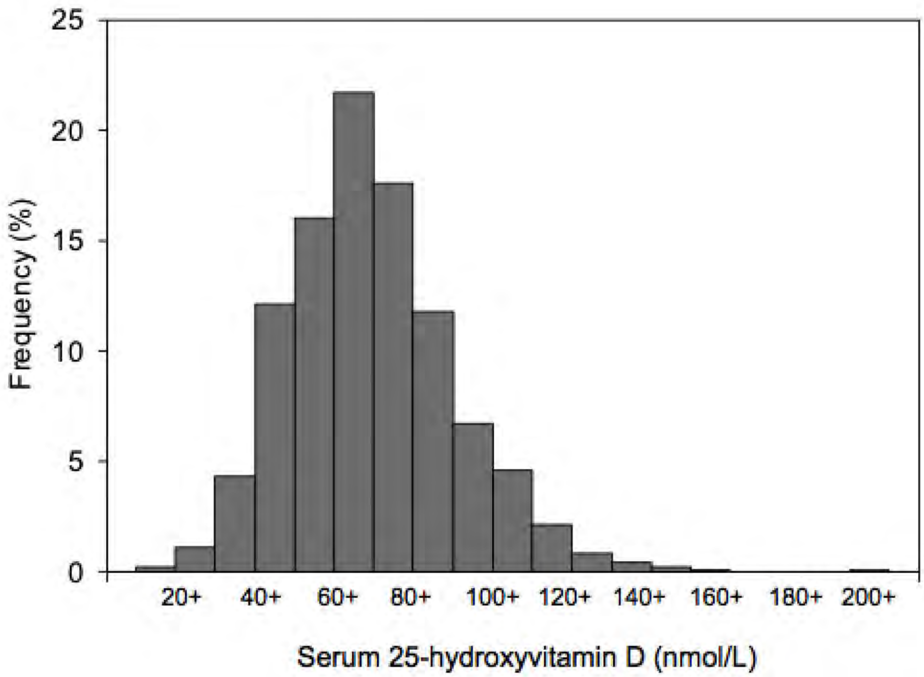

Fig. 2 Frequency distribution of 25-hydroxyvitamin D levels in Thais.

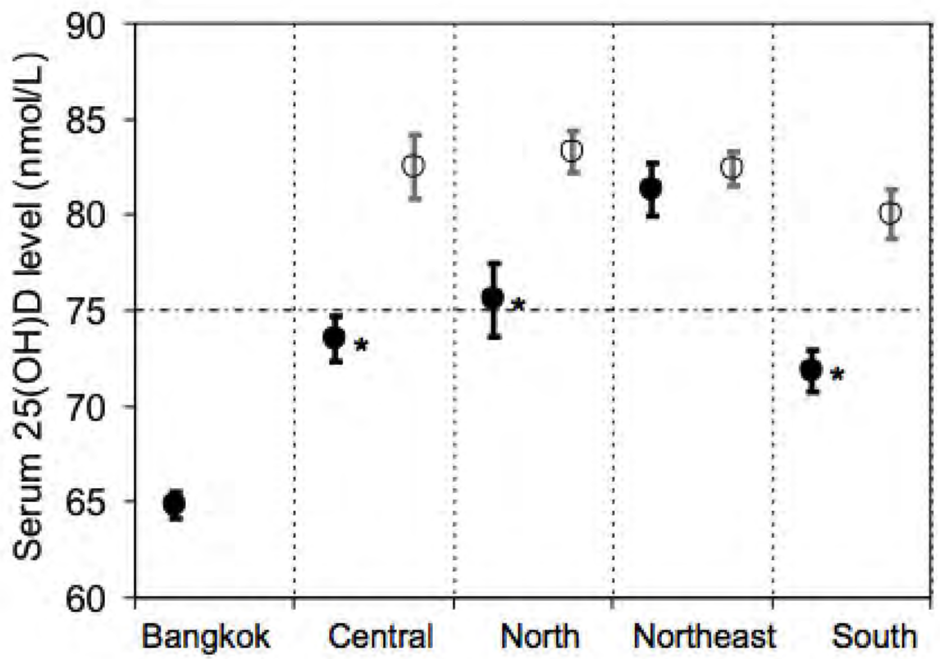

Fig. 3 Vitamin D status inside (closed circle) and outside (open circle) the municipal area in each region. Values are mean $\pm$ SE. * = significant compared to outside the municipal area within region.

THE ASSOCIATION BETWEEN VITAMIN D STATUS AND TYPE 2 DIABETES IN A THAI POPULATION, *A CROSS-SECTIONAL STUDY.*

Abstract

Objective To explore vitamin D status in relation to diabetes, based on data from a national health examination survey in Thailand.

Design and Methods A total of 2,641 adults, aged 15–98 years, were randomly selected according to geographical region from the Thai 4^{th} National Health Examination Survey sample. Logistic regressions were used to examine the *cross-sectional* association between diabetes status and level of 25(OH)D separately by age groups and areas area of residence.

Results Fifty percent of the subjects were male and 5.8% had diabetes. The mean level of 25(OH)D was 79.3 ± 0.8 nmol/L. Six percent and 45% had vitamin D insufficiency based on cut-off values of 50 and 75 nmol/L, respectively. *In a regression model, it was found that 25(OH)D3 and total 25(OH)D were positively associated with diabetes. In addition,* logistic regression analysis showed low circulating 25(OH)D3 but not 25(OH)D2 levels was significantly associated with an increased odds of diabetes in older persons (aged >=70 years) in urban areas. *However, for subjects residing in rural areas, no association between serum 25(OH)D3 or total 25(OH)D and diabetes was found. Furthermore, vitamin D insufficiency was associated with a higher risk of diabetes (OR 1.56, 95%CI 1.10, 1.12) only in the urban elderlys*

Conclusion Low vitamin D status is modestly associated with a small increase in the risk of diabetes in urban Thai elderly. The observation that higher vitamin D status is associated with increased diabetic risk in young adults needs to be further explored and confirmed.

Introduction

Vitamin D possesses myriad biological functions. Observational studies have demonstrated the association of vitamin D status and a number of chronic disorders besides osteoporosis, including cardiovascular diseases, certain cancers, and diabetes mellitus. Both type 1 and type 2 diabetes have been found to be related to vitamin D status [1, 2]. For type 1 diabetes, vitamin D modulates immunity; and increased vitamin D intake in children results in a decreased risk of type 1 diabetes [1, 3]. Although a number of studies have found an association between circulating 25-hydroxyvitamin D (25(OH)D) levels and type 2 diabetes [4], interventional studies looking at the effect of the repletion of vitamin D on incident type 2 diabetes have provided less clear results [5]. The causal role of vitamin D in type 2 diabetes is still in question.

Epidemiological data regarding vitamin D and type 2 diabetes have so far been generated mostly from Caucasian populations. It is unclear if there is a relationship between vitamin D status and type 2 diabetes for populations residing close to the equator. Although vitamin D insufficiency is highly prevalent, geographical location and distance from the equator can partly influence the extent of the problem. Moreover, type 2 diabetes is pathophysiologically heterogeneous. While heredity influences the risk of type 2 diabetes, environmental factors such as urbanization, modern lifestyle, with a high-fat diet and little exercise can also influence its risk. Toward that end, the purpose of the present study is to explore vitamin D status in relation to diabetes in the Thai population, based on the results of a recent national health examination survey.

Patient and Methods

Population

This study used data from the Thai 4th National Health Examination Survey (NHES-IV) conducted in 2008–2009 by the National Health Examination Survey Office, Health Systems Research Institute [6]. Subjects aged 15–98 years were randomly selected from 21 provinces in four geographical regions of Thailand as well as the capital city, Bangkok, using stratified, multistage probability sampling of the population aged ≥15 years, with a sample size of 21,960 individuals. Demographic data such as age, gender, and religion were included. *Urban was defined according to the administration area, the urban are municipal area where rural are non-municipal area. In general, municipal areas were more likely to be economically developed than rural areas*. Body mass index (BMI) was measured using standard procedure. Fasting blood samples were obtained and transferred to a freezer at a central laboratory in Ramathibodi Hospital, a university hospital in Bangkok, where they were kept at -80 Cº. The present study used a subsample of the NHES-IV serum samples to measure serum levels of 25(OH)D. The subjects were randomly selected according to age group (15–29, 30–44, 45–59, 60–69, 70–79, and ≥80 years), gender, region, and area of residence. In each stratum, 25 individuals were randomly selected using statistical software. A total of 2,700 were sampled, of which 2,641 serum samples were available. The study was approved by the ethics committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Serum 25-hydroxyvitamin D (25(OH)D) measurement

Serum 25(OH)D2 and 25(OH)D3 were analyzed by LC-MS/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) using a MassChrom® 25-OH-Vitamin D3/D2 diagnostics kit (ChromSystems, Munich, Germany). The summation of serum 25(OH)D2 and 25(OH)D3 was used to reflect vitamin D status. The inter-assay and intra-assay coefficients of variation of serum total 25(OH)D level were 6.3% and 5.0%, respectively.

Assessment of diabetes mellitus

Individuals with diabetes was defined as 1) individuals with a previous diagnosis of diabetes by physician and intake of hypoglycemic drug during two weeks prior to the study or 2) individuals who had a fasting plasma glucose concentration of ≥ 7.0 mmol/L at time of the present study [7].

Statistical analysis

Level of 25(OH)D2 and 25(OH)D3 were expressed as mean (SE) and compared between those having diabetes and those without diabetes according to other independent variables including gender, age groups (15-44, 45-69 and ≥70 years), area of residence, BMI categories (<25 and >=25 kg/m^2), smoking status (current smoker vs non-smoker), alcohol drinking (assessed by graduated frequency questionnaire and categorized into 2 groups at cut-off points: >=41 gm/d for men and >=21 gm/d for women) and leisure time physical activity status (assessed by using global physical activity questionnaire), categorized into 2 groups as those having moderate or higher >=150 min/week and those <150 min/week using t-test statistics. Logistic regression analyses were conducted to examine the association of levels of vitamin D (25(OH)D2 and 25(OH)D3) with diabetes status controlling for other covariates including age, gender, BMI and levels of, area of residence, smoking, alcohol drinking, and physical activity. Initially, the regression model included independent variables and several product terms including between 25(OH)D3 and gender; 25(OH)D3 and age; 25(OH)D3 and BMI, 25(OH)D3 and smoking, 25(OH)D3 and alcohol drinking, age and area of residence as well as between area of residence and gender to test for interactions. The results indicated that there was an interaction between area of residence and age group, but not for the others. Subsequently, the regression models were separately analyzed for area of residence and the three age groups. There was no correlation between serum 25(OH)D2 and 25(OH)D3, (r=0.004), but the serum 25(OH)D3 was highly correlated with serum total 25(OH)D,(r=0.99), hence we include 25(OH)D2 and 25(OH)D3 in the same model and run a separate model for total 25(OH)D. In the logistic regression model, the odds ratios for serum 25(OH)D2, 25(OH)D3 and total 25(OH)D were calculated as per one unit change of their corresponding standard deviation (SD); 3 nmol/L,22.3 nmol/L and 22.5 nmol/L respectively. Additional logistic regression analysis was also done using total 25(OH)D as a dichotomous variable with a cut-off point at 75 nmol/l (25(OH)D <75 nmol/l coded as 1 and >=75 nmol/l as 0). A P value less than 0.05 was considered statistically significant and a P value less than 0.10 was considered significance for interaction. All analyses were weighted to the probability of sampling using Stata version 10.1 (StataCorp LP, College Station, TX, USA), with a svy command to take into account the complex survey design.

Results

The mean age of the study population was 40.3 ± 0.3 years. Fifty percent of the subjects were males and 5.8% had diabetes. The mean level of 25(OH)D was 79.3 ± 0.8 nmol/L. Six percent and 45% were considered to have vitamin D insufficiency based on the cut-off values of 50 and 75 nmol/L, respectively.

Table 1 shows mean serum 25(OH)D2, 25(OH)D3 and total 25(OH)D in subjects with and without diabetes according to gender, age, area of residence, BMI, smoking, alcohol drinking and physical activity**.** In general, it was found that most subjects with diabetes had higher serum 25(OH)D2, 25(OH)D3 and total 25(OH)D than those without diabetes except that subjects aged >=70 years with diabetes had lower 25(OH)D3 and total 25(OH)D levels compared to those without diabetes. In a regression model investigating variables associated with diabetes adjusted for potential confounding factors, it was found that 25(OH)D3 and total 25(OH)D were positively associated with diabetes (Table 2). *In addition, it was found that age as well as area of residence but no others had an interaction with 25(OH)D status on the risk of diabetes*. Therefore, subjects were separately analyzed by area of residence and age groups. Table 3 shows mean serum 25(OH)D2, 25(OH)D3 and total 25(OH)D in subjects with and without diabetes by area of residence. There were no significant differences in 25(OH)D2 between individuals with diabetes and without diabetes in both urban and rural areas except in the urban 15-44 age group. With regard to vitamin D3 and total vitamin D, subjects in the 15-44 age group who had diabetes had higher levels of 25(OH)D3 and total 25(OH)D than those without diabetes in both urban and rural areas. In contrast, diabetic subjects aged ≥70 years residing in urban areas had a significant lower level of serum 25(OH)D3 and total 25(OH)D compared to those without diabetes. In addition, subjects without diabetes who resided in urban areas also had lower serum 25(OH)D2, 25(OH)D3 and total 25(OH)D levels compared to those without diabetes in rural area.

Table 4 shows the levels of 25(OH)D2, 25(OH)D3 and variables associated with diabetes according to age groups and areas of residence. Higher BMI was associated with an increased risk of diabetes in all age categories. The association of 25(OH)D3 with diabetes varied by age group and area of residence. Circulating 25(OH)D3 and total 25(OH)D levels was negatively associated with diabetes only in subjects aged ≥70 years in urban areas. However, for subjects residing in rural areas, no association between serum 25(OH)D3 or total 25(OH)D and diabetes was found. Table 5, model 2 shows a consistent result of an additional analysis for logistic regression using total 25(OH)D as a dichotomous variables (<75 nmol/l indicating vitamin D insufficiency) which indicating that vitamin D insufficiency in subjects aged 15-44 years residing in the urban or rural areas vitamin D insufficiency was associated with a lower risk of diabetes. In contrast, vitamin D insufficiency was associated with a higher risk of diabetes (OR 1.56, 95%CI 1.10, 1.12) only in the urban elderlys (Table 5).

Discussion

In this study, we found that low vitamin D status was modestly associated with a small increase in the risk of diabetes in urban Thai elderly. Most of the existing data regarding vitamin D status in relation to diabetes have been generated in Western countries; studies investigating the role of vitamin D in the modulation of the risk of diabetes in other populations are scarce[8, 9] and based on limited sample sizes [10-12]. To our knowledge, our study is the first population-based study to demonstrate the association of vitamin D status, as directly assessed from circulating 25(OH)D, and diabetes in the tropical region. *Thailand is situated in a geographic area (latitudes between 5°30′ N and 20°30′ N.) where sun always shines brightly all year round causing little of the seasonal fluctuations seen where there is in winter (the sunshine length during day time in summer and winter is between 4.0 – 8.1 hours and 3.1 -8.2).* Because of the cross-sectional nature of the study, our findings cannot delineate the causal role of vitamin D in the development of diabetes. However, some of the findings, particularly in the urban elderly, are in line with a number of previous studies demonstrating the association of vitamin D status, assessed by various methods, with incident type 2 diabetes [2, 13, 14]. This is not without dispute, since small interventional studies administering vitamin D supplements to individuals at risk in order to infer causality have provided controversial results [15-17]. If a causal role of vitamin D does exist, the discrepancy may be due in part to the lack of power, inadequate doses of vitamin D, and possibly the inclusion of subgroups of subjects in which vitamin D plays only a small pathophysiological role with regard to glucose metabolism. In light of the health and economic burdens of diabetes, further appropriately designed clinical studies to address the issue are very much warranted.

Multiple interacting factors play roles in the pathogenesis of type 2 diabetes. It is therefore conceivable that vitamin D can interact with other risk factors to alter type 2 diabetes susceptibility. In the present study, a statistical interaction among vitamin D levels, age and area of residence for the diabetes status was found. With regard to the interaction between age and vitamin D on the risk of type 2 diabetes, our results are in line with a recent cross-sectional study in the US population, in that the association between vitamin D and HbA1c was observed only in certain age groups (35-74 years) [18].

Urbanization is well recognized to be associated with increased risk of type 2 diabetes [19, 20]. The relationship is generally believed to be due to lifestyle changes associated with improved socioeconomic status and modern living conditions. Urban residents have less sun exposure, which can predispose individuals to lower vitamin D status. Besides lower vitamin D status, urbanization is associated with a number of shared risk factors for certain non-communicable age-related diseases such as lack of physical activity and increased exposure to air pollution. It is of note that in the present study we demonstrated a statistical interaction between vitamin D insufficiency and urbanization on diabetes, in that the influence of vitamin D insufficiency was evident only in the older subjects residing in an urban environment.

In contrast to the association between lower vitamin D status and diabetes in older subjects residing in urban area, the direction of the relationship reversed in the younger age group. Although the strength of the statistical association was relatively weak and the underlying basis unclear, the finding is intriguing. Despite the fact that lower circulating vitamin D is generally associated with increased adiposity and calcitriol inhibits lipogenesis [21], studies in vitamin D receptor (VDR) knockout animals have yield opposite results in that VDR knockout animals are lean [22] and there is an increase in brown adipose tissue. Brown adipose tissue has recently been confirmed to exist and functional in human adults [23] It might be that vitamin D adversely influence glucose tolerance in some individuals through it potentially adverse effect on brown fat. Further study to explore the comparative effects of vitamin D on brown and white adipose tissues is warranted.

Although controversial, the biological effect of vitamin D2 versus D3 is generally considered to be equivalent in humans[24, 25]. This notion, however, was mainly derived from a study regarding calcium and bone metabolism, and has never been explored in the context of other biological functions of vitamin D. In the present study, we observed that the relationship between vitamin D status and diabetes existed in the case of 25(OH)D3 but not for 25(OH)D2. A number of possibilities may account for this finding. It is likely that adding 25(OH)D2 to 25(OH)D3 for the derivation of total 25(OH)D introduces an additional source of *systematic* error and may render the result less accurate for reflecting vitamin D status. On the other hand, this probably suggests that 25(OH)D3 and 25(OH)D2 are not equivalent in terms of their biological effects. In fact, vitamin D3 has been shown to differ from vitamin D2 in terms of metabolism, affinity to vitamin D binding protein, and affinity of their active metabolites to vitamin D receptors [26]. Nevertheless, our study was not specifically designed to address the issue, and further more appropriate studies to better answer the question should be initiated. Furthermore, it is conceivable that the discrepancy in the results of the vitamin D intervention study may partly be due to the type of vitamin supplement administered.

There are a number of limitations in the present study. Oral glucose tolerance test was not performed which may misclassify diabetes status in some subjects. *Distinguishing between type 1 and type 2 diabetes, particularly in those younger than 35 years old, was not feasible in this large cross-sectional survey. However, type 1 diabetes is very rare in Thailand even in recent years* [27] *and it is very likely that almost all diabetic subjects in this age group had type 2 diabetes.* Data regarding vitamin D supplementation as well as the detailed dietary records were not *gathered* at the time of the survey. Such parameters may influence diabetes status or confound the association between vitamin D status and diabetes.

We concluded that low vitamin D status is associated with a modest increase in the risk of diabetes in urban Thai elderlys. The observation that higher vitamin D status is associated with increased diabetic risk in young adults needs to be further explored and confirmed.

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Table 1. Mean (SE) serum 25(OH)D2, 25(OH)D3 and total 25(OH)D in subjects with (n = 281) and without (n = 2360) diabetes according to gender, age, area of residence, BMI, smoking, alcohol drinking and physical activity

		Serum 25(OH)D2 (nmol/L)			Serum 25(OH)D3 (nmol/L)			Serum total 25(OH)D (nmol/L)		
Variables		Diabetes	No Diabetes	P value	Diabetes	No Diabetes	P value	Diabetes	No Diabetes	P value
Gender	Men	1.96 (0.09)	1.84 (0.04)	NS	83.89 (1.73)	84.10 (1.13)	NS	85.86 (1.77)	85.95 (1.13)	NS
	Women	2.48 (0.14)	2.07 (0.05)	0.01	77.64 (2.35)	70.49 (0.85)	<0.01	80.12 (2.32)	72.55 (0.87)	<0.01
Age (years)	15–44	1.78 (0.13)	1.79 (0.04)	NS	91.53 (2.86)	75.04 (0.88)	<0.001	93.31 (2.90)	76.84 (0.89)	0.001
	45–69	2.25 (1.0)	2.19 (0.07)	NS	75.52 (1.63)	80.02 (0.93)	<0.05	77.77 (1.68)	82.21 (0.94)	<0.05
	>=70	3.12 (0.5)	2.65 (0.17)	NS	80.16 (2.62)	86.71 (1.25)	<0.05	83.28 (2.44)	89.36 (1.23)	<0.05
Area of	urban	2.26 (0.16)	1.64 (0.04)	<0.01	73.3 (1.30)	70.34 (1.36)	<0.05	75.56 (1.29)	71.98 (1.36)	<0.05

residence	rural	2.26 (0.10)	2.08 (0.05)	NS	85.26 (1.98)	79.91 (0.63)	<0.05	87.52 (1.96)	81.99 (0.64)	<0.05
BMI (kg/m^2)	< 25	2.48 (0.18)	2.00 (0.05)	<0.05	83.17 (1.92)	78.06 (0.83)	<0.05	85.65 (1.91)	80.06 (0.85)	<0.05
	>= 25	2.10 (0.08)	1.87 (0.04)	<0.05	78.3 (2.32)	75.3 (1.18)	NS	80.41 (2.33)	77.14 (1.18)	NS
Smoking	no	2.26 (0.10)	1.97 (0.05)	<0.05	79.13 (1.72)	74.0 (0.87)	<0.01	81.39 (1.70)	75.97 (0.88)	<0.01
	yes	2.26 (0.10)	1.92 (0.06)	<0.05	86.71 (1.89)	86.93 (1.15)	NS	88.97 (1.92)	88.85 (1.16)	NS
Alcohol drinking	moderate	2.3 (0.10)	1.95 (0.04)	<0.01	79.32 (1.79)	76.58 (0.85)	NS	81.64 (1.78)	78.54 (0.85)	NS
	heavy	1.79 90.10)	1.82 (0.07)	NS	89.0 (2.01)	83.94 (1.65)	NS	90.78 (2.07)	85.76 (1.67)	NS
Physical activity	inactive	2.25 (0.11)	2.04 (0.05)	NS	80.85 (1.91)	77.05 (0.85)	0.05	83.10 (1.89)	79.08 (0.87)	0.04
	active	2.28 (0.14)	1.71 (0.05)	0.001	78.30 (1.81)	77.64 (1.26)	NS	80.58 (1.81)	79.35 (1.27)	NS

Table 2. Adjusted odds ratios of variables associated with diabetes.

	Model 1		Model 2	
Variables	**OR (95%CI)**	**P-value**	**OR (95%CI)**	**P-value**
Age (years)	1.15 (1.10 1.20)	<0.01	1.12 (1.09, 1.16)	<0.01
Male	2.40 (0.36, 15.8)	NS	2.27 (0.37, 13.98)	NS
BMI (kg/m^2)	1.19 (1.03, 1.37)	<0.05	1.10 (0.98, 1.24)	NS
Urban residence	1.24 (0.35, 4.44)	NS	1.13 (0.33, 3.85)	NS
25(OH)D2 (nmol/L)	1.02 (0.99, 1.05)	NS	-	
25(OH)D3 (nmol/L)	1.11 (1.04, 1.18)	<.01	-	
Total 25(OH)D (nmol/L)	-	-	1.06 (1.02, 1.11)	0.01
Smoking	1.29 (0.27, 6.08)	NS	0.91 (0.21, 4.00)	NS
Alcohol drinking	0.96 (0.14, 6.5)	NS	0.75 (0.11, 5.15)	NS
Physical activity	2.18 (0.61, 7.73)	NS	2.12 (0.6, 7.44)	NS
Urban residence – age*	1.01 (1.0, 1.02)	<0.1	1.01 (1.0, 1.03)	<0.1
Male -25(OH)D3*	0.98 (0.96, 1.0)	0.05	0.98 (0.96, 1.0)	<0.1
Age - 25(OH)D3*	1.00 (1.0,1.0)	<0.01	1.00 (1.0, 1.0)	<0.01

Model 1is for 25(OH)D2 and 25(OH)3 and Model 2 for total vitamin D. Both models were adjusted for physical activity, current smoking (yes/no) and moderate to heavy alcohol drinking estimated as >=40 gm/day in men or >=20 gm/day in women (yes/no).

The odds ratios for 25(OH)D2 and 25(OH)D3 were calculated per one unit change of their corresponding standard deviation (SD); 3 nmol/L, and 22.3 nmol/L respectively.

* Interaction terms, NS = non significance

Table 3. Mean (SE) of serum 25(OH)D2, 25(OH)D3 and total 25(OH)D levels according to diabetes status, age group and area of residence

	Age (years)	**Urban (n = 1458)**			**Rural (n = 1183)**		
		Diabetes (n = 193)	**No Diabetes (n = 1265)**	**P value**	**Diabetes (n = 88)**	**No Diabetes (n =1095)**	**P value**
Serum 25(OH)D2 (nmol/L)	15–44	1.6 (0.1)	1.5 (0.03)*	0.05	1.8 (0.2)	1.9 (0.1)	NS
	45–69	2.0 (.1)	1.8 (0.1)*	NS	2.5 (0.1)	2.3 (0.1)	NS
	>=70	3.7 (0.9)	2.3 (0.2)	NS	2.5 (.2)	2.8 (0.2)	NS
Serum 25(OH)D3 (nmol/L)	15–44	81.3 (0.03)	67.7 (1.4)*	<0.05	94.4 (3.1)	77.9 (0.8)	<0.01
	45–69	72.6 (1.8)	74.0 (1.5)*	NS	78.1 (2.2)	82.6 (0.8)	NS
	>=70	69.9 (1.7)	79.9 (1.4)*	<0.01	91.4 (3.2)	89.2 (1.5)	NS
Serum total 25(OH)D (nmol/L)	15–44	82.9 (4.9)	69.2 (1.5)*	<0.05	96.2 (3.2)	79.8 (0.8)	<0.01
	45–69	74.6 (1.8)	75.9 (1.5)*	NS	80.5 (2.2)	84.9 (0.8)	NS
	>=70	73.6 (1.6)	82.3 (1.5)*	<0.01	93.9 (3.2)	91.9 (1.5)	NS

* p < 0.01 compared between those without diabetes in urban and rural areas, NS = non significance

Table 4 Adjusted odds ratios (OR) for 25(OH)D2 , 25(OH)D3 and variables associated with having diabetes according to three age groups and area of residence

	Adjusted OR (95%CI)					
Age (years)	**15–44**	**P value**	**45–69**	**P value**	>=**70**	**P value**
Urban	n = 483		n = 498		n = 477	
Age (years)	1.11 (1.05, 1.18)	0.001	1.07 (1.04, 1.11)	0.001	0.96 (0.92, 0.99)	0.01
Men	3.32 (1.15, 9.6)	<0.05	1.07 (0.65, 1.79)	NS	0.93 (0.63, 1.36)	NS
BMI (kg/m^2)	1.25 (1.12, 1.41)	0.001	1.19 (1.11, 1.28)	0.001	1.07 (1.03, 1.12)	<0.01
25(OH)D2 (nmol/L)	0.79 (0.28, 2.20)	NS	1.13 (0.81, 1.57)	NS	1.12 (1.01, 1.24)	<0.05
25(OH)D3 (nmol/L)	2.49 (1.15, 5.37)	<0.05	1.0(0.82, 1.23)	NS	0.69 (0.55, 0.87)	<0.01
Rural	n = 399		n = 386		n =3 98	
Age (years)	1.07 (0.97, 1.17)	NS	1.07 (1.03, 1.13)	0.005	0.97 (0.92, 1.03)	NS
Men	0.43 (0.08, 2.24)	NS	0.71 (0.39, 1.29)	NS	1.43 (0.76, 2.70)	NS
BMI (kg/m^2)	1.09 (1.02, 1.16)	<0.05	1.08 (1.02, 1.15)	0.01	1.05 (1.0, 1.09)	<0.05
25(OH)D2 (nmol/L)	0.27 (0.05, 1.52)	NS	1.27 (0.81, 1.99)	NS	0.98 (0.87, 1.09)	NS
25(OH)D3 (nmol/L)	2.05 (1.24, 3.39)	<0.01	0.68 (0.45, 1.01)	NS	1.06 (0.74, 1.52)	NS

Adjusted for physical activity, current smoking (yes/no) and moderate to heavy alcohol drinking estimated as >=40 gm/day in men or >=20 gm/day in women (yes/no).

The odds ratios for 25(OH)D2 and 25(OH)D3 were calculated per one unit change of their corresponding standard deviation (SD); 3 nmol/L, and 22.3 nmol/L respectively.

NS = non significance

Table 5. Adjusted odds ratios (OR) for vitamin D insufficiency (total 25(OH)D < 75 nmol/l) and variables associated with having diabetes according to three age groups and area of residence.

Age (years)	15-44	P value	45-69	P value	>=70	P value
Urban	n = 483		n = 498		n = 477	
Age (years)	1.10 (1.03, 1.17)	<0.01	1.07 (1.04, 1.11)	<0.01	0.95 (0.92, 0.98)	<0.01
Men	3.82 (1.26, 11.58)	<0.05	1.08 (0.65, 1.78)	NS	0.86 (0.59, 1.24)	NS
BMI (kg/m^2)	1.24 (1.12, 1.37)	<0.01	1.19 (1.11, 1.28)	<0.01	1.07 (1.01, 1.12)	0.01
Total 25(OH)D <75 nmol/L	0.26 (0.09, 0.75)	<0.05	0.84 (0.56, 1.25)	NS	1.56 (1.10, 2.20)	0.01
Rural	n = 399		n = 386		n =3 98	**P value**
Age (years)	1.08 (0.99, 1.17)	NS	1.07 (1.02, 1.12)	<0.01	0.98 (0.93, 1.04)	NS
Men	0.69 (0.19, 2.49)	NS	0.63 (0.35, 1.11)	0.10	1.27 (0.66, 2.42)	NS
BMI (kg/m^2)	1.08 (1.02, 1.14)	0.01	1.08 (1.02, 1.15)	0.01	1.05 (101, 1.10)	<0.05
Total 25(OH)D <75 nmol/L	0.12 (0.03, 0.51)	<0.01	1.33 (0.78, 2.26)	NS	0.50 (0.16, 1.55)	NS

Adjusted for physical activity, current smoking (yes/no) and moderate to heavy alcohol drinking estimated as >=40 gm/day in men or >=20 gm/day in women (yes/no).

The odds ratios for total 25(OH)D was calculated per one unit change of their corresponding standard deviation (SD); 22.5 nmol/L.

NS = non significance

HIGH VITAMIN D STATUS IN YOUNGER INDIVIDUALS IS ASSOCIATED WITH LOW CIRCULATING THYROTROPIN

Abstract

***Background*:** Vitamin D is an immunomodulator and may affect autoimmune thyroid diseases. Vitamin D has also been shown to influence thyrocytes directly by attenuating the thyrotropin (TSH) stimulated iodide uptake and cell growth. However, it is unclear how vitamin D status is related to TSH at the population level. The goal of the present study was to investigate the relationship between vitamin D status and TSH levels according to thyroid autoantibodies in a population-based health survey in Thailand.

Methods: A total of 2,582 adults, aged 15–98 years, were randomly selected according to geographical region from the Thailand 4th National Health Examination Survey sample. Serum levels of 25-hydroxyvitamin D (25(OH)D), TSH, thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TgAb) were measured in all subjects.

Results: The mean age was 55.0 ± 0.4 (SE) years. Fifty percent of the subjects were males. In subjects positive for serum TgAb, serum TSH levels were higher whereas total serum 25(OH)D levels were lower. In addition, the prevalence of vitamin D insufficiency in TgAb-positive subjects was significantly higher than that observed in TPOAb and TgAb-negative subjects, whether based on cutoff values of 20 or 30 ng/mL: 8.3% vs 5.6%, $p < 0.05$; or 47.6% vs 42.0%, $p < 0.05$, respectively. However, vitamin D status was not associated with positive TPOAb and/or TgAb after controlling for gender and age. To explore the probable interaction between vitamin D status and age on serum TSH, analyses were performed according to age tertiles, it was found that higher 25(OH)D levels were independently associated with lower TSH, but only in subjects in the lowest age tertile.

Conclusions: This population-based study showed that high vitamin D status in younger individuals is associated with low circulating thyrotropin.

Introduction

Vitamin D is produced endogenously when the skin is exposed to sunlight, or obtained exogenously from nutrients or supplements. Despite the ability of dermal production, less sunshine or the tendency to avoid sun exposure can potentially lead to vitamin D insufficiency. Vitamin D insufficiency is now recognized to be highly prevalent and is observed to varying degrees in many different countries, regardless of geographical location (1-3). Despite the fact that Thailand is located near the equator, the prevalence of vitamin D insufficiency was found to be at least 45% in a population survey when using a 25(OH)D threshold of 75 nmol/L (4). Vitamin D enhances intestinal calcium absorption and vitamin D deficiency can lead to osteomalacia as well as an increased propensity for osteoporosis when vitamin D is inadequate (5). Besides its role in calcium and bone metabolism, vitamin D may also affect non-skeletal functions. Observational studies have demonstrated the association of vitamin D status with a number of common disorders including atherosclerosis (6), hypertension (7) and diabetes (8, 9). Whether vitamin D insufficiency is causally related to these disorders is still unclear. Recent studies in Thais also demonstrated that vitamin D insufficiency is associated with diabetes in older individuals (10). However, no relationship between vitamin D status and high blood pressure was found (11).

Vitamin D is well recognized as an immunomodulator (10), and vitamin D insufficiency has been associated with autoimmune thyroid disease (11) . Besides potentially affecting the thyroid gland through immune-mediated process, vitamin D has also been shown to influence thyrocytes directly by attenuating the thyrotropin (TSH) stimulated iodide uptake and cell growth of rat thyroid cells (12). On the other hand, it has been shown that vitamin D modulated TSH secretion of pituitary thyrotrophs by binding to specific binding sites (13). Moreover, increased TSH level has been observed after acute administration of 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) (14). It is unclear, however, how vitamin D status is related to TSH levels at the population level. Therefore the purpose of the present study is to investigate whether vitamin D status is associated with circulating TSH according to thyroid autoantibodies in a population-based health survey in Thailand.

Materials and Methods

Population

This study used data and blood samples from the Thai 4th National Health Examination Survey (NHES-IV) conducted in 2008–2009 by the National Health Examination Survey Office, Health Systems Research Institute. Subjects aged 15–98 years were randomly selected from 21 provinces in four geographical regions of Thailand as well as the capital city, Bangkok, using stratified, multistage probability sampling of the population aged

≥15 years, with a sample size of 21,960 individuals. Demographic data such as age, sex, and religion were included. Body mass index (BMI) was measured using standard procedure. Fasting blood samples were obtained and transferred to a freezer at a central laboratory in Ramathibodi Hospital, a university hospital in Bangkok, where they were kept at -80 ºC.

The present study used a subsample of the NHES-IV serum samples to measure serum levels of 25-hydroxyvitamin D (25(OH)D). The subjects were randomly selected in 1:1 sex ratio according to age group (15–29, 30–44, 45–59, 60–69, 70–79, and ≥80 years), region, and urban/rural place of residence from a sample size of 21,960. In each stratum, 25 individuals were randomly selected using statistical software. A total of 2,700 were sampled, of which 2,587 serum samples were available. Five subjects with marked hypothyroidism (serum TSH greater than 50 mIU/L) were excluded from the study. The study was approved by the ethics committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Serum 25-hydroxyvitamin D (25(OH)D) measurement

Serum $25(OH)D_2$ and $25(OH)D_3$ were analyzed by LC-MS/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Foster City CA, USA) using a MassChrom® 25-OH-Vitamin D_3/D_2 diagnostics kit (ChromSystems, Munich, Germany) . The 25(OH)D assay was performed according to the manufacturer's instructions. This method used a deuterated 25(OH)D3 as an internal standard to correct for sample and instrument variability. Samples were analyzed using an atmospheric pressure chemical ionization source for maximum sensitivity. The 25(OH)D separation was performed using Chromsystems precipitation reagent and trap column in conjunction with Agilent 1200 HPLC system configured for on-line sample preparation according to the configuration included in the documentation with this method. Briefly, 25(OH)D3 and 25(OH)D2 were extracted by mixing 100 µl of serum sample with 25 µl precipitation reagent and 200 µl of the internal standard solution. The mixture was vortexed for 20 seconds and incubated for 10 minutes at 4 °C. After centrifuged the mixture for 5 minutes at 9,000 g, the upper layer was transferred to an autosampler vial and 5 µl was injected to the LC-MS/MS. The summation of serum $25(OH)D_2$ and $25(OH)D_3$ was used to reflect vitamin D status. The inter-assay and intra-assay coefficients of variation of total serum 25(OH)D level were 6.3% and 5.0%, respectively.

Serum thyrotropin (TSH), Thyroid peroxidase antibody (TPOAb) and anti-thyroglobulin antibody (TgAb) measurements

Serum TSH, thyroid peroxidase antibody (TPOAb) and thyroglobulin antibody (TgAb) were measured by electrochemiluminescence immunoassay on a Cobas e 411 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The assays have intra-assay precision of 3.6%, 9.2% and 6.1%, respectively. Positive TPOAb and TgAb were defined as value greater than 34 IU/mL and 115 IU/mL, respectively.

Statistical analysis

Data were expressed as mean ± SE. Differences between two groups were assessed by Student's *t*-test. Logistic regression analysis was performed to identify the predictive variables. Linear regression analysis was used to examine the relationship between vitamin D status and serum TSH. A *p*-value less than 0.05 was considered statistically significant. All analyses were performed using SPSS statistical software package, version 16.0 (SPSS Inc., Chicago IL, USA).

Results

Table 1 demonstrates the clinical and laboratory characteristics of the remaining 2,582 subjects. The mean age was 55.0 ± 0.4 (SE) years. The mean level of total serum 25(OH)D (summation of 25(OH)D_2 and 25(OH)D_3) was 32.5 ± 0.18 ng/mL. Women had higher BMI, serum TgAb and TPOAb levels but lower serum 25(OH)D levels than men. Figure 1 shows the distribution of log-transformed TSH. Four percent had serum TSH levels above 5 mIU/L while 19.8% had serum TSH levels above 2.5 mIU/L. Of the 2,582 subjects, 425 (16.5%) and 315 (12.2%) subjects were positive for serum TPOAb and TgAb, respectively. When subjects with positive serum TPOAb and/or TgAb were excluded, the distribution of TSH did not change appreciably.

Table 2 compares the clinical and laboratory characteristics based on positive serum TPOAb (Table 2A), TgAb (Table 2B) and TPOAb and/or TgAb (Table 2C). Subjects with positive serum TPOAb and/or TgAb were older, had higher serum TSH level, and included a lower proportion of males. After excluding subjects with positive TPOAb and TgAb, the mean serum TSH levels decreased from 2.09 ± 0.04 to 1.94 ± 0.03 mIU/L. In addition, the prevalence of vitamin D insufficiency (whether based on cutoff values of 20 (15) or 30 (16) ng/mL) in TgAb-positive subjects was significantly higher than that observed in TgAb-negative subjects. Multivariate analysis using logistic regression revealed that independent determinants of positive TPOAb and/or TgAb were female gender and age. Serum 25(OH)D levels, however, were not associated with positive TPOAb and /or TgAb (Table 3), or positive TgAb alone (supplemental table was available in the online Data Supplement) after controlling for gender and age.

To account for the probable interaction between vitamin D status and age on serum TSH, further analyses were performed according to age tertiles. The mean age (± SD) of subjects in the first, second and third tertiles were 29.2 ± 9.2. 57.6 ± 7.3 and 78.66 ± 5.4 yeaars, respectively. Serum TSH levels were log-transformed before regression analysis. With regard to the relationship between vitamin D status and serum TSH, it was found that higher 25(OH)D levels were associated with lower TSH - independent of age, gender, BMI, serum TPOAb and/or TgAb – only in subjects in the lowest age tertile (Table 4). Age, BMI andthyroid autoantibodies, but not 25(OH) or gender, were associated with serum TSH in the upper age tertile.

Discussion

Similar to studies in other populations (17-19), the distribution of serum TSH in our study did not follow a true Gaussian distribution but skewed towards the higher values. In addition (also not unlike other previous studies), serum TSH levels were higher in subjects with evidence of autoimmune thyroid disease, as indicated by the presence of TPOAb and/or TgAb. After excluding subjects with positive TPOAb and/or TgAb, the mean serum TSH levels decreased, which is likely to more accurately reflect the mean reference value of serum TSH. It is now well established that serum TSH levels are ethnically related; hence the reference value of serum TSH should be determined specifically based on ethnicity. Besides thyroid autoimmunity, factors previously reported to be related to TSH levels or thyroid hormones include age, gender, smoke exposure and ethnicity (17, 18, 20). The association of circulating TSH with age and gender was also reflected in the present study.

With regard to the immune system, it has been found that vitamin D regulates the differentiation and activation of CD4+ T lymphocytes and can prevent the development of certain autoimmune disorders (21). Moreover, 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) reduces the number of antigen-presenting cells in vitro (22). *In vivo*, 1,25$(OH)_2$D has a direct immunosuppressive effect on dendritic cells and reduces the production of cytokines (23). Regarding autoimmune thyroid disease, previous studies have demonstrated lower vitamin D status in Graves' disease (GD) (24, 25), Hashimoto's thyroiditis (11) or thyroid autoimmunity (26). Moreover, an association between Graves' disease and vitamin D receptor (VDR) polymorphisms have been demonstrated in number of studies (27). The effect of vitamin D on Graves' disease may not be mediated through the inhibition of antibody production but by its effect on modulating autoimmune-induced thyroid hormone production (28).

In the present study, we could not demonstrate an independent relationship between vitamin D status and thyroid autoimmunity as assessed by the presence of TPOAb and/or TgAb. However, a number of limitations existed in the present study. First, anti-thyroid-stimulating hormone receptor (TSHR) was not measured in our study. Second, the study was cross-sectional in nature and the causative role of vitamin D, if any, on thyroid autoimmunity could not be readily determined. Lastly, in addition to environmental factors, genetics plays an important role in thyroid autoimmunity. Many genes have been proposed to be associated with GD (29), including genes encoding human leukocyte antigen (HLA) class II, cytotoxic T-lymphocyte antigen-4 (CTLA-4), protein tyrosine phosphatase-22 (PTPN22), TSHR, and more recently CD40 (30) and Fc receptor-like 3 (FCRL3) (31). It is possible that there might be an interaction between vitamin D status and genetic determinants of thyroid autoimmunity; this may render the effect of vitamin D less apparent if genetic variation in relation to thyroid autoimmunity is not concurrently determined.

We found in the present study that higher circulating 25(OH)D was associated with lower TSH levels in younger individuals. Besides affecting the function of the thyroid gland through autoimmunity, it is likely that vitamin D may influence the thyroid gland through its action on the central nervous system and the thyrotrophs. There is evidence that vitamin D affects CNS function. Low vitamin D status may adversely affect brain development (32) and neurocognitive function (33). Moreover, vitamin D can enhance the responsiveness in TRH-induced TSH secretion of rat pituitary thyrotrophs (34). On the other hand, vitamin D receptors are present in thyrocytes and modulate their differentiation and function (13). It is therefore conceivable that the lower TSH in the presence of higher vitamin D status observed in the present study could also be the result of increased thyroid hormones caused by the stimulatory effect of vitamin D on thyrocytes. (35). However, the underlying basis of the observed high vitamin D status was associated with low serum TSH only in the younger age group is unclear. Further clinical investigation of the effect of vitamin D supplementation on circulating TSH and thyroid hormones, particularly in younger individuals, is needed to address the causal role of vitamin D on either the thyrotrophs or the thyroid gland.

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TABLE 1. BASELINE CHARACTERISTICS OF THE POPULATION STUDY

Characteristics	Men (n = 1,290)	Women (n = 1,292)	Total (n = 2,582)
Age (years)	54.7 ± 0.61	55.2 ± 0.60	55.0 ± 0.42
BMI (kg/m^2)	22.7 ± 0.11	23.9 ± 0.14*	23.3 ± 0.09
Serum 25(OH)D (ng/mL)	35.0 ± 0.26	30.0 ± 0.22*	32.5 ± 0.18
Serum TSH (mIU/L)	1.99 ± 0.05	2.18 ± 0.06	2.09 ± 0.04
Serum TPOAb (IU/mL)	39.4±3.7	75.3±9.3*	57.4±5.0
Serum TgAb (IU/mL)	82.7 ± 11.4	143.3 ± 12.7*	113.0 ± 8.5

Values are mean ± SE

*Significantly different from men (p < 0.001)

TABLE 2A. COMPARISON OF CLINICAL AND LABORATORY CHARACTERISTICS BASED ON THE POSITIVITY OF SERUM TPOAb (CUTOFF VALUE = 34 IU/ml)

	Serum TPOAb		
	Positive (n = 426)	Negative (n = 2,156)	*P* value
Age (years)	61.4 ± 0.93	53.7 ± 0.47	< 0.001
BMI (kg/m^2)	23.4 ± 0.23	23.3 ± 0.10	NS
Male gender [n (%)]	178 (41.9%)	1112 (51.6%)	< 0.001
Serum TSH levels (mIU/L)	2.7 ± 0.18	2.0 ±0.03	< 0.001
Serum 25(OH)D (nmol/L)	32.4 ± 0.45	32.5 ± 0.19	NS
Prevalence of vitamin D insufficiency [n (%)]			
25(OH)D < 20 nmol/L	30 (7.1%)	124 (5.7%)	NS
25(OH)D < 30 nmol/L	183 (43.1%)	919 (42.6%)	NS

Values are mean ± SE or number (percent) , NS = non significance

TABLE 2B. COMPARISON OF CLINICAL AND LABORATORY CHARACTERISTICS BASED ON THE POSITIVITY OF SERUM TgAb (CUTOFF VALUE = 115 IU/ml)

	Serum TgAb		
	Positive (n = 315)	Negative (n = 2,267)	*P* value
Age (years)	57.9 ± 1.12	54.6 ± 0.46	< 0.01
BMI (kg/m^2)	23.9 ± 0.25	23.3 ± 0.10	NS
Male gender [n (%)]	87 (27.6%)	1203 (53.1%)	< 0.001
Serum TSH levels (mIU/L)	2.9 ± 0.19	2.0 ±0.04	< 0.001
Serum 25(OH)D (nmol/L)	31.5 ± 0.52	32.6 ± 0.19	NS
Prevalence of vitamin D insufficiency [n (%)]			
25(OH)D < 20 nmol/L	26 (8.3%)	128 (5.6%)	< 0.05
25(OH)D < 30 nmol/L	150 (47.6%)	952 (42.0%)	<0.05

Values are mean ± SE or number (percent) , NS = non significance

TABLE 2C. COMPARISON OF CLINICAL AND LABORATORY CHARACTERISTICS BASED ON THE POSITIVITY OF SERUM TPOAb and/or TgAb (CUTOFF VALUE = 34 IU/ml and 115 IU/ml, respectively)

	Serum TPOAb and/or TgAb		
	Positive (n = 564)	Negative (n = 2,018)	*P* value
Age (years)	59.9 ± 0.84	53.6 ± 0.49	< 0.05
BMI (kg/m^2)	23.4 ± 0.19	23.31 ± 0.10	NS
Male gender [n (%)]	208 (36.9%)	1082 (53.6%)	< 0.001
Serum TSH levels (mIU/L)	2.6 ± 0.14	2.0 ±0.03	< 0.001
Serum 25(OH)D (nmol/L)	32.3 ± 0.39	32.54 ± 0.20	NS
Prevalence of vitamin D insufficiency [n (%)]			
25(OH)D < 20 nmol/L	39 (6.9%)	115 (5.7%)	NS
25(OH)D < 30 nmol/L	249 (44.1%)	853 (42.3%)	NS

Values are mean ± SE or number (percent) , NS = non significance

TABLE 3 DETERMINANTS OF POSITIVE SERUM TPOAb and/or TgAb

	Adjusted OR (95% CI)	*P* value
Age (years)	1.02 (1.01–1.02)	< 0.001
Male gender	0.51 (0.42–0.62)	< 0.001
BMI (kg/m^2)	1.00 (0.98–1.03)	NS
Serum 25(OH)D (ng/ml)	1.00 (0.99–1.01)	NS

OR = odds ratio, CI = confidence interval, NS = non significance

TABLE 4. STANDARDIZED REGRESSION COEFFICIENTS OF VARIABLES IN RELATION TO SERUM TSH ACCORDING TO AGE TERTILES

	Age (years)					
	15–44 (n = 871)		45–69 (n = 864)		≥ 70 (n = 847)	
	Beta	*p*	Beta	*p*	Beta	*p*
Age (years)	-0.05	NS	0.05	NS	0.13	<0.001
Male gender	-0.02	NS	-0.03	NS	0.01	NS
BMI (kg/m^2)	0.04	NS	0.07	<0.05	0.10	<0.01
Presence of TPOAb and/or TgAb	-0.03	NS	-0.04	NS	0.09	<0.01
Serum 25(OH)D (ng/ml)	-0.14	0.001	-0.05	NS	-0.01	NS

NS = non significance

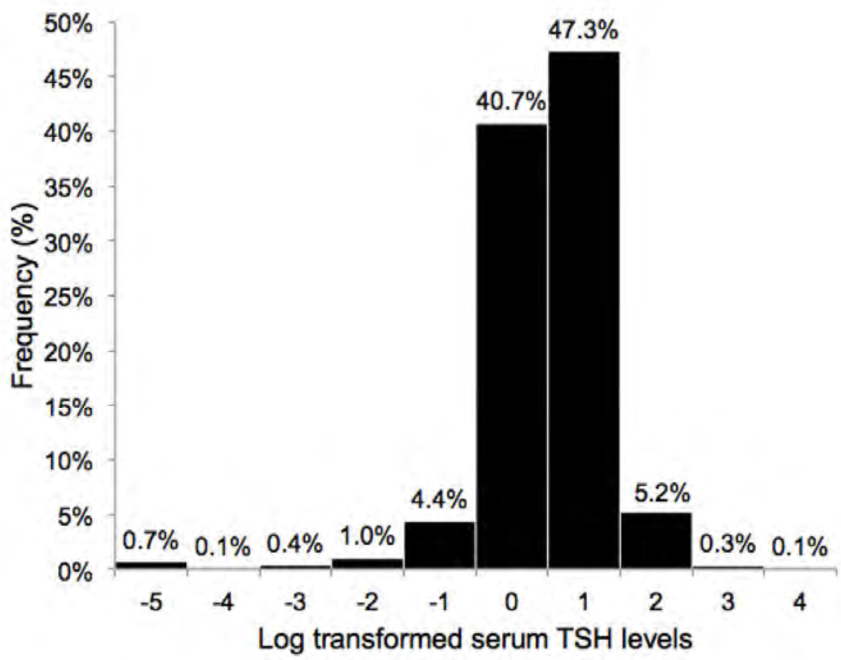

Figure 1 Distribution of log-transformed serum TSH levels

BONE MORPHOGENETIC PROTEIN 6 GENE POLYMORPHISM IS ASSOCIATED WITH SKELETAL RESPONSIVENESS TO CALCIUM SUPPLEMENTATION IN ELDERLY WOMEN

Abstract

Calcium supplementation is widely used either in isolation or in combination with antiresorptive or bone-forming agents for the treatment of osteoporosis. Bone morphogenetic protein 6 (BMP6) has been shown to increase bone mass in ovariectomized animals. BMP6 also modulates intestinal iron absorption. The role of BMP6 in the absorption of other elements of physiological importance is currently unknown. It is the purpose of the present study to explore the role of BMP6 gene in the skeletal responsiveness to calcium supplementation.

gene.

Subjects consisted of 171 postmenopausal women with lumbar spine (L2-L4) bone mineral density (BMD) T-score greater than -2.5. All were given 500 mg/day of elemental calcium supplementation via oral calcium carbonate. PTH and BMD were measured as baseline and after 2 years. High throughput single-nucleotide polymorphism (SNP) screening was performed by comparing the estimated allele frequency derived from hybridization signal intensities of pooled DNA samples from tertile 1 and 3 of PTH responsiveness on Affymetrix's 10K SNP genotyping chips. SNP rs1112482 in ME1 gene was chosen for validation in the present study. Fractional calcium absorption was measured in a separate group of 19 subjects. Data were expressed as mean +/- SEM.

Clinical characteristics of the study subjects are shown in Table 1. The mean (±SD) rate of compliance during the two-year study period was 89.3 ± 4.9 percent. Twenty three (12.9%) had vitamin D deficiency at baseline according to a 25(OH)D cutoff of 20 ng/mL. Table 2 shows the changes in BMD and PTH after calcium supplements according to vitamin D status. Lumbar spine BMD increased significantly both in those with and without vitamin D deficiency. However, significant reduction in serum PTH was apparent only in those who were vitamin D sufficient at baseline.

The BMP6 rs267202 genotype distribution was 55.1% AA, 34.3% AG and 19% GG which conforms to the Hardy –Weinberg equilibrium. In subjects who were vitamin D sufficient, those without the A allele had significantly higher increase in femoral neck BMD (+0.92 ± 0.63 vs. -0.23 ± 0.27%, $P < 0.05$), but no difference in the change in lumbar BMD was detected (+4.05 ± 0.80 vs. +2.62 ± 0.33%, $P = 0.15$) . In line with the response in femoral neck BMD, there was a significant higher suppression of PTH after calcium in

subjects without the A allele (-15.82 ± 4.83 vs. -1.95 ± 0.2.39%, $P < 0.05$). On the contrary, no relationship between BMP6 genotype and the response in BMD or PTH was found in subjects who were vitamin D deficient. No difference in serum hepcidin concentrations in relation to the rs267202 genotype was found.

Introduction

Osteoporosis is in part genetically determined. A number of studies have attempted to elucidate the genes associated with osteoporosis by candidate gene or genome-wide approach which has resulted in the findings helpful in improving understanding and elucidating relevant pathways for therapeutic purposes (1). With regard to current available therapeutic modalities for osteoporosis, calcium supplementation is widely used either in isolation or in combination with antiresorptive or bone-forming agents. The effect of calcium supplementation alone on bone mineral density is generally small and can be transient (2, 3). A systematic review has shown that calcium supplementation reduces fracture risk only in those with very good adherence to calcium (4). Although genetic factors are likely to play role in the skeletal responsiveness to calcium, studies investigated this issue have been scarce.

Bone morphogenetic proteins (BMP) are essential in bone biology. Of all the BMPs, only recombinant human BMP2 and BMP7 are currently approved for clinical use in promoting fracture healing (5). On the other hand, with regard to osteoporosis, only BMP6 has been shown to increase bone mass in ovariectomized experimental animals (6). Besides its role in bone biology, BMP6 also modulates body iron hemeostasis through its regulation of hepcidin, an inhibitor of intestinal iron absorption (7). The role of BMP6 in the absorption of other elements of physiological importance is currently unknown. Given the functions of BMP6 in bone physiology and the regulation of the intestinal absorption of iron, it is conceivable that BMP6 might play role in the responsiveness of bone to calcium supplementation. Therefore it is the purpose of the present study to explore the role of BMP6 gene in the skeletal responsiveness to calcium supplementation.

Materials and Methods

Subjects

Physically active healthy postmenopausal women over the age of 60 years were recruited by public advertisements. Demographic data such as age, sex, occupation and clinical variables including reproductive history were assessed. Each woman was screened by medical history and physical examination. Exclusion criteria included history of metastatic or non-osteoporotic metabolic disease, history of kidney stone within previous five years, vertebral fractures as confirmed by radiographs of the thoracolumbar spines at the beginning of the study, thyroid or parathyroid diseases, the use of calcium or vitamin D supplementation within the previous two months, the use of hormone replacement therapy including estrogens, estrogen receptor modulator or testosterone within the previous six months. Subjects taking glucocoticoids, anticonvulsants or fluoride within the previous year and those with lumbar spine (L2-L4) bone mineral density (BMD) T-score less than or equal -2.5 were also excluded. On the basis of these criteria, 197 women were enrolled in the study. They were assigned to receive 500 mg of elemental calcium in the form of calcium carbonate (British dispensary (L.P.) Co., Ltd., Thailand). Subjects were told to avoid other calcium supplementation during the study period. Compliance to calcium supplementation was assessed by tablet counting every 6 months. Fasting blood samples were obtained at baseline and after 2 years of treatment. The study was approved by local institutional review board and written informed consent was obtained from each participant before entering the study.

Bone mineral density (BMD)

BMD of lumbar spine (L2 –L4) and femoral neck was measured by dual energy x-ray absorptiometry (Lunar Corp, Madison, Wisconsin) at baseline and 2 years after treatment. Quality assurance was maintained by daily calibration and use of one phantom.

Biochemical Measurement

Serum and plasma samples were kept frozen at –80°C until analysis. Serum calcium was analyzed on an automated biochemical analyzer (Dimension RxL, Dadebehring Co Ltd, USA). Plasma intact parathyroid hormone (PTH) was determined by electrochemiluminescence immunoassay on an Elecsys 2010 analyzer (Roche Diagnostic GmbH, Mannheim, Germany). The assay has an intra-assay precision of 3.6 %. CTX**, Hepcidin…

DNA pooling experiment

DNA was extracted from peripheral blood samples by conventional phenol-chloroform method. DNA samples were diluted in TE buffer (0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and quantified using fluorometry (Hoefer DyNa Quant 200, Hoechst dye; Hoefer, Holliston, MA) to a target concentration of 100 ng/μl and then to 50 ng/μl. DNA pools were constructed separately for subjects with change in femoral neck BMD in tertiles 1 and 3 by combining the 50 ng/μl DNA in equal volume. Each pool was purified and again quantified exactly to 50 ng/μl. Allele frequency estimation from pooled DNA was performed as described in a previous report (8). Briefly, subjects in were divided into tertiles based on femoral the change in femoral neck BMD after 2 years of calcium supplementation. Triplicates of pooled DNA samples were formed, each consisting of DNA from individuals with the change in femoral neck BMD in the lower tertile, together with another triplicates of pooled DNA samples from subjects with the change in femoral neck BMD in the higher tertile. Pooled DNA samples were then genotyped using an Affymetrix GeneChip® Human Mapping 500K Array set (Santa Clara, CA). Signal intensity among the 46 SNPs in BMP6 gene were compared between subjects in the lowest and the highest tertiles. The intronic SNP rs267202 in which there was the highest difference in signal intensity was then genotyped individually in all subjects.

Individual SNP genotyping

Individual genotyping of all subjects was performed using real-time PCR (TaqMan® MGB probes): 10 ng of DNA was added into the PCR reaction, consisting of TaqMan Universal Master Mix (1x) and TaqMan MGB probes for intronic A/T SNP rs267202 (1x) in a total volume of 10 μL. The real-time PCR reaction protocol was 10 min at 95 °C, 40 cycles of 15 s at 92 °C, and 1 min at 60 °C using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Statistical Analysis

Data were expressed as mean +/- SEM unless stated otherwise. The unpaired Student's *t* test (**Kolmogorov–Smirnov) was used to analyze differences between groups. A p value of < 0.05 was considered statistically significant.

Results

Clinical characteristics of the study subjects are shown in Table 1. The mean (±SD) rate of compliance during the two-year study period was 89.3 ± 4.9 percent. Twenty three (12.9%) had vitamin D deficiency at baseline according to a 25(OH)D cutoff of 20 ng/mL. Table 2 shows the changes in BMD and PTH after calcium supplements according to vitamin D status. Lumbar spine BMD increased significantly both in those with and without vitamin D deficiency. However, significant reduction in serum PTH was apparent only in those who were vitamin D sufficient at baseline.

The BMP6 rs267202 genotype distribution was 55.1% AA, 34.3% AG and 19% GG which conforms to the Hardy –Weinberg equilibrium. Clinical characteristics according to the BMP6 genotype are shown in Table 3. There was no significant difference in age, body weight, height, serum PTH and BMDs among genotypes.

Figure 1 shows the percent changes in lumbar BMD, femoral BMD and PTH after 2 years of calcium supplementations in relation to the BMP6 genotype in subjects who were vitamin D sufficient. When subjects with having the A allele were combined (AA+AG), it was found that subjects without the A allele had significantly higher increase in femoral neck BMD (+0.92 ± 0.63 vs. -0.23 ± 0.27%, $P < 0.05$), but no difference in the change in lumbar BMD was detected (+4.05 ± 0.80 vs. +2.62 ± 0.33%, $P = 0.15$) . In line with the response in femoral neck BMD, there was a significant higher suppression of PTH after calcium in subjects without the A allele (-15.82 ± 4.83 vs. -1.95 ± 0.2.39%, $P < 0.05$). On the contrary, no relationship between BMP6 genotype and the response in BMD or PTH was found in subjects who were vitamin D deficient (Figure 2).

It is now known that BMP6 regulate the expression of hepcidin, an intestinal iron absorption inhibitor. To explore if the rs267202 SNP is functional in term of the regulation of circulating hepcidin, association study of serum hepcidin levels and the rs267202 SNP was performed. There was no difference in serum hepcidin concentrations in relation to rs267202 genotype as shown in Figure 2.

Discussion

In the present study, we demonstrated that the response in BMD after calcium supplementation is related to a genetic variant in BMP6. Moreover, in agreement with the finding on BMD, the suppression of PTH was also related to the BMP6 genotype. BMPs including BMP6 are important in osteogenesis when studied in vitro. However, BMP6 knockout mice show no gross skeletal abnormality except for a delay in sternal ossification (9). Our study suggests that besides possessing direct positive effect on bone formation, BMP6 may also mediate the influence of calcium on bone formation or mineralization. Despite the better responsiveness of BMD in relation to BMP6 genotype, no difference in baseline BMD was detected. This may partly be explained in that calcium intake is generally low in our population and the effect of calcium according to the BMP6 genotype, if any, is less likely to be discerned without calcium supplementation.

In the present study, we also demonstrated the influence of BMP6 genotype on the suppression of PTH after calcium supplementation. Besides directly affect bone formation, BMP6 also influences bone formation induced by PTH (10). On the other hand, the reciprocal influence of BMP6 on PTH synthesis and secretion has not been reported. The parathyroid glands are controlled mainly through the calcium sensing and vitamin D receptors (11, 12). It is unclear at present if BMP6 affects the expression of calcium sensing and vitamin D receptors. Given the observation from the present study, further studies to evaluate this issue are warranted.

Recently it has been demonstrated that BMP6 controls the expression of hepcidin, an inhibitor of intestinal calcium absorption produced by the liver. BMP6 knockout mice have marked increase in iron absorption and hemochromatosis (13). Hepcidin inhibit iron absorption through its binding to ferropontin, the main iron exporter in mammals (14). Although the main biological role of hepcidin is in iron metabolism, it also possesses functions related to innate immunity. It is unknown at present if hepcidin plays any role in the regulation of calcium and bone metabolism. Our results suggest that such role of hepcidin in less likely since there was no relationship between circulating hepcidin and the responsiveness to calcium supplementation. Moreover, the skeletal responsiveness in relation to the BMP6 genotype is not likely to be mediated through hepcidin since the SNP under study is not associated with circulating hepcidin levels.

It is of note that our results suggest interactions among BMP6 genotype and vitamin D status in the skeletal responsiveness to and calcium. Other potential mechanisms for the relationship between skeletal responsiveness to calcium may involve the influence of BMP6 on the induction of osteogenesis by PTH and vitamin D. BMPs by themselves are potent stimulators of bone formation. Furthermore, in the presence of BMP6, there is a potent effect of PTH and vitamin D in inducing bone development by human mesenchymal stem cells (15). The SNP studied is located in the intron with unclear biological function although there is possibilty the studied SNP is in linkage disequilibrium with nearby SNPs with more direct functional role. It is unknown if the intronic SNP studied affects circulating BMP6 since BMP6 levels were not measured in the present study.

In conclusion, we demonstrated for the first time in the present study that cytosolic malic enzyme 1 gene polymorphism is associated with the degree of suppression of parathyroid hormone after long-term calcium supplementation. The effect is probably mediated through increased intestinal calcium absorption.

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Table 1 Baseline characteristic of the subjects

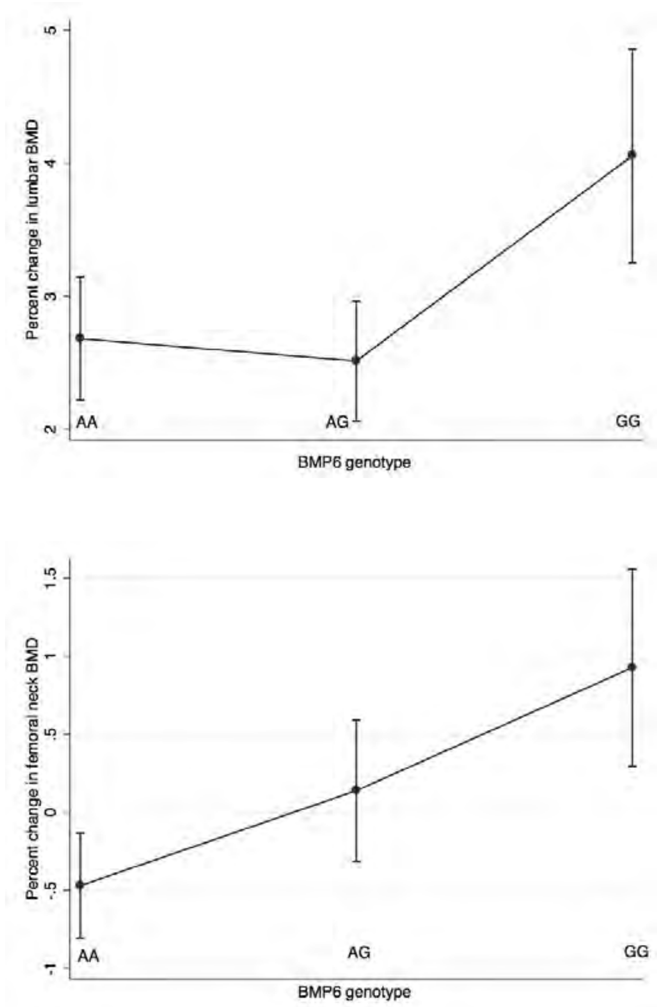
Characteristics	**Mean ± SD**
Age (year)	66.0 ± 4.5
Weight (kg)	59.3 ± 8.8
Height (cm)	153.0 ± 5.0
BMI (kg/m^2)	25.3 ± 3.5
Serum calcium (mmol/L)	2.37 + 0.11
Plasma PTH (pmol/L)	4.7 ± 1.9
Serum 25(OH)D (nmol/L)	69.4 ± 18.5
Lumbar spine L2-L4 BMD (g/cm^2)	1.01 ± 0.12
Femoral neck BMD (g/cm^2)	0.79 ± 0.10

	Vitamin D deficient (n = 31)			Vitamin D sufficient (n = 147)		
Lumbar BMD (g/cm^2)	0.99±0.02	1.01± 0.02	< 0.05	1.01±0.01	1.04±0.01	< 0.001
Femoral neck BMD (g/cm^2)	0.80±0.02	0.79 ±0.02	NS	0.79±0.01	0.79±0.01	NS
PTH (pg/mL)	46.5±2.7	44.3 ± 3.1	NS	43.8 ±1.5	40.6 ± 1.3	< 0.01

Table 2 Changes in BMD and PTH after calcium supplementation according to vitamin D status.

	AA (n = 98)	AG (n = 61)	GG (n = 19)
Age (year)	65.7±4.4	66.1±4.6	66.8±4.3
Body weight (kg)	59.3±8.9	58.5±7.8	59.9±10.0
Height (cm)	153.2± 4.9	153.1±5.3	152.6±4.1
PTH (pg/mL)	43.3 ±17.7	46.1±20.1	43.0±9.8
L2-4 BMD (g/cm^2)	1.01±0.11	1.01±0.13	0.99±0.14
Femoral neck BMD (g/cm^2)	0.80±0.10	0.80±0.09	0.76±0.09

Table 3 Clinical characteristics according to the BMP6 genotype.



<u>Fig 1</u> Percent changes in lumbar BMD and femoral BMD after 2 years of calcium supplementations in relation to the BMP6 genotype in subjects who were vitamin D sufficient.

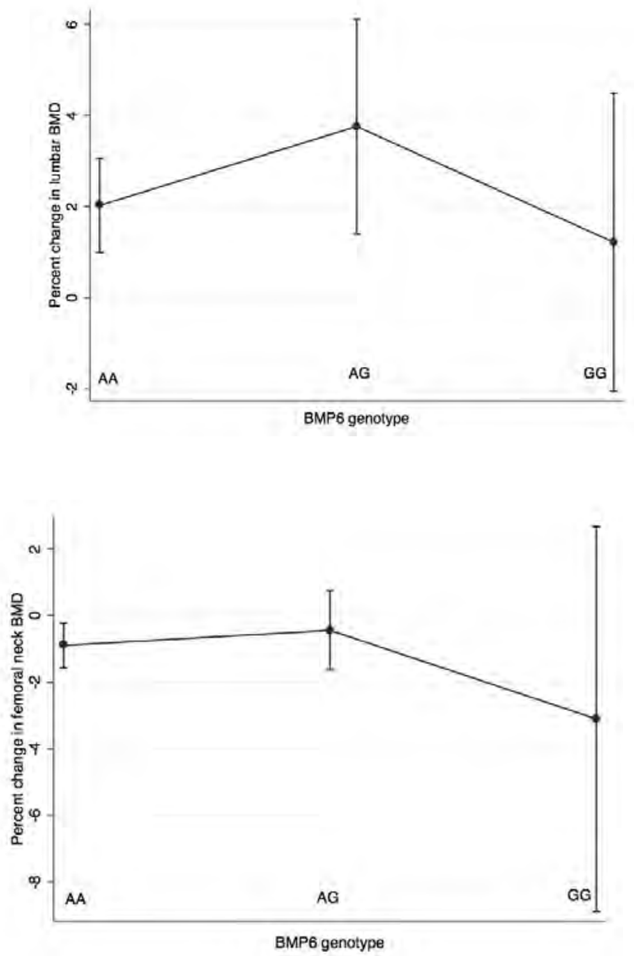


Fig 2 Percent changes in lumbar BMD and femoral BMD after 2 years of calcium supplementations in relation to the BMP6 genotype in subjects who were vitamin D insufficient.

THAI TRADITIONAL MASSAGE INCREASES BIOCHEMICAL MARKERS OF BONE FORMATION IN POSTMENOPAUSAL WOMEN: A RANDOMIZED CROSSOVER TRIAL

Abstract

Background: The effect of massage therapy on bone metabolism in adults has only scarcely been explored. In a randomized crossover trial, we investigated the skeletal effect of Thai traditional massage by examining the changes in biochemical markers of bone turnover.

Methods: Forty-eight postmenopausal women participated in the study. All volunteers were randomized to a 2-hour session of Thai traditional massage twice a week for 4 weeks and a 4-week control period after a 2-week washout, or vice versa.

Twenty-one subjects were allocated to receiving Thai traditional massage first, followed by the control period, while 27 were initially allocated to the control period.

Results: Serum P1NP increased significantly after Thai traditional massage ($P < 0.01$), while there was no change in serum osteocalcin or CTX. During the control period, there was no significant change in P1NP, osteocalcin or CTX compared to baseline. When age and height were taken into account, P1NP in postmenopausal women whose ages were in the middle and higher tertiles and whose heights were in the lower and middle tertiles (n = 22) had a 14.8 ± 3.3% increase in P1NP after massage ($P < 0.001$), while no change in P1NP was found in the rest of the women (n = 26).

Conclusions: Thai traditional massage results in an increase in bone formation as assessed by serum P1NP, particularly in postmenopausal women who are older and have a smaller body build.

Background

Mechanical loading favorably influences bone mass. Active exercise, as well as passive exercise through low-amplitude whole-body vibration, have been demonstrated to improve bone mass or delay bone loss [1,2]. Massage therapy has been shown to alleviate bone pain [3] and improve bone growth in both animals [4] and humans [5] during the postnatal period. However, the effect of massage therapy on bone metabolism in adults has only scarcely been explored.

Thai Traditional massage exerts pressure on the body in a rhythmic fashion. The massage performer uses the outstretched heels of both hands to exert pressure on the body of the subject approximately once every 1-2 seconds for 2 hours. It is likely that the physical load from Thai traditional massage may induce strain in the skeleton and affect bone, similar to other means of applying mechanical load. We have demonstrated in a previous study that Thai traditional massage results in an acute anabolic effect on bone, as assessed by biochemical markers of bone turnover [6]. It is unclear if a longer term of massage therapy would result in similar effects. Toward this end, a randomized crossover study was employed to investigate the skeletal effect of Thai traditional massage by examining the changes in biochemical markers of bone turnover.

Methods

Subjects

A randomized crossover design was used. A total of 48 postmenopausal women participated in the study. All were non-diabetic, as defined by a 2-hour plasma glucose level <200 mg/dL on a 75-g oral glucose tolerance test. Subjects having disorders that could affect bone metabolism – such as hyperparathyroidism, thyrotoxicosis, diabetes, rheumatoid arthritis and cancer, as well as those who were taking glucocorticoids or medications for osteoporosis – were excluded. The study was approved by the Institutional Review Board of Ramathibodi Hospital. Signed informed consent was obtained from each subject prior to the study.

Thai traditional massage

All volunteers were randomized to either the treatment or the control group. Subjects in the treatment group underwent a 2-hour session of Thai traditional massage twice a week for 4 weeks, while no intervention was given to subjects in the control group. After a 2-week washout period, subjects were switched to the other arm of intervention for 4 weeks.

Thai traditional massage was performed by a single masseuse throughout the study. Subjects were requested to change into comfortable, loose-fitting clothes and to lie flat on a firm mattress on the floor. The procedure consisted of the masseuse applying firm, rhythmic pressure over the volunteer's body through the heels of her hands. The 2-hour procedure started with massaging the feet, and then the legs, arms, hands, back and neck, ending with a head massage.

Biochemical measurement

Subjects were requested to refrain from exercise for 24 hours and to fast for at least 10 hours before blood was drawn. Blood samples were collected on the day of the initiation of the massage or control period, and on the day following each massage or control period. Serum C-terminal telopeptide of type I collagen (CTx-I), total procollagen type 1 amino-terminal propeptide (P1NP), N-MID osteocalcin, and insulin were determined by electrochemiluminescence immunoassay on a Cobas e 411 analyzer (Roche Diagnostics, Mannheim, Germany). The intra-assay precision was 3.8%, 3.8%, 1.4% and 1.9%, respectively

Bone densitometry and measurement of body composition

Bone mineral density (BMD) and body composition were measured by dual-energy X-ray absorptiometry (DEXA) (Lunar Prodigy; GE Healthcare, Little Chalfont, UK). Daily calibration and quality control were performed regularly according to the manufacturer's recommendations. Body composition and BMD of lumbar spine 1-4, femoral neck and total hip were measured in each subject.

Statistical analysis

Changes in biochemical markers of bone turnover for both Thai traditional massage and control periods were assessed by paired Student's *t*-test. Crossover statistical analysis was performed by the pkcross routine in Stata 12 software (StataCorp LP, College Station, TX), assuming no carryover effects. A *P*-value of less than 0.05 was considered statistically significant.

Results

Table 1 demonstrates the clinical characteristics of the 48 study subjects. The mean age was 59.1 ± 4.3 years. All were postmenopausal women with an average of 8.9 ± 5.6 years since menopause. Seventeen (35.4%) were obese, based on BMI >25 kg/m^2. Seven (14.6%) had osteoporosis either at the spine, femoral neck or total hip, according to a DEXA T-score of −2.5 or less at the corresponding sites.

Twenty-one subjects were allocated to have Thai traditional massage first, followed by the control period, while 27 were initially allocated to the control period. When combined data from all subjects were analyzed, it was found that serum P1NP increased significantly after Thai traditional massage while there was no change in serum osteocalcin or CTX. During the control period, there was no significant change in P1NP, osteocalcin or CTX compared to baseline (Table 2). In a linear mixed model looking at the effect of massage and the sequence of intervention, it was found that Thai traditional massage significantly increased serum P1NP. No influence of the sequence of treatment allocation was found (Table 3).

It is likely that body size as well as bone and fat mass may affect the responsiveness to the externally applied mechanical loading from Thai traditional massage. We investigated if there were associations between the percent change in P1NP after massage and age, body weight, height, and body composition. Table 4 demonstrates the change in serum P1NP according to body height tertiles. There was a significant increase in serum P1NP post-massage in subjects in both the lower and middle height tertiles. However, no change in serum P1NP was detected in subjects in the upper body height tertile. When age and body height were considered in combination, it was found that P1NP in postmenopausal women whose ages were in the middle and upper tertiles and whose heights were in the lower and middle tertiles (n = 22) had a 14.8 ± 3.3% increase in P1NP after massage (P <0.001), while no change in P1NP was found in the rest of the women (n = 26).

Discussion

Massage has been widely utilized for the alleviation of a number of musculoskeletal disorders, including low back pain and bone pain from metastatic malignancy [3]. Despite its common utilization, studies showing evidence of the beneficial effects of massage therapy are still limited. Thai traditional massage has been shown to reduce pain and muscle tension in patients with scapulocostal syndrome [7]. Moreover, in a preliminary study on the acute effects of Thai traditional massage on biochemical markers of bone turnover, we demonstrated that a single 2-hour session can acutely increase serum P1NP, a marker of bone formation [6]. In keeping with our previous study, using a randomized crossover design we demonstrated in the present study that two sessions per week of Thai traditional massage for 4 weeks resulted in an increase in circulating P1NP without affecting CTX, a marker of bone resorption. It is of note that the effect of Thai traditional massage on bone formation was more apparent in postmenopausal women of older age. While the improvement in the marker of bone formation cannot readily be extrapolated to an enhancement of bone mass or reduced fracture risk, our finding at least suggests that Thai traditional massage is likely to be beneficial to bone, particularly in women of advancing age among whom osteoporosis is a common health problem.

The effect of massage on bone metabolism in adults has scarcely been explored. However, a number of studies have investigated the influence of massage on the alteration of bone growth, particularly during the postnatal period. For example, massage in the early postnatal period was found to promote lean mass and bone growth in experimental animals [4]. In humans, when combined with physical activity, massage during the peri-neonatal period improves bone formation without changes in bone resorption [8]. Our findings are in keeping with those in infants, where massage therapy results in an increase in serum P1NP but not CTX.

It is well established that mechanical load affects bone cells. The strain characteristics that determine skeletal responses include strain magnitude [9], strain frequency [10] and strain rate [11]. There appears to be an inverse relationship between strain magnitude and frequency for inducing osteogenic effects. Low-magnitude mechanical load needs to be applied at high frequency in order to have an effect equivalent to high-magnitude mechanical load at lower frequency [12]. In line with our previous study, we demonstrated in the present study that external periodic mechanical loading applied through Thai traditional massage is likely to have an anabolic effect on bone; this suggests that Thai traditional massage could be another option for enhancing bone health through mechanical loading. Besides its direct effect on bone through mechanical loading, it is conceivable that Thai traditional massage may affect bone indirectly through the central nervous system. The adipokine leptin inhibits bone formation through a central nervous system delay [13]. Mechanical tactile stimulation reduces stress hormones and improves bone mineralization in rats [14]. Moreover, a study using functional MRI has shown that types of massage can influence brain cortical areas differently [15]. To what extent the effect of Thai traditional massage is due to its effect on stress hormones and the central nervous system is unknown. One of the other possible mechanisms involves ghrelin. It has been shown that ghrelin enhances bone formation [16], and that massage therapy in infants increases circulating ghrelin [17].

There are a number of limitations in the present study. The sample size is relatively small and may not be able to detect small effects on bone resorption. Moreover, although the current results are consistent with those of our previous study and of other studies in infants, it is still unclear if the increase in bone formation as reflected by bone markers will result in higher bone mass or reduced fractures. Further studies to confirm our results, as well as to investigate the effect of Thai traditional massage on bone mass or fractures, are warranted.

Conclusions

The present study demonstrated that Thai traditional massage results in an increase in bone formation as assessed by serum P1NP, particularly in postmenopausal women who are older and have a smaller body build.

Table 1 Characteristics of the study population.

Parameters	Mean ± SD (range) (n = 48)
Age (years)	59.1 ± 4.4 (49.8–66.6)
Years since menopause	8.9 ± 5.6 (1.2–24.3)
Body weight (kg)	57.1 ± 7.4 (40.0–73.8)
Height (cm)	153.1 ± 5.0 (141.5–163.5)
Body mass index (kg/m^2)	24.3 ± 2.9 (18.1–30.9)

Table 2 Serum P1NP (median (range)), osteocalcin (mean ± SE) and CTX (median (range)) before and after massage or the control period. Serum P1NP increased significantly after massage.

	Baseline	**After massage**	***P***
P1NP	45.2 (40.2–50.3)	48.5 (44.2–53.3)	<0.01
Osteocalcin	22.8 ± 1.0	22.9 ± 0.8	NS
CTX	0.38 (0.33–0.43)	0.39 (0.34–0.43)	NS

	Baseline	**After control period**	***P***
P1NP	45.8 (41.9–50.1)	46.0 (42.0–50.4)	NS
Osteocalcin	23.0 ± 0.9	23.0 ± 0.9	NS
CTX	0.38 (0.34–0.43)	0.38 (0.34–0.43)	NS

Table 3 Effects of treatment and its sequence on the change in P1NP after massage. The effect of massage was statistically significant, while the sequence of treatment did not have a significant effect.

	MS	**F**	***P***
Sequence effect	43.76	0.26	NS
Treatment effect	1294.95	5.69	<0.05

Table 4 Changes in P1NP (%) after massage according to body height and age tertiles. Serum P1NP increased significantly in the middle and upper body height tertiles. Likewise, serum P1NP increased significantly in the middle age tertile. The increase in P1NP in the upper age tertile almost reached statistical significance.

Body height	**Post-massage change in P1NP**	***P***
Tertile 1	+12.3 ± 3.9	<0.01
Tertile 2	+9.7 ± 4.0	<0.05
Tertile 3	+3.5 ± 3.5	NS

Age	**Post-massage change in P1NP**	***P***
Tertile 1	+3.9 ± 3.2	NS
Tertile 2	+13.6 ± 3.8	<0.01
Tertile 3	+8.1 ± 4.3	0.08

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EFFECTS OF WHOLE BODY VIBRATION ON SERUM OSTEOCALCIN AND GLUCOSE HOMEOSTASIS

Abstract

Objective To assess the effects of whole body vibration on serum osteocalcin and glucose homeostasis in premenopausal and postmenopausal women.

Research design and methods Thirty premenopausal and thirty postmenopausal women (age, 20-70 years) were randomly assigned to a whole body vibration (WBV) group or a control (CON) group. Baseline serum osteocalcin, P1NP, C-telopeptide, fasting plasma glucose and fasting plasma insulin were measured. In the WBV group, subjects were assigned to train three times weekly for 12 weeks. After 3-month intervention, all parameters were measured again. HOMA-B%, HOMA-S% and HOMA-IR were calculated by using HOMA2 Calculator v2.2 of Diabetes Trials Unit, University of Oxford. Data were analyzed by means of paired-samples T test and independent-samples T test.

Results In WBV group, none of the baseline to 3-month differences of both premenopausal and postmenopausal women were statistically significant. None of mean changes of all parameters in both premenopausal and postmenopausal women who were assigned to vibration training or no training showed statistically significant differences between groups.

Conclusion Whole body vibration training has no effect on serum osteocalcin and glucose homeostasis.

Osteoporosis is an important risk factor for fractures leading to a higher rate of morbidity and mortality compared to those without a fractures (1). Increased economic burden both for the caring families and the governments involved can ensue as a result of osteoporotic fractures(2). Current therapeutic measures for osteoporosis focus on using antiresorptive medications and the promotion of exercise to improve bone strength (1). However, exercise has its own limit since it may cause injury and increase the risk of fracture (3). It is therefore crucial to search for alternatives to active exercise which are safer and beneficial. Recently the use of low magnitude mechanical signals to inhibit bone loss has been described (4). During intense activity, small magnitude strain signals are evident in bone from muscle contraction and persist over long durations. These signals are useful for the function and strength of the bone. In immobilized persons and the elderly people with limited mobility, decreased strain signals may result in a higher suscepibilty to osteoporosis (4). The use of vibration to deliver low-magnitude, high-frequency signals can produce similar results as those occuring during active exercise which may be able to ameliorate the deterioration of bone mass in such patients.

There has been an increasing interest in the relationship between serum osteocalcin and glucose homeostasis in recent years. There are studies showing that elevated level of serum osteocalcin was associated with improved glucose tolerance and enhanced ß-cell function (5,6,7). Furthermore, the carboxylated form of serum osteocalcin was found to be associated with improved insulin sensitivity (5). It is likely that deliver low-magnitude, high-frequency vibration may result in higher serum osteocalcin through the stimulation of osteoblasts and might affect glucoe homeostasis favorably. Toward this end, we investigated in the present study whether low-magnitude, high-frequency whole body vibration training can increase serum osteocalcin and improve glucose homeostatis in pre- and postmenopausal women.

Materials and method

Thirty premenopausal and thirty postmenopausal women, aged 20 to 70 years, who worked at Ramathibodi hospital volunteered to participate in the study. All subjects were screened by a questionnaire and physical examination. The inclusion criteria were (1) women, aged 20 to 70 years; (2) normal nutritional status; (3) free from diseases or medications known to affect bone metabolism or muscle strength.The exclusion criteria were (1) a history of malignancy; (2) never been treated with glucose lowering agents; (3) the taking of medications known to affect bone or glucose metabolism, such as, glucocorticoids; (4) smoking; (5) alcohol drinking > 2 drinks/ day; (6) a history of liver, renal or thyroid diseases; (7) a previous fracture; (8) psychiatric disorder; (9) hyperparathyroidism; (10) pregnancy/breast feeding; (11) frequent exercise ≥ 3 times/week. All subjects were randomly assigned to a whole body vibration (WBV) group or a control (CON) group according to a confidential, randomized number sequence generated by an independent statistical consultant. Fifteen subjects each from premenopausal and postmenopausal women who were assigned to the WBV group were trained for 3 months on a vibrating platform (Model my5^{TM}; PowerPlate). Training frequency was three times a week and the duration was 20 min/day. Thirty subjects in CON group did not participate in any training. All subjects provided written informed consent before enrollment, and the study protocol was approved by local Institutional Review Board.

At Baseline and at 3 months, serum osteocalcin, P1NP, C-telopeptide, fasting plasma glucose and fasting plasma insulin were measured. Serum osteocalcin, P1NP, C-telopeptide and fasting plasma insulin were determined by electrochemiluminescence immunoassay on a Cobas e411 (Roche Diagnostic GmbH, Mannheim, Germany). Fasting plasma glucose levels were determined by the hexokinase method. To estimate ß-cell function, insulin sensitivity and insulin resistance, HOMA was used based on fasting plasma glucose and fasting plasma insulin concentrations. Beta-cell function, insulin sensitivity and insulin resistance were estimated using HOMA-B%, HOMA-S% and HOMA-IR, respectively. All HOMA values were calculated by using HOMA2 Calculator v2.2 of Diabetes Trials Unit, University of Oxford.

Statistical analysis

The independent-sample T test was used to test for differences in baseline characteristics between the WBV group and CON group both in premenopausal and postmenopausal women. Baseline to 3-month changes of premenopausal and postmenopausal women in the WBV and the CON groups were evaluated by the paired T test. Mean changes of premenopausal and postmenopausal women in both groups were also analyzed by independent-sample T test. All data are presented as means ± SDs. Analysis was performed using SPSS version 17.0 and P values of < 0.05 were considered significant.

Results

One patient denied to complete the study because of the big flood that occurred during the study period> Many subjects discontinued the study for a while but came back to continue the study when the situation resolved. Because of this, compliance rate of subjects varied. data Subjects who could not participate in the training ≥ 50% were then excluded from analyses. This resulted in the exclusion of 1 premenopausal subject who was assigned to the WBV group and 4 postmenopausal subjects who were assigned to the WBV group.

Baseline characteristics of subjects are shown in Table 1. Age, weight, BMI, osteocalcin and C -telopeptide levels of postmenopausal women in both the WBV and the CON groups were significantly higher than those of premenopausal women in each group. P1NP and HOMA-

IR of postmenopausal women in the CON group were also significantly higher than those in premenopausal women in the same group (55.92±12.33 vs 40.38±15.36, $P < 0.05$ and 1.35±0.74 vs 0.84±0.25, $P < 0.05$). No significant differences were observed at baseline between the WBV and the CON group of both premenopausal and postmenopausal women in terms of age, height, weight, BMI, P1NP, osteocalcin, C-telopeptide, HOMA-B%, HOMA-S% or HOMA-IR (Table 2 and Table 3).

In the WBV and CON group, none of the changes from baseline of both premenopausal and postmenopausal women were statistically significant. Mean changes of all parameters in both premenopausal and postmenopausal women are shown in Table 6 and Table7. No significant between-group differences were observed in all parameters. Similarly, none of the between-group differences of mean changes in WBV and CON group were statistically significant (Table 8 and Table 9). Two subjects developed diabetes during this study. One patient was premenopausal woman assigning in WBV group which her data was excluded from the study because of poor compliance and another subject was postmenopausal woman also assigning in WBV group. Both were advised to start the treatment.

Discussion

Whole body vibration is a mechanical acceleration that uses low–magnitude, high-frequency mechanical stimuli to induce muscle activity. Normally, during vigorous activity, in addition to large amplitude mechanical forces that have already associated with, smaller magnitude strain signals are also evident in bone, and it is these signals that whole body vibration can produce similarly. These signals are useful for muscle strength and the function and strength of the bone. So, there is a hypothesis that whole body vibration may have an osteogenic effect and can inhibit bone loss which may have an efficacy for preventing and treating osteoporosis. Moreover, this vibration training is increasingly being interested because of its safety that has been more than strenuous load-bearing exercises which may increase the risk for injuries. This makes the whole body vibration serving as an alternative strategy for low-risk exercise program.

Due to the decrease of high-frequency muscle-based signals which usually occurs in elderly individuals who have decreasing muscle movement, many studies of whole body vibration training were done in this group of population who are most at risk of osteoporotic fractures. Verschueren et al. showed that hip BMD significantly increased 0.9% in postmenopausal women who were randomly assigned to a whole body vibration training group which trained three times weekly for 6 months (8). There were no changes in serum osteocalcin in any group of this study.

In premenopausal women, there are few studies of whole body vibration. Torvinen et al. studied in young healthy adults and showed no effect of whole body vibration training on mass, structure, and strength of bone (9). The authors suggested that this was because of the musculoskeletal tissues of these young adults had no physiological need to adapt to the vibration loading. Moreover, in this study, the duration of vibration was only 4 minutes that was quite short and might have been insufficient to require adaptation. So in our study, we designed to train 20 min/day which had been a study supporting enough effect on postmenopausal women.

The results of our study showed that no significant baseline to 3-month differences of both premenopausal and postmenopausal women who were assigned to WBV group were observed. Remarkably, HOMA-B% of postmenopausal women in this group decreased after 3-month of intervention even though this change was not significant but almost ($p = 0.053$), after comparing with postmenopausal women in the control group in which the HOMA-B% also decreased, no significant between-group differences were observed. Moreover, none of mean changes of all parameters in both premenopausal and postmenopausal women who were assigned to vibration training or no training showed statistically significant differences between groups and our study did not show association between serum osteocalcin and glucose homeostasis. The observed negative results might in part be due to the discontinuation of most subjects because of the big flood in Thailand last year and poor compliance of subjects which varied between 50-90%. No adverse reactions were reported in the WBV group in our study. More studies are needed to prove the effect of whole body vibration training on bone and its safety in long-term usage.

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<u>Table 1</u>. Baseline characteristics

parameter	WBV group		CON group		p Value#	p Value##
	pre	post	pre	post		
Age (years)	32.82±8.82	57.56±2.24	32±8.21	56±2.77	< 0.001*	< 0.001*
Height (cm.)	155.82±5.86	155.67±7.63	155.58±4.60	155.77±5.23	0.960	0.926
Weight (kg.)	51.45±6.22	62.22±9.40	50±6.18	63.54±9.49	0.007*	< 0.001*
BMI (kg/m^2)	21.09±2.17	25.78±3.83	20.58±2.19	26.08±3.09	0.003*	< 0.001*
Osteocalcin (ng/ml)	18.10±5.71	26.21±7.64	20.21±7.70	28.41±9.40	0.007*	0.014*
C-telopeptide (ng/ml)	0.28±O.12	0.41±0.17	0.32±0.21	0.45±0.11	0.030*	0.039*
PINP (ng/ml)	42.96±15.30	56.21±16.68	40.38±15.36	55.92±12.33	0.056	0.005*
HOMA-B%	113.94±47.03	104.94±33.94	101.23±19.14	106.15±39.16	0.612	0.665
HOMA-S%	266.06±525.46	107.85±53.48	127.70±33.77	100.61±58.55	0.356	0.132
HOMA-IR	0.99±0.70	1.23±0.74	0.84±0.25	1.35±0.74	0.42	0.021*

Pre = premenopausal women, Post = postmenopausal women

p Value# = pre-post difference in WBV group

p Value## = pre-post difference in CON group

Table 2. Baseline characteristics in premenopausal group

parameter	Baseline in Pre[#] group		Between-group difference	
	WBV group	CON group	Mean	p Value
Age (years)	32.82±8.82	32±8.21	0.82	0.820
Height (cm.)	155.82±5.86	155.58±4.60	0.23	0.916
Weight (kg.)	51.45±6.22	50±6.18	1.45	0.580
BMI (kg/m^2)	21.09±2.17	20.58±2.19	0.51	0.583
PINP	42.96±15.30	40.38±15.36	2.59	0.654
Osteocalcin	18.10±5.71	20.21±7.70	-2.11	0.411
CTx	0.28±O.12	0.32±0.21	-0.04	0.483
HOMA-B%	113.94±47.03	101.23±19.14	12.70	0.36
HOMA-S%	266.06±525.46	127.70±33.77	138.36	0.317
HOMA-IR	0.99±0.70	0.84±0.25	0.15	0.457

Pre[#] = premenopausal women group

Table 3. Baseline characteristics in postmenopausal group

parameter	Baseline in Post# group		Between-group difference	
	WBV group	CON group	Mean	p Value
Age (years)	57.56±2.24	56±2.77	1.56	0.178
Height (cm.)	155.67±7.63	155.77±5.23	-0.10	0.970
Weight (kg.)	62.22±9.40	63.54±9.49	-1.32	0.752
BMI (kg/m^2)	25.78±3.83	26.08±3.09	-0.30	0.842
PINP	56.21±16.68	55.92±12.33	0.28	0.961
Osteocalcin	26.21±7.64	28.41±9.40	-2.21	0.543
CTx	0.41±0.17	0.45±0.11	-0.04	0.505
HOMA-B%	104.94±33.94	106.15±39.16	-1.21	0.937
HOMA-S%	107.85±53.48	100.61±58.55	7.24	0.757
HOMA-IR	1.23±0.74	1.35±0.74	-0.12	0.687

Post# = postmenopausal women group

Table 4.

parameter	WBV group				Baseline-3-month difference within group (p Value)[#]	Baseline-3-month difference within group (p Value)[##]
	pre		post			
	Baseline	3 months	Baseline	3 months		
PINP	42.96±15.30	42.44±15.93	56.21±16.68	57.29±17.06	0.857	0.733
Osteocalcin	18.10±5.71	18.25±5.61	26.21±7.64	25.58±5.83	0.845	0.763
CTx	0.28±O.12	O.27±0.11	0.41±0.17	0.47±0.20	0.670	0.107
HOMA-B%	113.94±47.03	118.94±56.84	104.94±33.94	88.10±30.72	0.710	0.053
HOMA-S%	266.06±525.46	136.74±73.16	107.85±53.48	135.62±107.84	0.350	0.265
HOMA-IR	0.99±0.70	1.05±0.80	1.23±0.74	1.20±0.82	0.755	0.815

p Value[#] premenopausal women group

p Value[##] postmenopausal women group

Table 5.

parameter	CON group				Baseline-3-month difference within group (p Value)#	Baseline-3-month difference within group (p Value)##
	pre		post			
	Baseline	3 months	Baseline	3 months		
PINP	40.38±15.36	40.82±12.53	55.92±12.33	54.56±10.42	0.824	0.459
Osteocalcin	20.21±7.70	19.44±6.49	28.41±9.40	27.53±6.13	0.296	0.562
CTx	0.32±0.21	0.35±0.24	0.45±0.11	0.46±0.13	0.307	0.567
HOMA-B%	101.23±19.14	106.21±31.22	106.15±39.16	98.58±35.58	0.428	0.275
HOMA-S%	127.70±33.77	123.31±44.74	100.61±58.55	115.64±68.62	0.697	0.355
HOMA-IR	0.84±0.25	0.91±0.35	1.35±0.74	1.14±0.60	0.422	0.052

p Value# premenopausal women group

p Value## postmenopausal women group

Table 6. Mean changes and between-group differences in premenopausal group

parameter	Mean changes in Pre# group		Between-group difference	
	WBV group	CON group	Mean	p Value
PINP	-0.53	+0.45	-0.98	0.779
Osteocalcin	+0.16	-0.77	0.92	0.388
CTx	-0.01	+0.03	-0.03	0.266
HOMA-B%	+5	+4.97	0.03	0.999
HOMA-S%	-129.33	-4.39	-124.94	0.343
HOMA-IR	+0.06	+0.07	-0.01	0.967

Pre# = premenopausal women group

Table 7. Mean changes and between-group differences in postmenopausal group

parameter	Mean changes in Post# group		Between-group difference	
	WBV group	CON group	Mean	p Value
PINP	+1.08	-1.36	2.44	0.469
Osteocalcin	-0.62	-0.88	0.26	0.917
CTx	+0.052	+0.011	0.04	0.221
HOMA-B%	-16.84	-7.57	-9.27	0.375
HOMA-S%	+27.77	+15.03	12.74	0.642
HOMA-IR	-0.03	-0.21	0.18	0.263

Post# = postmenopausal women group

Table 8. Mean changes and between-group differences in WBV group

parameter	Mean changes in WBV group		Between-group difference	
	pre	post	Mean	p Value
PINP	-0.53	+1.08	-1.60	0.710
Osteocalcin	+0.16	-0.62	0.78	0.723
CTx	-0.01	+0.052	-0.06	0.093
HOMA-B%	+5	-16.84	21.84	0.209
HOMA-S%	-129.33	+27.77	-157.10	0.337
HOMA-IR	+0.06	-0.03	0.09	0.722

Pre = premenopausal women

Post = postmenopausal women

Table 9. Mean changes and between-group differences in CON group

parameter	Mean changes in CON group		Between-group difference	
	pre	post	Mean	p Value
PINP	+0.45	-1.36	1.81	0.503
Osteocalcin	-0.77	-0.88	0.11	0.945
CTx	+0.03	+0.011	0.02	0.595
HOMA-B%	+4.97	-7.57	12.55	0.176
HOMA-S%	-4.39	+15.03	-19.42	0.321
HOMA-IR	+0.07	-0.21	0.29	0.041

Pre = premenopausal women

Post = postmenopausal women

SERUM URIC ACID LEVELS IN RELATION TO BONE-RELATED PHENOTYPES IN MEN AND WOMEN

Abstract

Introduction Serum uric acid levels has recently been found to be associated with bone mineral density (BMD) in elderly males. It was the purpose of the present study to investigate the relationship between bone-related phenotypes and serum uric acid levels in young and middle-aged males and females.

Methods Subjects consisted of 1320 males and 485 females aged 25-54 years. Bone densitometry and quantitative ultrasonometry (QUS) were performed in each subject. Serum uric acid and biochemical markers of bone turnover were measured in fasting serum samples.

Results When adjusted for covariates including age and body weight in multiple linear regression models, it was found that there was a positive association between uric acid levels and bone mineral density (BMD) in males at the lumbar spine ($P < 0.05$) and total femur ($P < 0.05$). The association between uric acid levels and BMD was found in females after controlling for age and body weight only at the femoral neck but in the opposite direction ($P < 0.05$). Uric acid levels were related to stiffness index (SI) as assessed by QUS in males independently of age and body weight ($P < 0.05$). No association between uric acid and SI in females was found.

Conclusions We demonstrated in the present study a positive association in males of serum uric to BMD and SI from QUS suggesting a beneficial influence of uric acid on both the quantity and quality of bone in males.

Introduction

Uric acid is traditionally believed to be a metabolic waste of purine metabolism with no significant biological function in humans. However, it has been suggested that uric acid possesses antioxidant property [1, 2]. Oxidative stress plays important role in a number of illnesses including hypertension [3], diabetes [4] and chronic kidney disease [5]. A number of antioxidants, including vitamin C and E, have been shown to potentially affect such disorders favorably. Likewise, there are evidences that uric acid also favorably affects stroke [6, 7]

Oxidative stress may increase the propensity for age-related bone loss [8]. In this regard, a recent study in elderly males has demonstrated a positive association between serum uric acid levels and bone mineral density (BMD) independent of other associated factors including age and body mass index [9]. The finding is intriging and suggests a probable role of uric acid in bone metabolism. Nevertheless, it is unclear at a present if there is an association of uric acid with bone related phenotypes in younger age groups and if there is any gender difference. Toward this end, we investigated in the present study the association between bone-related phenotypes and serum uric acid levels in young and middle-aged males and females.

Materials and methods

The study was a part of the heath survey of the employees of the Electricity Generating Authority of Thailand (EGAT). Institutional Review Board approval was obtained prior to the commencement of the study and all subjects gave informed consent. Survey data was collected by using self-administered questionnaire, physical examination, electrocardiography, chest radiography, and blood analysis. Fasting blood samples of were obtained and sent for uric acid analysis. Blood samples for P1NP and CTX were kept frozen until the time of analysis.

BMD measurements

After changing to light clothing without dense object, each volunteer underwent DXA (Hologic QDR 4500W) to obtain BMD values of the lumbar spine (L1-L4) and left proximal femur (femoral neck and total hip). The DXA procedure complied with the ISCD Position Statement [2]. For lumbar spine DXA, each volunteer lay flat on the midline of the imaging table with legs elevated by a supporting pillow so that the spine was straight. The scan included T12 and L5 vertebrae and both iliac crests. For the proximal femur DXA, the volunteer lay supine with the left foot fixed to a positioning device to keep the hip internally rotated and adducted so that the femoral shaft was straight with minimal visualization of the lesser trochanter while the ischium and the greater trochanter were included on the scan. The scans were then analyzed according to the manufacturer's recommendation. A daily quality control procedure was performed according to the manufacturer's recommendation every morning of the survey dates prior to taking measurement to ensure a machine precision of less than 1.5%. The BMD coefficients of variation were 0.82%, 2.52%, and 1.51% for the lumbar spine, femoral neck, and total hip, respectively.

Quantitative ultrasonometry (QUS)

Each volunteer underwent QUS measurement at the left calcaneus with a Lunar Achilles ultrasound machine. The stiffness index (SI), QUS T-score, and QUS Z-score were obtained. The SI describes bone quality by combining and standardizing BUA and SOS into a single clinical quantity using the formula: SI = (0.67BUA + 0.28SOS)-420. The formula is derived such it has half of the contribution from BUA and the other half from SOS over the adult age range and that young adult value is 100 [3]. Each morning of the survey, the quality assurance procedure was carried according to the manufacturer recommendation to ensure the machine precision of less than 1.5%. The stiffness index coefficient of variation was 1.33%.

Serum uric acid measurement

Serum uric acid levels were determined using the uricase method (Siemens Healthcare Diagnostic Inc, Newark, DE, USA). The assay range is 0-20 mg/dl with reference ranges of 2.6-6.0 mg/dl and 3.5-7.2 mg/dl for females and males, respectively.

Bone marker measurement

Serum C-terminal telopeptide of type I collagen (CTx) and serum total procollagen type I amino-terminal propeptide (PINP) levels were determined by electrochemiluminescence immunoassay on a Cobas e 411 (Roche Diagnostic GmbH, Mannheim, Germany). The assays have intra-assay precision of 5.4% and 3.8%, respectively.

Results

Table 1 demonstrates the clinical characteristics of the study population. Subjects were mostly males (73.1%) largely because of the demographic structure of the EGAT. When comparing males and females, it was found that males were slightly older, had significantly higher BMD at the femoral neck and total femur but not at the lumbar spine. No difference was found in terms of SI as measured by QUS. Serum uric acid levels were significantly higher in males.

There were correlations between uric acid levels with BMD at various skeletal sites in males but not in females as shown in Table 2. When adjusted for covariates including age and body weight in multiple linear regression models, it was found that the positive association between uric acid levels and BMD was still statistically significant in males at the lumbar spine and total femur. The association between uric acid levels and BMD was found in females after controlling for age and body weight only at the femoral neck but in an opposite direction (Table 3). To explore the probable underling mechanism of the observed relationship between BMD and uric acid, we further examined the association between uric acid levels and biochemical markers of bone turnover. As shown in Table 4, there was a significant relationship between serum CTX, but not P1NP, with uric acid in males. In females, the relationship between uric acid and serum CTX almost reach statistical significance. Similar to the finding in males, no association between uric acid and P1NP was found.

With regard to bone quality as assessed by QUS, the results were similar to the findings with BMD. Uric acid levels were related to SI in males independently of age and body weight (Table 5). No association between uric acid and SI in females was found.

Discussion

In the present study, we demonstrated in males a relationship between serum uric acid levels and BMD at the lumbar spine, a skeletal site rich in trabecular bone. Although our study population was younger in age, our finding is in keeping with a previous study in elderly males (9). This suggests that the influence of uric acid on bone, if causal in nature, is likely to begin at a younger age and could probably affect the attainment of peak bone mass. However, whether uric acid is causally related to bone mass is not entirely clear at present since studies so far were cross-sectional in nature and causality cannot be readily established. Hyperuricemia is a common disorder and uric acid lowering agents are frequently used. To our knowledge, no study has been conducted looking at the effect of uric acid lowering on bone mass. On the other hand, hypouricemia due to mutations in the renal uric acid transporters is not uncommon. These individuals have very low uric acid levels usually < 2 mg/dL, mostly asymptomatic but no bone-related phenotype has been described. However, monosodium urate monohydrate crystal can directly inhibits osteoblasts [10] which may partly explain the bone erosion observed in chronic gout. Taken together, although it is biologically probable that uric acid may directly affect bone, more studies to delineate the causal role of uric acid in bone metabolism need to be performed.

We demonstrated as well in the present study that not only was uric acid levels related to BMD, it was also associated with SI derived from bone QUS. In men, SI was found to be positively related to serum uric acid. Discrepancy between results from QUS and bone densitometry is not infrequent and not necessary reflects technical error [11]. QUS of bone may capture determinants of bone quality and combining the results from QUS and densitometry has been shown to be able to better predict fractures than either method alone [12]. It is believed that bone quality is a composite measure of multiple factors including bone microarchitecture, bone quality, material property as well as bone turnover [13]. In this regard, our findings appear to suggest that uric acid levels are related not only to bone quantity, as reflected in BMD, but likely bone quality as well since a positive association of serum uric acid with stiffness index as well as a negative association with bone turnover, as assessed by serum CTX, were found in the present study.

In the present study, we demonstrated that although uric acid was related to BMD in males, its relationship to bone mass in females was not as apparent, being seen only at the femoral neck but in a reverse direction. What the underlying basis is for the observed difference in the association between bone mass and uric acid in females is unclear. Although bone mass decreases with age in both genders and predispose both men and women to osteoporosis, a number of differences exist and underlie the sexual dimorphism observed. For example, bone structures are different in elderly men as compared to women and osteoporosis in men and women is likely to be pathophysiolocally distinct [14-16]. Furthermore, although established treatment for osteoporosis in women works as well in men [17-19], some modalities show gender-related difference in responsiveness. Vitamin K2, for example, was demonstrated to be mildly effective in increasing bone mass only in women but not in men in a recent meta-analysis [20]. Uric acid levels in general are lower in pre-menopausal women as compared to men but the degree of difference decreases after menopause. Since the female study population in our study was predominantly premenopausal, the degree of variability of uric acid levels may not be high enough for the association of uric acid and bone mass in women, if exists, to be discerned.

Conclusion

We demonstrated in the present study a positive association in males of serum uric to BMD and stiffness index from QUS suggesting a beneficial influence of uric acid on both the quantity and quality of bone in males.

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Table 1 Clinical characteristics of the study population

	Males (n = 1320)	Females (n = 485)	P value
Age (year)	41.7 ± 0.2 (25-54)	40.2 ± 0.3 (25-54)	< 0.001
Body weight (kg)	70.4 ± 0.3 (40.9-115.0)	55.1 ± 0.4 (37.3-95.4)	< 0.001
Height (cm)	169.0 ± 0.2 (144.3-187.5)	157.7 ± 0.2 (145.0-180.0)	< 0.001
Lumbar spine BMD (g/cm^2)	0.974 ± 0.003 (0.639-1.684)	0.970 ± 0.005 (0.616-1.298)	NS
Femoral neck BMD (g/cm^2)	0.817 ± 0.003 (0.472-1.30)	0.75 ± 0.005 (0.427 1.083)	< 0.001
Total femur BMD (g/cm^2)	0.946 ± 0.003 (0.590-1.442)	0.856 ± 0.005(0.477-1.318)	< 0.001
Stiffness index	101.8 ± 0.5 (38-169)	102.7 ± 1.6 (23-172)	NS
Uric acid (mg/dL)	6.12 ± 0.03 (1.2-11.3)	4.07 ± 0.04 (1.7-7.6)	< 0.001

Table 2A Correlation coefficients (P values) of BMD, uric acid levels and covariates in males.

	Uric acid	Age	Body weight	Lumbar BMD	Femoral neck BMD	Total femur BMD
Uric acid	1					
Age	0.03 (NS)	1				
Body weight	0.24 (<0.001)	-0.07 (< 0.05)	1			
Lumbar BMD	0.12 (< 0.001)	-0.08 (< 0.01)	0.24 (<0.001)	1		
Femoral neck BMD	0.1178 (<0.001)	-0.21 (< 0.001)	0.38 (<0.001)	0.62 (<0.001)	1	
Total femur BMD	0.14 (< 0.001)	-0.1305 (<0.001)	0.3867 (<0.001)	0.6398 (<0.001)	0.8861 (<0.001)	1

Table 2B Correlation coefficients (P values) of BMD, uric acid levels and covariates in females.

	Uric acid	Age	Body weight	Lumbar BMD	Femoral neck BMD	Total femur BMD
Uric acid	1					
Age	0.11 (< 0.05)	1				
Body weight	0.40 (<0.001)	0.14 (<0.01)	1			
Lumbar BMD	0.07 (NS)	-0.02 (NS)	0.30 (<0.001)	1		
Femoral neck BMD	0.09 (<0.05)	0.01 (NS)	0.43 (<0.001)	0.63 (<0.001)	1	
Total femur BMD	0.12 (<0.05)	0.07 (NS)	0.45 (<0.001)	0.64 (<0.001)	0.87 (<0.001)	1

Table 3A Multivariate analysis of the relationship between uric acid levels and BMD in males. Uric acid levels were associated with BMD at the lumbar spine and the total femur independently of age and body weight.

	Lumbar BMD		Femoral neck BMD		Total femur BMD	
	Beta	P value	Beta	P value	Beta	P value
Age (yr)	-0.07	< 0.001	-0.19	< 0.001	-0.11	< 0.001
Body weight (kg)	0.22	< 0.001	0.36	< 0.001	0.37	< 0.001
Uric acid (mg/dL)	0.07	< 0.05	0.04	NS	0.05	< 0.05

Table 3B Multivariate analysis of the relationship between uric acid levels and BMD in females. Uric acid levels were associated with lower rather than higher BMD independently of age and body weight only at the femoral neck.

	Lumbar BMD		Femoral neck BMD		Total femur BMD	
	Beta	P value	Beta	P value	Beta	P value
Age (yr)	-0.06	NS	-0.05	< 0.001	0.01	NS
Body weight (kg)	0.33	< 0.001	0.48	< 0.001	0.48	< 0.001
Uric acid (mg/dL)	-0.05	NS	-0.09	< 0.05	-0.08	NS

Table 4 The relationship of bone markers to uric acid, age and body weight in males and females. Uric acid was independently associated with CTX, a bone resorption marker, in males.

	Males				Females			
	CTX		P1NP		CTX		P1NP	
	Beta	P value	Beta	P value	Beta	P value	Beta	P value
Uric acid	-0.07	< 0.01	-0.01	NS	0.09	0.06	0.04	NS
Age	-0.23	< 0.001	-0.17	< 0.001	0.07	NS	0.03	NS
Body weight	-0.09	< 0.001	-0.05	NS	-0.09	NS	-0.05	NS

Table 5 The relationship between uric acid and SI from QUS. Uric acid was independently related to SI in males but not in females.

	SI			
	Males		Females	
	Beta	P value	Beta	P value
Uric acid (mg/dL)	0.06	< 0.05	0.05	NS
Age (year)	-0.25	< 0.001	-0.14	< 0.001
Body weight (kg)	-0.10	< 0.001	0.05	NS

CAUSAL INFERENCE OF THE EFFECT OF ADIPOSITY ON BONE MINERAL DENSITY IN ADULTS

Abstract

Objective The causal effect of adipose tissue on bone mass and the direction of its net influence have not been directly assessed in adult humans. Using the Mendelian randomization analysis, we assessed the causality of adiposity in measurements of bone mass in adult males and females.

Design and Methods Subjects consisted of 2,154 adults aged 25–54 years from a cross-sectional cohort of the employees of the Electricity Generating Authority of Thailand. Body composition was determined after at least 3 h of fasting using multi-frequency bioelectrical impedance analysis. Bone mineral density was assessed by dual energy X-ray absorptiometry. A polymorphism in the fat mass and obesity-associated gene (*FTO* rs9939609) was used as an instrument in the Mendelian randomization analysis.

Results The genotype distribution of the *FTO* rs9939609 polymorphism was 61.1% TT, 33.9% AT and 5.0% AA. The average body mass index (BMI) , body fat mass and percent body fat were 23.9 kg/m^2 (SD = 3.6), 17.9 kg (SD = 6.6) and 26.8 % (SD = 7.2) , respectively. The *FTO* rs9939609 polymorphism was significantly correlated with BMI (coefficient = 0.673 kg/m^2, $P < 0.001$), body fat mass (coefficient = 0.948 kg, $P < 0.001$) and percent body fat (coefficient = 0.759 %, $P < 0.01$) . An instrumental variable (IV) regression model, using BMI as the intermediate phenotype, suggested that *FTO* was a strong IV. Also, the *FTO*-BMI polymorphism was significantly associated with total hip and femoral neck bone mineral density (BMDs) but was not correlated with total spine BMD, with estimated correlation coefficients of 0.0189 (95% CI: 0.0046, 0.0332), 0.0149 (95% CI: 0.0030, 0.0268) and 0.0025 (95% CI: -0.0131, 0.0136) g/cm^2, respectively. The variances of BMDs explained by the *FTO*-BMI were 19.0%, 21.3%, and 1.1%, respectively. Similar trends were also observed for the *FTO*-body fat mass and *FTO*-percent body fat correlations.

Conclusions Mendelian randomization analysis suggests that adiposity might be causally related to bone mineral density at the femur but not at the spine.

Introduction

Osteoporosis is a health problem worldwide. A number of risk factors are related to susceptibility to osteoporosis, including estrogen deficiency, sedentary lifestyle and reduced adiposity. Adipose tissue is now considered an endocrine organ. In addition to its conventional role in energy storage, adipose tissue secretes a number of bioactive proteins which influence a variety of biological processes, including energy homeostasis and inflammation. As far as bone is concerned, leptin, an adipokine secreted from adipose tissue, has been shown to diminish bone formation through a central nervous system delay in animal models. In humans, a number of adipokines, including leptin,[1,2] adiponectin[3–5] and omentin-1,[6] have been shown in observational studies to be variably related to bone mineral density (BMD).

Although results from animal models and observational studies in humans suggest that adiposity influences bone mass, the effects of adipokines on bone mass are different. Results from observational studies can be confounded by factors which influence both adiposity and bone mass, such as body size and weight. Moreover, the causality of adipose tissue on bone mass and the direction of net influence have not been directly assessed in adult humans.

The fat mass and obesity-associated (*FTO*) locus on chromosome 16 (16q12.2) has been identified from genome–wide association studies as a major candidate gene for obesity in children and adults.[7–9] The *FTO* rs9939609 polymorphism is of particular interest, since it was found to be associated with obesity in different ethnic groups.[8,10–13] The finding of common genetic variants of *FTO,* which have been consistently associated with adiposity, provided an opportunity to conduct a Mendelian randomization study of obesity and bone outcomes. Therefore, utilizing the Mendelian randomization analysis, we assessed the causality of adiposity in the attainment of BMD in adults.

Materials and methods

The study design was a cross-sectional cohort of the employees of the Electricity Generating Authority of Thailand (EGAT). Prior to commencement, the study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. All subjects gave written informed consent. Survey data were collected using a self-administered questionnaire, physical examination, electrocardiography, chest radiography and blood analysis.

BMD measurements

After changing into lightweight clothing without dense objects, each participant underwent dual-emission X-ray absorptiometry (DXA) (Hologic QDR 4500W; Bedford, MA) to obtain BMD values of the lumbar spine (L1–L4) and left proximal femur (femoral neck and total hip). The DXA procedure complied with the ISCD Position Statement.[14] For lumbar spine DXA, each participant lay flat on the midline of the imaging table, with legs elevated by a supporting pillow so that the spine was straight. The DXA scan included T12 and L5 vertebrae and both iliac crests. For the proximal femur DXA, the participant lay supine with the left foot fixed to a positioning device to keep the hip internally rotated and adducted so that the femoral shaft was straight, with minimal visualization of the lesser trochanter while the ischium and the greater trochanter were included on the scan. The scans were then analyzed according to the manufacturer's recommendations. Each morning prior to the scheduled DXA scans, a quality–control procedure was performed as recommended by the manufacturer to ensure machine precision of more than 98.5%. The BMD coefficients of variation were 0.82%, 2.52% and 1.51% for the lumbar spine, femoral neck and total hip, respectively.

Adiposity measurements

Anthropometric variables including weight, height and waist circumferences were measured using standard techniques in all studies. Body mass index (BMI) was derived by weight (kg)/height $(m)^2$. Body composition was determined after at least 3 h of fasting using multi-frequency bioelectrical impedance analysis with eight-point tactile electrodes (InBody 720; Biospace, Seoul, Korea).

FTO genetic analysis

DNA was extracted by phenol-chloroform method.[15] Individual genotyping of all subjects was performed using real-time PCR (TaqMan® MGB probes): 10 ng of DNA was added into the PCR reaction, consisting of TaqMan Universal Master Mix (1x) and TaqMan MGB probes for *FTO* rs9939609 SNP (1x) in a total volume of 10 μL. The real-time PCR reaction protocol was 10 min at 95 °C, 40 cycles of 15 s at 92 °C, and 1 min at 60 °C using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were expressed as mean (or median, where appropriate) and frequency for continuous and categorical data, respectively. Hardy–Weinberg equilibrium was assessed using an exact test. [16] Relationships between the *FTO* polymorphism and variables were assessed for both males and females using linear regression and a chi-square test (or exact test, where appropriate) for continuous and categorical data, respectively.

Mendelian randomization analysis[17] was applied to assess causal relationships between *FTO*, adiposity (i.e. BMI and body fat mass) and BMD. Instrumental variable (IV) regression with two-stage least squares method was applied to explore these causal relationships, using the *FTO* polymorphism with additive effect as the IV and BMI/body fat mass as the endogenous variables.[18,19] These models were also adjusted for confounding variables (i.e. alcohol, age and gender), since univariate analysis suggested that they were associated with intermediate phenotype and/or BMD. In the first-stage regression, the F–statistic (hereafter called F-First) was used to assess whether the *FTO* polymorphism was sufficiently strong to be an IV. A value of F-First greater than 10 indicated that the *FTO* was a strong IV, and thus the estimated causal relationships should be valid. In addition, linear regression with ordinary least squares (OLS) method was also applied to directly assess the association between *FTO*, adiposity and BMD. The Durbin–Wu–Hausman statistic was applied to compare the results between the IV and OLS regression approaches. All analyses were performed using STATA version 12.0. A *P*-value of less than 0.05 was considered statistically significant.

Results

Table 1 describes the clinical characteristics, adiposity and BMD of the study subjects (n = 2,154). The mean age of the subjects was 40.2 years (SD = 6.9). The average body mass index (BMI) , body fat mass and percent body fat were 23.9 kg/m^2 (SD = 3.6), and 17.9 kg (SD = 6.6) and 26.8 % (SD = 7.2)), respectively. The correlation matrix between parameters of adiposity and BMD is shown in Table 2. Measures of adiposity, including BMI, body fat mass and percent body fat, were all significantly related to BMD at all skeletal sites.

The association of measures of adiposity and BMD can be confounded by variables such as age, gender, alcohol consumption and cigarette smoking. For being an IV, the *FTO* polymorphism should not be associated with these potential confounders. Therefore, associations between the subject characteristics and the *FTO* genotypes were assessed. As described in Table 3, none of these potential confounders were associated with the *FTO* polymorphism. The *FTO* genotype frequencies complied with Hardy–Weinberg equilibrium rules (*P* = 0.510).

Relationships between adiposity (i.e. BMI, body fat mass and percent body fat) and the *FTO* polymorphism were explored (Table 4). The mean BMI was significantly higher in minor homozygous and heterozygous genotypes compared to the major homozygous genotype (*P* < 0.001). Applying linear regression analysis by fitting *FTO* as an additive effect suggests that the *FTO* polymorphism was significantly correlated with BMI (coefficient = 0.637 kg/m^2, p < 0.001); indicating that carrying an A allele would increase BMI of 0.637

kg/m^2. This trend was also observed with body fat mass and percent body fat, i.e. mean body fat mass or percent body fat in minor homozygous and heterozygous genotypes was significantly higher than in the major homozygous genotype ($P < 0.001$).

Table 5 describes the results from OLS and IV regression analyses by measures of adiposity and skeletal sites. In the IV regression model using BMI as the intermediate phenotype, *FTO* was a strong IV for total hip, femoral neck, and total spine BMD, with F-statistics of 25.7, 21.9, and 21.8, respectively. The *FTO*-BMI was also significantly associated with total hip and femoral neck BMDs but not with total spine BMD, with estimated correlation coefficients of 0.0189 (95% CI: 0.0046, 0.0332), 0.0149 (95% CI: 0.0030, 0.0268) and 0.0025 (95% CI: −0.0131, 0.0136) g/cm^2, respectively. The variances of BMDs explained by the *FTO*-BMI were 19.0%, 21.3%, and 1.1%, respectively. Similar trends were also observed for *FTO*-body fat mass, i.e. the *FTO*-body fat mass was significantly associated with total hip and femoral neck BMDs but not with total spine BMD, with correlation coefficients of 0.0122 (95% CI: 0.0023, 0.0221), 0.0086 (95% CI: 0.0005, 0.0167) and 0.0012 kg (95% CI: −0.0074, 0.0098), respectively. Similar results for percent body fat was obtained. The F-First statistics showed that *FTO* was a strong IV for all BMDs.

The results of OLS regression suggest that both BMI and body fat mass correlate significantly with all BMD sites after adjusting for *FTO* and other covariables. Although the magnitude of adiposity effects from IV estimates were higher than the OLS estimates for all BMDs except total spine BMD, Durbin–Wu–Hausman tests did not reach statistical significance (Table 5).

Discussion

In the present study, using a Mendelian randomization analysis, we have demonstrated that adiposity is likely to play a causal role in the determination of bone mass. The magnitude of the effect, however, is relatively small. Although causality is suggested by these analyses, the underlying mechanism(s) still require further investigation. In addition to being the source of adipokines, adipose tissue is able to convert androgens to estrogens through the *CYP19* aromatase enzyme,[20] which may account for the observation that obese postmenopausal women have lower bone turnover, higher bone mass and fewer fractures.[21] Given the myriad of possible mechanisms affecting the influence of adiposity on bone mass, it remains unclear which one plays a predominant role; and it is likely that additional underlying possibilities still remain to be discovered. On the other hand, it is well accepted that body size is also associated with higher areal BMD. It is therefore unclear whether adiposity directly leads to high bone mass or is simply related to BMD because of the confounding effect of body size and weight. Using the instrumental variable analysis, our results suggest that there is a net positive effect of adiposity on BMD which is not likely to be confounded by body size and weight, a finding that is in agreement with a previous study using a different approach.[22]

It is noteworthy that in the present study the causal role of adiposity in the determination of bone mass is only apparent in the femur. Of the skeletal sites used for BMD measurement, the vertebra is comprised of more trabecular bone compared to the femur, which suggests that adiposity may predominantly influence cortical bone. The underlying reason for this phenomenon is not entirely clear. Metabolic differences exist between skeletal sites rich in trabecular bone and cortical bone. Trabecular BMD changes more (in either direction) in response to bone stimuli, including exercise[23] and antiresorptive[24] or bone–forming agents such as intermittent teriparatide.[24] However, unlike other bone active stimuli, parathyroid hormone (PTH), particularly when continuously elevated, affects cortical bone to a greater extent than trabecular bone.[25] Since a number of studies[27–29] have found that adiposity and serum PTH are correlated, it is conceivable that the influence of adiposity predominantly on cortical bone may be partly mediated through the effect of PTH. Such a hypothesis cannot be readily tested in the present study since serum PTH data are lacking. Further investigations involving the potential causal relationships among adiposity, PTH and bone mass are warranted.

The Mendelian randomization analysis has been increasingly utilized in health research to assess causality based on genetic observational studies. A proper Mendelian randomization study must comply with a number of assumptions.[24,30] In the present study the *FTO* genotype was strongly associated with adiposity, which satisfies one of the assumptions for a proper instrumental variable. The associations between the *FTO* gene and osteoporosis phenotypes have recently been described.[31] The effect is likely to be mediated through the influence of adiposity. However, since the function of the *FTO* gene is not entirely known, the possibility remains that there might be other *FTO* gene–determined confounders of adiposity and bone mass; this would render the use of the *FTO* gene as an instrumental variable in this case less valid.

In conclusion, the Mendelian randomization analysis suggested that adiposity might be causally related to bone mineral density in the femur but not in the spine.

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Table 1. Clinical characteristics of cohort

Characteristic	Mean (SD)
Age (years)	40.0 (7.4)
Body weight (kg)	66.1 (12.5)
Height (cm)	166.2 (7.8)
BMI (kg/m^2)	23.9 (3.6)
Body fat mass (kg)	17.9 (6.6)
Percent body fat (%)	26.8 (7.2)
Cigarettes smoking/day, median (range)	0 (0–50)
Alcohol consumption, number (%)	982/2,325 (42.2)
Lumbar spine BMD (g/cm^2)	0.975 (0.118)
Femoral neck BMD (g/cm^2)	0.801 (0.121)
Total femur BMD (g/cm^2)	0.925 (0.129)

Table 2. Correlation matrix between measures of adiposity and BMD

	BMI	Body fat mass(kg)	Body fat mass(%)	Lumbar BMD	Femoral neck BMD	Total femur BMD
BMI	1.0000					
Body fat mass(kg)	0.85	1.0000				
	(< 0.001)					
Body fat mass(%)	0.51	0.82				
	(<0.001)	(<0.001)				
Lumbar BMD	0.19	0.14	0.03	1.0000		
	(< 0.001)	(< 0.001)	(0.13)			
Femoral neck BMD	0.39	0.23	-0.02	0.62	1.0000	
	(< 0.001)	(< 0.001)	(0.39)	(< 0.001)		
Total femur BMD	0.39	0.22	-0.03	0.57	0.81	1.0000
	(< 0.001)	(< 0.001)	(0.162)	(< 0.001)	(< 0.001)	

Table 3. Description and comparison of characteristics of subjects, according to *FTO* genotypes

Characteristic	*FTO* genotype			*P*-value
	TT (n = 1315)	AT (n = 731)	AA (n = 108)	
Age, mean (SD)	40.1 (6.9)	40.0 (6.9)	40.3 (6.8)	0.500
Gender				
Male	949 (72.2%)	532 (72.8%)	79 (73.2%)	0.955
Female	366 (27.8%)	199 (27.2%)	29 (26.8%)	
Alcohol consumption				
Yes	565 (43.0%)	305 (41.7%)	46 (42.6%)	0.941
No	749 (57.0%)	426 (58.3%)	62 (57.4%)	
Cigarette smoking, median (range)	0 (0,50)	0 (0,30)	0 (0,15)	0.526

Table 4. Relationship between adiposity and *FTO* genotypes

Adiposity	*FTO* rs9939609			B (linear regression**)	P value
	TT	AT	AA		
BMI	23.7(3.7)*	24.3(3.6)	25.4(4.2)	0.637	< 0.001
Body fat mass (kg)	17.6(6.6)	18.3(6.9)	20.2(7.9)	0.948	< 0.001
Percent body fat (%)	26.6(7.2)	27.1(7.1)	28.6(8.2)	0.759	0.006

*mean (SD), **additive effect of *FTO* locus

Table 5. Linear and IV regression analysis of the relationships between BMD and BMI (A), body fat mass (B) and percent body fat (C)

A

BMI (kg/m^2)	Linear regression			IV regression				
	β	95% CI	*P*-value	β	95% CI	*P*-value	F-First*	WH *P*-value**
Total hip BMD (g/cm^2)	0.0138	0.0124, 0.0153	< 0.001	0.0189	0.0046, 0.0332	0.010	25.734	0.486
Femoral neck BMD (g/cm^2)	0.0119	0.0107, 0.0132	< 0.001	0.0149	0.0030, 0.0268	0.014	21.864	0.629
Total spine BMD (g/cm^2)	0.0069	0.0056, 0.0083	< 0.001	0.0025	−0.0131, 0.0136	NS	21.826	0.313

B

Body fat mass (kg)	Linear regression			IV regression				
	β	95% CI	*P*-value	β	95% CI	*P*-value	F-First*	WH *P*-value**
Total hip BMD (g/cm^2)	0.0052	0.0043, 0.0061	<0.001	0.0122	0.0023, 0.0221	0.016	15.378	0.142
Femoral neck BMD (g/cm^2)	0.0045	0.0041, 0.0055	<0.001	0.0086	0.0005, 0.0167	0.037	15.377	0.348
Total spine BMD (g/cm^2)	0.0026	0.0019, 0.0034	<0.001	0.0012	-0.0074, 0.0098	0.790	15.303	0.725

C

* β =Regression coefficient, **First-stage regression F-statistic, *** Durbin–Wu–Hausman test comparing ordinary least square vs. instrumental variable regression

Percent body fat (%)	Linear regression			IV regression				
	β*	95% CI	*P*-value	β	95% CI	*P*-value	F-First**	WH *P*-value***
Total hip BMD (g/cm^2)	0.0035	0.0026,0.0044	<0.001	0.0134	0.0019, 0.0250	0.023	17.188	0.067
Femoral neck BMD (g/cm^2)	0.0032	0.0024,0.0040	<0.001	0.0094	0.0002, 0.0187	0.046	17.188	0.168
Total spine BMD (g/cm^2)	0.0013	0.0004, 0.0021	0.002	0.0013	-0.0079, 0.0104	0.784	17.090	0.997

Outputs **จากโครงการ**

ชื่อ-นามสกุล	**เมื่อเข้าร่วมโครงการ**			**ปัจจุบัน**		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
1. พญ. สมโชดก ชาครีย์รัตน์		คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล	แพทย์ผู้ช่วยอาจารย์		โรงพยาบาลภูมิพล	สำเร็จการศึกษาแล้ว
2. พญ. เพ็ชรงาม เต็งพฤทธิ์ธนากร		คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล	แพทย์ผู้ช่วยอาจารย์	อาจารย์	มหาวิทยาลัยบูรพา	สำเร็จการศึกษาแล้ว
3. พญ. จิตรา ตันติวัฒนเสถียร		คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล	แพทย์ผู้ช่วยอาจารย์		โรงพยาบาลสมิติเวช	สำเร็จการศึกษาแล้ว

ชื่อ-นามสกุล	เมื่อเข้าร่วมโครงการ			ปัจจุบัน		
	ตำแหน่งวิชาการ	สังกัด	ตำแหน่งในโครงการ	ตำแหน่งวิชาการ	สังกัด	สถานภาพปัจจุบัน
4. พญ. ปราณี ทองไทย		คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล	แพทย์ผู้ช่วยอาจารย์			กำลังศึกษาอยู่
5. พญ. รัตนพรรณ สมิทธารักษ์		คณะแพทยศาสตร์ โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล	แพทย์ผู้ช่วยอาจารย์			กำลังศึกษาอยู่

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Appendix

Endocr (2011) 39:278–282
DOI 10.1007/s12020-010-9426-1

ORIGINAL ARTICLE

Elevated vitamin D status in postmenopausal women on thiazolidinediones for type 2 diabetes

Somchodok Chakreeyarat · Sunee Saetung · La-or Chailurkit · Sasivimol Rattanasiri · Suparat Ditbanjong · Niyata Chitrapazt · Supaneewan Jaovisidha · Boonsong Ongphiphadhanakul

Received: 15 July 2010 / Accepted: 24 September 2010 / Published online: 11 November 2010

Abstract Thiazolidenediones (TZD) have been reported to lead to non-vertebral bone loss in postmenopausal women with diabetes, but the true incidence of vertebral fractures has been under-detected because two-thirds of vertebral fractures are silent. TZD is also related to increased adiposity, with a consequently greater risk of vitamin D deficiency—both of which seem to aggravate the untoward effect of TZD on bone. The aim of this study is to determine whether TZD use is associated with prevalence of vertebral fractures and low vitamin D status in postmenopausal women with type 2 diabetes. A group of 102 postmenopausal women with type 2 diabetes, 52 TZD users for at least 12 months, and 50 non-TZD users were enrolled in the study. Any data regarding diabetes, age at menopause, co-morbidities, and drug use were recorded. Blood sampling and thoraco-lumbar radiography were performed. Bone mineral density (BMD) of L2–L4 and the femur were measured by dual-energy X-ray absorptiometry (DXA). The occurrence of vertebral fractures at one level or more in subjects on TZD was higher than those not on TZD, but did not reach statistical significance (19.2 vs. 14.0%, $P = 0.5$). Total hip BMD in subjects on TZD was significantly lower than those not on TZD (0.96 $\pm$ 0.15 vs. 1.02 $\pm$ 0.11; $P < 0.05$). Levels of 25(OH)D in TZD users were significantly higher (35.3 $\pm$ 1.5 vs. 25.9 $\pm$ 1.2 ng/dl; $P < 0.001$). The prevalence of vitamin D deficiency was 75.5% in subjects not on TZD compared to 34.6% in those on TZD (OR 6.4, 95% CI 2.6–15.6). Higher circulating 25(OH)D was observed in TZD users. TZD use was associated with lower total hip BMD but not with vertebral fracture.

Keywords Thiazolidenediones · Vertebral fractures · Bone mineral density · Osteoporosis · Postmenopausal · Morphometry · Vitamin D

S. Chakreeyarat · S. Saetung · L. Chailurkit · B. Ongphiphadhanakul (✉)
Division of Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand
e-mail: rabpk@mahidol.ac.th

S. Rattanasiri · S. Ditbanjong
Faculty of Medicine, Research Center, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand

N. Chitrapazt · S. Jaovisidha
Department of Radiology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand

Introduction

Thiazolidinediones (TZD) are widely used in the treatment of type 2 diabetes. TZD use is associated with accelerated bone loss (0.6–1.2% per year) at the trochanter, whole body, and lumbar spine in diabetic women [1]. Concerns have recently been raised about a small increase in the risk of fractures associated with TZD at peripheral skeletal sites [2]. It is of note that increases in fractures at common sites for fragility fractures, such as the spine and hip, were not reported. Vertebral fractures are frequently undetected since only 30–40% are symptomatic and come to clinical attention. Nevertheless, asymptomatic vertebral fractures are also associated with subsequent adverse health outcomes [3]. A recent study performed on Caucasian males focused specifically on the risk of vertebral fractures associated with TZD, and found a significant increase in vertebral fractures in subjects on TZD [4]. It remains unclear whether and how TZD might affect the risk of vertebral fractures in females of other ethnicity.

Calcium and vitamin D play important roles in the maintenance of bone health. At present, vitamin D deficiency is highly prevalent [5]. Obesity is a recognized risk factor of vitamin D deficiency, which may be related to the sequestration of vitamin D in adipose tissue [6]. The relationship between the use of TZD and vitamin D status is currently unknown. We hypothesized that TZD, by increasing adiposity, may adversely affect vitamin D status and lead to further aggravation of the untoward effect of TZD on bone. To that end, we investigated, in a case–control study, the difference in the prevalence of vertebral fractures and vitamin D status between postmenopausal women on TZD and those not on TZD.

Materials and methods

Subjects

We enrolled by consecutive medical record review 102 postmenopausal women, as defined by cessation of menstruation for at least 12 months, with type 2 diabetes at the outpatient clinic of Ramathibodi Hospital, Thailand, between January 2009 and December 2009. Of the 102 subjects, 52 had been on TZD for at least 12 months; the other 50 subjects, matched for age and body mass index (BMI), had never been on TZD. All were ambulatory and non-alcoholic. Subjects were excluded in cases of: a history of cancer, hyperthyroidism, hyperparathyroidism, surgical menopause, use of oral or parenteral glucocorticoid ($\geq$5 mg prednisolone or equivalent/day) for more than 1 month within 6 months before study entry, or an estimated glomerular filtration rate <60 ml/min. No subjects had been receiving medications known to interfere with bone metabolism—including estrogen, vitamin D, antiresorptive agents, or thyroxine—within the past 12 months. The study protocol was approved by the local Institutional Review Board. All patients gave signed informed consent before participating in the study.

BMD measurements

Body weight was measured with subjects wearing light clothes. Bone mineral density (BMD) at the lumbar spine 2–4 (L2–L4) and the femur were measured by dual-energy X-ray absorptiometry (DXA) (Lunar Corp., USA) by a single experienced technician. Quality control was achieved by daily calibration and phantom scans. The coefficient of variation for the phantom scans was 0.6%; these values were 1.2 and 1.6% at L2–L4 and the femoral neck, respectively.

Ascertainment of vertebral fractures

Ascertainment of vertebral fractures was performed according to a previously described method [7]. Lateral and anteroposterior thoraco-lumbar radiographs centered at the T12 level were performed on all subjects. Vertebral fractures were evaluated by a single radiologist who was blind to the subjects' clinical characteristics. Anterior and posterior heights of vertebral levels T10–L3 were measured, and the anterior/posterior height ratio was calculated. Vertebral fracture was defined as present if the height ratio was 0.8 or lower.

25(OH)D measurement

Serum 25(OH)D levels were measured by high performance liquid chromatography, with an intra-assay precision of 4.8% for 25(OH)D2 and 4.9% for 25(OH)D3.

Statistical analysis

The sample size was determined based on a power of 0.8 to detect a 2-fold increase in vertebral fracture risk from a baseline prevalence of 15%. Pearson's chi-square or Fisher's exact tests were used to compare characteristics between groups for categorical data. Student's t test was used for continuous variables to test for any statistical differences between groups. Linear relationships between BMI and vitamin D status were assessed by linear regression analysis. Independent risk factors for vitamin D insufficiency were determined by stepwise logistic regression. Statistical significance was set at $P < 0.05$. Data were presented as mean ± SE unless stated otherwise.

Results

Baseline characteristics of the subjects are shown in Table 1. There were no significant differences in age, menopausal age, and body mass index (BMI) between the two groups. With regard to biochemical and hormonal tests, there were no significant differences in baseline HbA1c level, serum TSH, calcium, LDL cholesterol, and eGFR between the two groups. The duration of TZD use was 42.3 ± 3.2 months. The mean doses were 23.8 ± 1.2 mg ($n = 41$) and 4.4 ± 0.4 mg ($n = 11$) for pioglitazone and rosiglitazone, respectively. Subjects using TZD had a longer duration of diabetes than those not using TZD. Pioglitazone was prescribed in 41 (78.8%) subjects, while rosiglitazone was used in the remaining 11 (21.2%) subjects. The mean duration of TZD use was 3.47 ± 1.89 years (median 3.22 years). Sulfonylurea was more

Table 1 Characteristics of study subjects stratified by TZD use

	TZD users ($n = 52$)	Non-TZD users ($n = 50$)	P value
Age (year)	59.3 ± 0.9	58.4 ± 0.9	NS
BMI (kg/m^2)	29.1 ± 0.7	29.2 ± 0.6	NS
Duration of diabetes (year) median (min, max)	11.3 (2.0, 29.0)	6.8 (1.3, 26.6)	<0.01
Age of menopause (year)	50.3 ± 0.5	50.2 ± 0.5	NS
A1C (%); mean(SE)	7.8 ± 0.1	7.5 ± 0.2	NS
GFR (ml/min/1.73 m^2); mean(SE)	89.2 ± 3.4	84.9 ± 2.7	NS
Serum calcium (mg/dl); mean(SE)	9.4 ± 0.1	9.4 ± 0.0	NS
Serum TSH (μIU/ml); median(min, max)	1.5 (0.4, 4.8)	1.5 (0.4, 5.7)	NS
Serum LDL (mg/dl)	104.0 ± 4.6	111.8 ± 4.0	NS
Serum total 25(OH)D level (ng/ml)	35.3 ± 1.5	25.9 ± 1.2	0.000
Subjects using other antidiabetic medications			
Insulin	12 (23.1%)	12 (24.0%)	NS
Metformin	49 (94.2%)	45 (90.0%)	NS
Sulfonylurea	43 (82.7%)	30 (60.0%)	<0.01
Alpha-glucosidase inhibitor	5 (9.6%)	5 (10.0%)	NS

NS not significant

widely used than other antidiabetic agents by patients treated with TZD (82.69 vs. 60.0%; $P = 0.01$).

Table 2 shows BMD at the lumbar spine (L2–L4), femoral neck, and total hip, and the prevalence of vertebral fractures. There was no difference in BMD of L2–L4 and the femoral neck between the two groups. However, total hip BMD in subjects on TZD was significantly lower than in those not on TZD ($P < 0.05$). The prevalence of vertebral fractures at one level or more in subjects on TZD tended to be higher, but did not reach statistical significance (19.2 vs. 14.0%, $P = 0.5$).

The 25(OH)D levels in TZD users were significantly higher (35.3 ± 1.5 vs. 25.9 ± 1.2 ng/dl, $P < 0.001$), as shown in Fig. 1. The prevalence of vitamin D insufficiency, as defined by 25(OH)D less than 30 ng/dl, was 34.6% in subjects on TZD compared to 75.5% in those not on TZD ($n = 49$). In a stepwise logistic regression model, including TZD, BMI, age, and HbA1c as independent variables, it was found that use of TZD and BMI were both independent risk factors for vitamin D insufficiency (Table 3). To further explore the influence of TZD on vitamin D status, we evaluated the relationship of vitamin D to BMI according to TZD usage. There was a negative association between 25(OH)D levels and BMI, but the relationship did not reach statistical significance. At each level of BMI, however, 25(OH)D was approximately 10 ng/dl higher in the presence of TZD (Fig. 2).

Discussion

It is currently recognized that TZD is associated with an increase, albeit small, in the risk of fractures. Fractures associated with TZD are mostly peripheral, such as hand and foot fractures. The underlying basis for the increase in fractures at the periphery rather than at more typical sites for osteoporotic fractures is not entirely clear. However, more common types of osteoporotic fractures, particularly vertebral fractures, could be underreported since such fractures are mostly asymptomatic.

In this study, we demonstrated that asymptomatic vertebral fractures were not more common in patients on TZD as compared to those on other anti-diabetic agents. This is in contrast to a previous study showing increased vertebral

Table 2 BMD and vertebral fractures in TZD and non-TZD users

	TZD users ($n = 52$)	Non-TZD users ($n = 50$)	P value
L2–L4 BMD (g/cm^2)	1.14 ± 0.19	1.18 ± 0.20	NS
Femoral neck BMD (g/cm^2)	0.88 ± 0.14	0.92 ± 0.14	NS
Total hip BMD (g/cm^2)	0.96 ± 0.15	1.02 ± 0.11	<0.05
Subjects with vertebral fractures at one level or more	10 (19.2%)	7 (14.0%)	NS
Subjects with vertebral fractures at two levels or more	2 (3.8%)	3 (6.0%)	NS

NS not significant

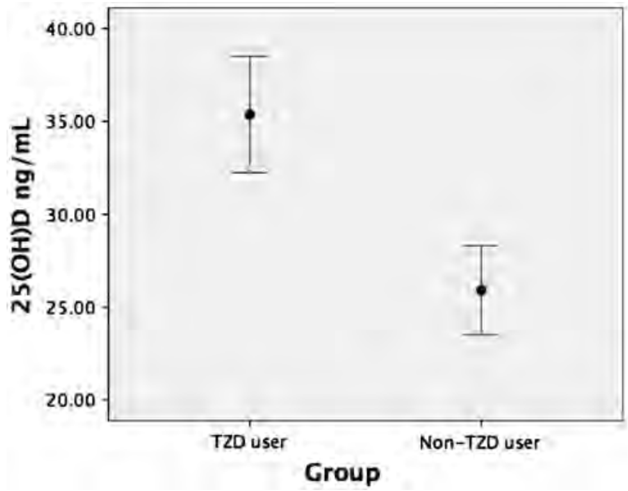

Fig. 1 25(OH)D levels in TZD versus non-TZD users (mean ± SE). *TZD* thiazolidinediones

Table 3 Characteristics associated with vitamin D insufficiency

	Odds ratio (95% CI)
Non-TZD user	6.4 (2.6–15.6)
BMI (kg/m^2)	1.110 (0.998–1.234)
Age (years)	NS
HbA1c (%)	NS

CI confidence interval, *NS* not significant

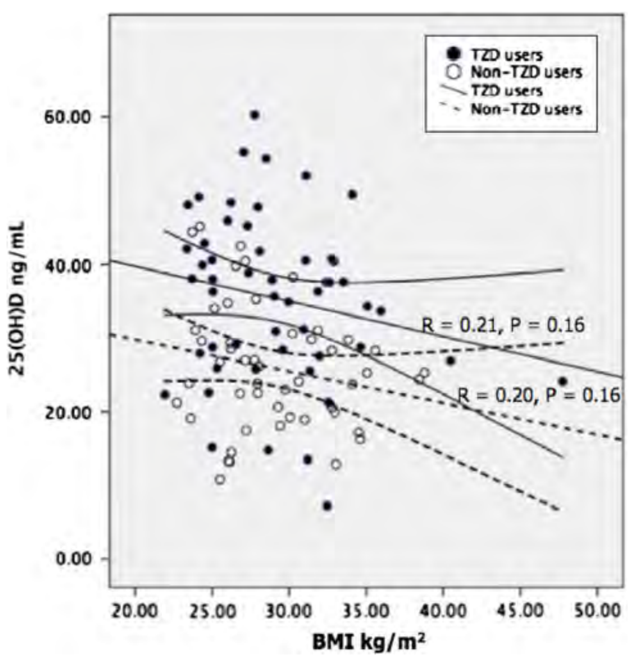

Fig. 2 Relationship between 25(OH)D levels and BMI in TZD and non-TZD users by linear regression

fractures in male TZD users [4]. A number of differences in study design may be accountable for the contradictory results. Our study group consisted of postmenopausal females, whereas only males were included in the other study. It has been demonstrated that TZD can affect the bone of both sexes differently, in that females tend to have a greater risk of fractures associated with TZD [8]. This may be due to the influence of estrogen deficiency which makes postmenopausal more susceptible to osteoporosis and osteoporotic fractures in general. It therefore seems counterintuitive that an increased propensity for vertebral fractures was not observed in this study. However, our study population was relatively young, which may render the increased risk, if any, less apparent [9].

Pioglitazone was more frequently used in our study. Both pioglitazone and rosiglitazone act through peroxisome proliferator-activated receptor gamma (PPARG) activation, but may possess differential biological functions due to the difference in the activation of other receptors. Although it has been suggested that pioglitazone causes a greater incidence of adverse skeletal effects than rosiglitazone [10], this notion is not without dispute [11]. Therefore, the lack of effect on vertebral fractures in this study is not likely to be attributed to the more common use of pioglitazone versus rosiglitazone.

There were a number of limitations in this study. Our sample size may not have enough statistical power to significantly demonstrate a small increase in vertebral fracture risk. Although subjects were well matched for age and body weight, medications for diabetes were not controlled and there was an imbalance in diabetic medications used between the two groups. In particular, sulfonylureas were used more often in the TZD group. However, it is less likely that this would affect our results since sulfonylureas, despite its long history of use in the management of diabetes, have not been associated with increased fracture risk. Lastly, TZD users had longer duration of diabetes which may more adversely affect bone metabolism.

In this study, we found that 25(OH)D levels in patients with type 2 diabetes were higher in TZD users. It is well recognized that fat mass affects vitamin D status, and low 25(OH)D levels are more commonly found in subjects with higher adiposity [12–14] as well as in those with metabolic syndrome [15]. This is likely due to sequestration of most vitamin D, a fat-soluble vitamin, in adipose tissue [16], although alteration in the metabolism of vitamin D in adipose tissue cannot be entirely ruled out. The difference in fat mass is less likely to be the cause of higher 25(OH)D associated with TZD use in this study since both the groups of study subjects were well matched for BMI. Moreover, the direction of association was opposite to what would be expected, in that TZD users had higher rather than lower vitamin D status. Although a difference in sun exposure could account for the difference in vitamin D status, the degree of difference is not likely to be enough to be attributable to the higher vitamin D status in the TZD group. Both the groups of subjects were active, ambulatory, and without other major complications which could lead to limited sun exposure. Moreover, sun exposure behavior as assessed by questionnaires, although interpretable in certain studies [17], only explain a minor part of the variation in vitamin D status together with a number of clinical risk factors [18]. It is also likely that TZD may affect dermal vitamin D metabolism. Vitamin D synthesis takes place in the dermal/epidermal junction. It is of note that besides being present in the liver, dermal fibroblasts also express 25-hydroxylase enzymes [19] and produce 25(OH)D upon UVB exposure [20]. PPARG is ubiquitous, with myriad biological effects. It is present in dermal fibroblasts and affects a number of their biological functions [21–23]. We therefore hypothesize that the increase in 25-hydroxylation of vitamin D synthesized from the skin in TZD users may be the underlying basis of the higher vitamin D status observed with TZD use. Further studies to explore the issue are warranted and could lead to increased understanding of the metabolism of vitamin D in the skin.

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Osteoporos Int (2011) 22:2159–2164
DOI 10.1007/s00198-010-1439-3

ORIGINAL ARTICLE

The relationship of fetuin-A and lactoferrin with bone mass in elderly women

L. Chailurkit · A. Kruavit · R. Rajatanavin · B. Ongphiphadhanakul

Received: 20 June 2010 / Accepted: 13 September 2010 / Published online: 21 October 2010

Abstract

Summary The relationships of fetuin-A and lactoferrin to bone-related phenotypes were investigated in elderly women. Fetuin-A was associated not only with bone mineral density (BMD) but also with bone resorption marker suggesting an influence of fetuin-A on osteoclasts.

Introduction The aim of this study is to investigate the relationship of bone-related phenotypes in elderly women with circulating fetuin-A and lactoferrin.

Methods Eighty-two elderly women were studied. Serum fetuin-A, lactoferrin, C-terminal telopeptide of type I collagen (CTx), total procollagen type 1 amino-terminal propeptide, and plasma intact parathyroid hormone (PTH) were analyzed. BMD of the lumbar spine at L2–4 and at the femoral neck was measured.

Results Serum fetuin-A was significantly associated with L2–4 BMD (r=0.23, P<0.05). After controlling for age and body weight, the association remained statistically significant. There was a significant association between serum fetuin-A and serum CTx (r=−0.37, P<0.001). The association between fetuin-A and L2–4 BMD no longer existed after controlling for serum CTx. There were positive associations of circulating lactoferrin with plasma PTH (r=0.24, P<0.05) and serum CTx (r=0.26, P<0.05). No association between serum lactoferrin and BMD at the lumbar spine or femoral neck was detected.

Conclusions Circulating fetuin-A is related to bone mass and bone resorption markers in elderly women. Lactoferrin, in contrast, is associated only with bone resorption markers.

Keywords Bone mineral density · Fetuin-A · Lactoferrin

Introduction

Fetuin-A and lactoferrin are both highly abundant circulating proteins with pleiotropic actions and are likely to affect bone in humans. Fetuin-A, also known as alpha-2-HS glycoprotein, is a serum glycoprotein of hepatic origin. It plays an important role in tissue mineralization by complexing with calcium and phosphate, leading to precipitation of hydroxyapatite in various tissues. In contrast to its inhibitory effect on tissue mineralization, it has recently been found that fetuin-A promotes bone mineralization in vitro [1]. In humans, a clinical study has shown the association of circulating fetuin-A levels with bone mineral density [2]. The effect is limited to women and is more apparent in subjects of advancing age. Biochemical markers of bone turnover were not measured, and the underlying basis for the observation is not entirely clear. Lactoferrin, an iron-containing protein with anti-inflammatory and antioxidant properties, has recently been found to be a potent stimulator of bone formation in vitro [3]. Ovariectomized animals fed with lactoferrin have preserved bone mass [4]. Whether and how lactoferrin might affect bones in humans is currently unknown. To further explore the probable influence of fetuin-A and

L. Chailurkit (✉) · R. Rajatanavin · B. Ongphiphadhanakul
Division of Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University,
Rama 6 Rd., Rajthevi,
Bangkok 10400, Thailand
e-mail: ralcl@mahidol.ac.th

A. Kruavit
Medical Health Maintenance Organization, Eastern Health, Box Hill Hospital,
Nelson Road, Box Hill,
Melbourne, VIC 3128, Australia

lactoferrin on bone, we investigated the relationship of bone-related phenotypes in elderly women with circulating fetuin-A and lactoferrin.

Methods

Subjects

Sample size for linear regression analysis with one predictive factor was calculated in order to have an 80% power to detect an association of small effect ($r^2=0.1$) at 95% significance level. Eighty-two elderly women were recruited from elderly care institutions within the vicinity of Bangkok (Ban-Bangkae 1 and Ban-Bangkae 2). Subjects who were unwilling to join the study or not well enough to come to the hospital were excluded. All subjects were apparently healthy. Subjects were also excluded if they had any diseases known to affect bone metabolism. Demographic features such as age, height, and weight were determined. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. The recruitment process was shown in Fig. 1. The study was approved by the Ethics Committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Biochemical measurement

Blood samples for biochemical analysis were drawn in the morning between 8:00 and 10:00 a.m. after an overnight fast. Serum calcium, creatinine, and inorganic phosphorus were analyzed on an automated biochemical analyzer (Dimension RxL; Dade Behring, Newark, DE, USA). Estimated glomerular filtration rate (GFR) was calculated as $(140-\text{age})\times\text{weight}\times1.23\times0.85$ divided by serum creatinine in millimole per liter. Serum fetuin-A and lactoferrin levels were measured by sandwich enzyme-linked immunosorbent assay (Epitope Diagnostics, Inc., San Diego, CA, USA and Assaypro, St. Charles, MO, USA, respectively). Plasma intact PTH, serum C-terminal telopeptide of type I collagen (CTx) and serum total procollagen type 1 amino-terminal propeptide (PINP) levels were determined by electrochemiluminescence immunoassay on an Elecsys 2010 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The assays have an intra-assay precision of 3.6%, 5.4%, and 3.8%, respectively.

Bone mineral density

Bone mineral density (BMD) of the lumbar spine at L2–4 and at the femoral neck was measured by dual-energy X-ray

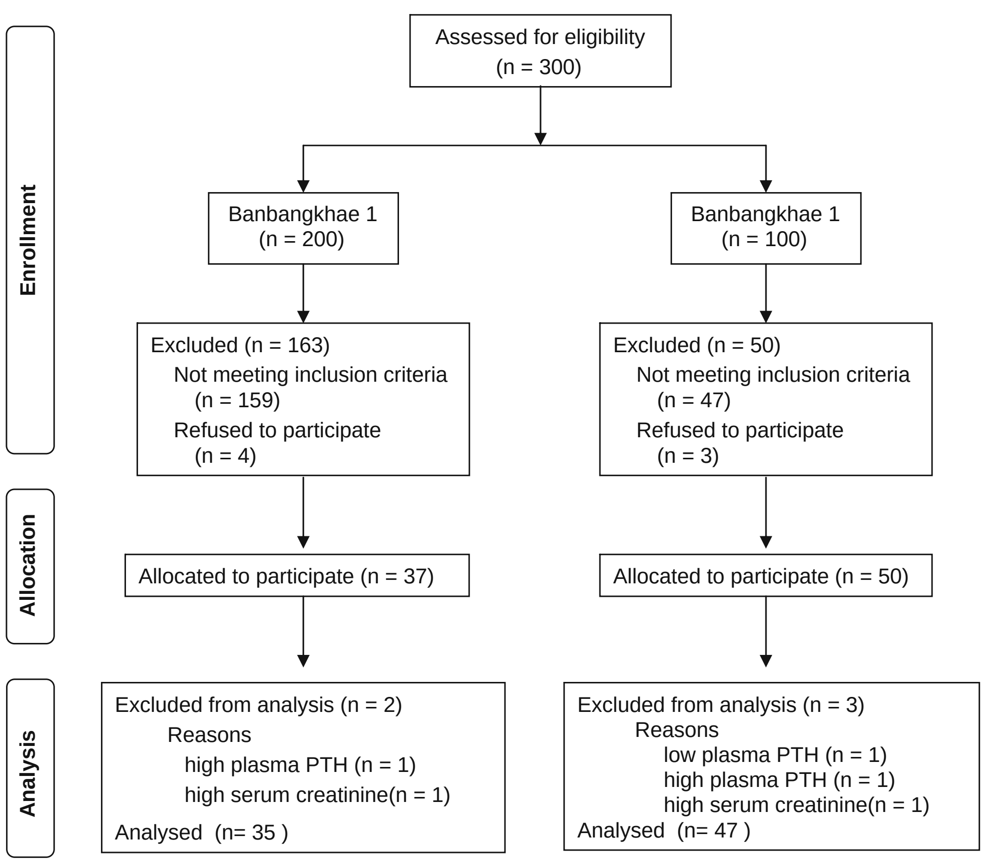

Fig. 1 Flow diagram of recruited subjects

absorptiometry (Lunar Expert XL; Lunar Corp., Madison, WI, USA). Subjects were classified as having osteoporosis if the BMD T-score was ≤−2.5 or as having osteopenia if the BMD T-score was >−2.5 and <−1.0 or as normal BMD if the BMD T-score was ≥−1.0 (according to the World Health Organization criteria) [5].

Statistical analysis

Normality of data was analyzed using the Kolmogorov–Smirnov test. Data normally distributed are presented as the mean ±SD. Non-normally distributed variables are presented as the median (range) and were log-transformed before a parametric test. Pearson's correlation coefficient was used to calculate the linear relationship between two variables. Stepwise multiple linear regression analyses were used to adjust for potential covariates. All analyses were performed using an SPSS statistical software package (version 17.0, SPSS Inc., Chicago, IL, USA). A P value <0.05 was considered statistically significant.

Results

Clinical characteristics of the study population are shown in Table 1. Ages of the women ranged between 65 and 97 years, with an average of 75.6 years. Only 3.7% of them had a previous history of hip fracture and 3.7% had a previous history of wrist fracture, whereas most subjects (92.7%) had no previous fractures.

Table 2 shows the correlation matrix of serum fetuin-A and variables related to bone and mineral metabolism. Serum fetuin-A was related to serum calcium but not to serum phosphorus levels. Serum fetuin-A levels were significantly associated with L2–4 BMD ($P<0.05$). After controlling for other potential covariates such as age and body weight, the association between serum fetuin-A and L2–4 BMD remained statistically significant (Table 3). In contrast, no association between serum fetuin-A and femoral neck BMD was demonstrated. To explore the underlying basis of the observation, we further analyzed the relationship between serum fetuin-A and biochemical markers of bone turnover. There is a significant association between serum fetuin-A and serum CTx. The association between serum fetuin-A and L2–4 BMD no longer existed after serum CTx was taken into account (Table 4). In addition, when the subjects were divided into high and low bone turnover according to the CTx level (by using the cut-off CTx level at 0.28 ng/ml (premonopausal level) [6]), the associations between serum fetuin-A and BMD also were not observed (Fig. 2). These data suggested that the effect of serum fetuin-A on BMD may be partly modulated through bone resorption. No association between serum fetuin-A and serum P1NP was detected.

There were positive associations of circulating lactoferrin with plasma PTH ($r=0.24$, $P<0.05$) and serum CTx ($r=0.26$, $P<0.05$) but not P1NP (Table 2); however, no association between serum lactoferrin and BMD at the lumbar spine or femoral neck was detected. With regard to body composition, there was no association between serum lactoferrin and BMI, body weight, or height. Nevertheless, a significant correlation between serum lactoferrin and serum creatinine was found ($r=0.28$, $P<0.05$) while there was no correlation between serum lactoferrin and estimated GFR.

There was a significant negative association between serum fetuin-A and serum lactoferrin ($r=-0.31$, $P<0.01$). Since both serum fetuin-A and serum lactoferrin were correlated to serum CTx, we examined whether the relationship between serum fetuin-A and serum lactoferrin was independent of serum CTx. After controlling for serum CTx, the correlation between serum fetuin-A and serum lactoferrin remained statistically significant ($r=-0.25$, $P<0.05$).

Table 1 Clinical characteristics of subjects in the study

Characteristics	Mean±SD
Age (years)	75.6±6.0
Weight (kg)	52.1±8.4
Height (cm)	147.5±6.1
BMI (kg/m^2)	23.9±3.7
Serum calcium (mmol/L)	2.4±0.09
Serum creatinine (μmol/L)	71.5 (49–151)[a]
Serum inorganic phosphorus (mmol/L)	1.16±0.14
Serum alkaline phosphatase (U/L)	81.8±26.7
Plasma intact PTH (pmol/L)	4.3±1.6
Serum CTx (ng/ml)	0.41±0.20
Serum PINP (ng/ml)	47.7 (12.5–193.7)[a]
Serum fetuin-A (μg/ml)	435.4±76.2
Serum lactoferrin (μg/ml)	3.3 (0.8–11.8)[a]
Lumbar spine L2–4 BMD (g/cm^2)	0.91±0.18
Z-scores lumbar spine L2–4 BMD	0.20±8.02
Femoral neck BMD (g/cm^2)	0.64±0.10
Z-scores femoral neck BMD	0.37±1.46

PINP procollagen type 1 amino-terminal propeptide

[a] Median (range)

Discussion

Whereas a number of in vitro and animal studies have demonstrated the role of fetuin-A in bone mineralization, studies in humans remain scarce. In a recent study, serum fetuin A is associated with BMD. The effect, however, is more evident in elderly women [2] relative to men. Our

Table 2 Correlation matrix of variables

Parameters	Age	Weight	Height	BMI	Calcium	Creatinine	Estimated GFR	Inorganic phosphorus	Intact PTH	CTX	Fetuin-A	Lactoferrin	PINP	Lumbar spine L2–4 BMD	Femoral neck BMD
Age	1														
Weight (kg)	−0.107	1													
Height (cm)	−0.124	0.42[a]	1												
BMI (kg/m^2)	−0.047	0.86[a]	−0.09	1											
Serum calcium (mmol/L)	−0.081	−0.01	0.08	−0.06	1										
Serum creatinine (μmol/L)	0.239[b]	−0.01	−0.11	0.05	−0.06	1									
Estimated GFR	−0.541[a]	0.50[a]	0.32[a]	0.36[a]	0.05	−0.79[a]	1								
Serum inorganic phosphorus (mmol/L)	−0.270[b]	−0.20	0.01	−0.23[b]	−0.98	−0.01	−0.02	1							
Plasma intact PTH (pmol/L)	0.179	−0.18	−0.10	−0.14	−0.11	0.29[a]	−0.27[b]	−0.16	1						
Serum CTX (ng/ml)	0.043	0.30[a]	−0.07	−0.29[a]	−0.33[a]	0.14	−0.24[b]	0.07	0.53[a]	1					
Serum fetuin-A (μg/ml)	0.105	0.02	0.00	0.02	0.26[b]	−0.12	0.06	0.02	−0.15	−0.37[a]	1				
Serum PINP (ng/ml)	−0.117	−0.08	−0.01	−0.10	−0.31[a]	0.03	−0.03	0.23[b]	0.28[b]	0.67[a]	−0.03	1			
Serum lactoferrin (μg/ml)	0.010	0.18	0.06	0.16	−0.04	0.28[b]	−0.09	0.06	0.24[b]	0.26[b]	−0.32[a]	0.14	1		
Lumbar spine L2–4 BMD (g/cm^2)	0.140	0.27[b]	0.09	0.25[b]	0.19	0.08	0.00	0.02	−0.11	−0.34[a]	0.23[b]	−0.07	−0.05	1	
Femoral neck BMD (g/cm^2)	−0.139	0.30[a]	0.33[a]	0.15	0.05	0.01	0.12	0.10	−0.17	−0.23[b]	0.17	−0.12	−0.06	0.46[a]	1

[a] Significant at 0.01 level (two-tailed)

[b] Significant at 0.05 level (two-tailed)

Table 3 Association of serum fetuin-A to L2–4 BMD after controlling for confounders

	Standardized coefficients	*P*
Fetuin-A (μg/ml)	0.22	<0.05
Body weight (kg)	0.26	<0.05
Age (year)	–	NS

Table 4 Lack of association of serum fetuin-A, body weight, and age to L2–4 BMD after controlling serum CTx

	Standardized coefficients	*P*
CTx (ng/ml)	−0.34	<0.01
Fetuin-A ((μg/ml)	–	NS
Body weight (kg)	–	NS
Age (year)	–	NS

study is in keeping with the influence of serum fetuin-A on BMD found in the above study. Moreover, our results suggest that serum fetuin-A may affect bone resorption since serum CTx, a biochemical marker of osteoclast activity, was significantly related to circulating levels of fetuin-A. Fetuin-A demonstrates many biological effects, some of which are probably unrelated to mineralization. For example, fetuin-A is related to insulin resistance [7], incident diabetes [8], and increased risk of myocardial infarction as well as stroke [9]. The underlying basis for these effects besides mineralization is not entirely clear; but fetuin-A has been shown to stimulate cytokine expression and reduce adiponectin production in adipocytes [10]. For bone cells, the direct effects of fetuin-A on osteoblasts, osteoclasts, or osteocytes have never been reported; however, an in situ hybridization study of bone biopsies from hemodialysis patients has suggested the production of fetuin-A by osteoblasts [11]. Our results thus raise the possibility that serum fetuin-A affects BMD in elderly women not only through enhancing bone mineralization but probably also through direct effect on bone cells.

Recently, lactoferrin has been found to possess a potent stimulatory effect on bone formation. In fact, this property of lactoferrin exceeds those of other bone regulators described so far, including PTH, transforming growth factor beta, and amylin [12]. In addition to its effect on osteoblasts, lactoferrin also affects osteoclasts by inhibiting osteoclast differentiation and bone resorption activity in vitro [13]. The role of lactoferrin in the regulation of the skeleton in vivo is less clear. Injection of lactoferrin in animals results in increased new bone formation [3]. In addition, oral administration of bovine lactoferrin to ovariectomized mice improves BMD [14]. In contrast, in

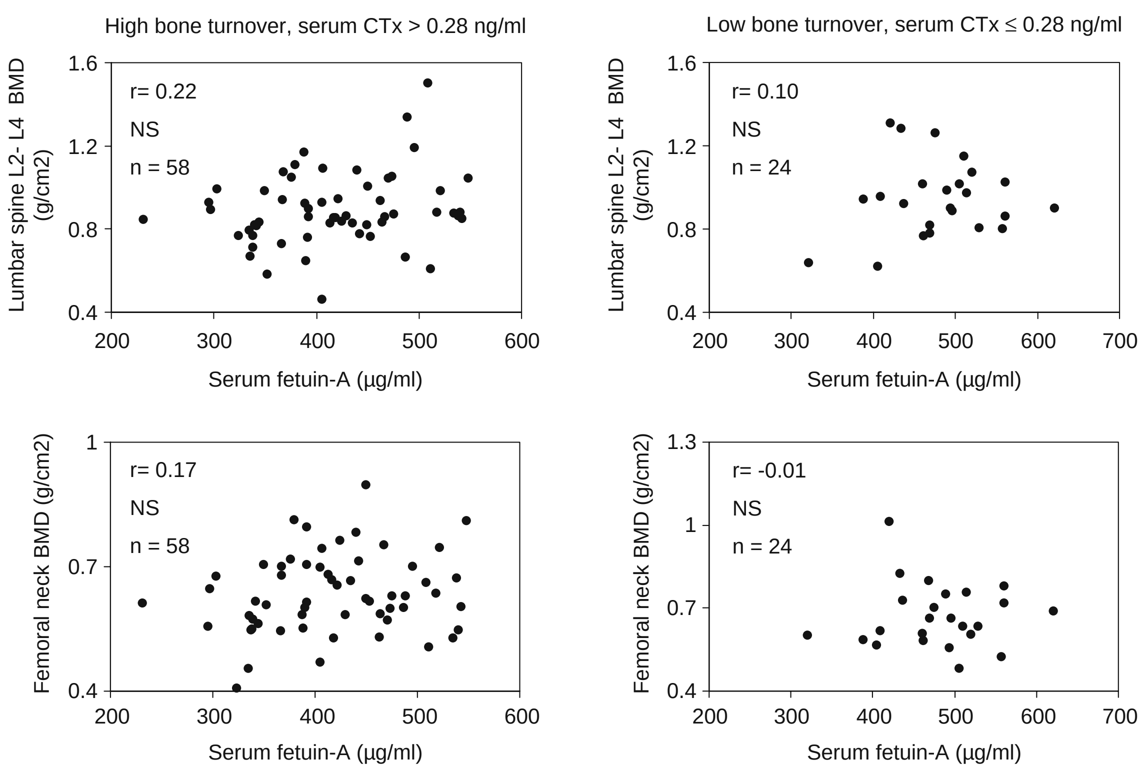

Fig. 2 Association between fetuin-A and BMD in the presence of high or low bone turnover markers. *CTx* C-terminal telopeptide of type I collagen, *NS* non-significance

a lactoferrin knockout animal model, no abnormality in bone-related phenotypes was described [15]. Our findings partly confirmed the influence of lactoferrin on bone resorption; however, no correlation between serum lactoferrin and serum P1NP, a marker of bone formation, was found, suggesting that circulating lactoferrin does not reflect the local stimulatory effect of lactoferrin on bone formation found in vitro and in vivo in animals. The net effect of lactoferrin did not translate into increased bone mass in elderly women, which likely reflects the complexity of skeleton regulation and suggests that circulating lactoferrin is not a significant biomarker of bone mass in humans.

In the present study, we found a strong association between serum fetuin-A and serum lactoferrin which persisted after controlling for bone resorption markers, suggesting that the demonstrated relationship is not the result of the association of bone resorption with both serum fetuin-A and serum lactoferrin. The underlying mechanism of the association is still unclear. Both fetuin-A and lactoferrin are multifunctional proteins affecting several biological processes, and it is still likely that the association between lactoferrin and fetuin-A is not causal but the result of a confounder not examined in the present study. For example, in a porcine model of hemodialysis venous graft stenosis, a proteomic study revealed elevation of both lactoferrin and fetuin-A in the stenosed tissue [16]. It is as yet unknown whether lactoferrin directly affects fetuin-A and vice versa. Further investigation to explore the issue is warranted and is likely to advance our understanding of the regulation of these two abundant and multifunctional proteins.

Conclusion

Circulating fetuin-A is related to bone mass and bone resorption markers in elderly women. Lactoferrin, in contrast, is associated only with bone resorption markers.

Conflicts of interest None.

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J Bone Miner Metab (2011) 29:606–614
DOI 10.1007/s00774-011-0263-y

ORIGINAL ARTICLE

Effect of *GGCX* gene polymorphism on the responses of serum undercarboxylated osteocalcin and bone turnover markers after treatment with vitamin K2 (menatetrenone) among postmenopausal Thai women

Thawee Songpatanasilp · La-or Chailurkit · Suwannee Chantprasertyothin · Boonsong Ongphiphadhanakul · Nimit Taechakraichana

Received: 31 August 2010 / Accepted: 12 January 2011 / Published online: 23 February 2011

Abstract The purpose of this study was to evaluate the influence of gamma-glutamyl transferase (*GGCX*) gene polymorphisms on the response of serum undercarboxylated osteocalcin (ucOC) and bone turnover markers 3 months after treatment with menatetrenone. One hundred and forty postmenopausal Thai women were enrolled and assigned to receive 45 mg/day treatment of menatetrenone (MK-4) concurrently with calcium 1.2 g and vitamin D 400 IU for 3 months. Demographic characteristics, *GGCX* genotyping, serum bone turnover markers and ucOC levels were obtained from all participants at baseline. We evaluated the reduction of ucOC at 3 months and the reduction of beta-CTx and P1NP at 1 and 3 months. The responses were compared between the different genotypes of GG and GA + AA groups. There was a significant reduction of serum ucOC, beta-CTx and P1NP from the baseline at 3 months ($p < 0.001$) though there was no significant difference between genotypes (GG vs. GA + AA; $p > 0.05$). Nonetheless, a subgroup analysis of postmenopausal women who 65 years of age or over ($N = 37$) revealed a significant difference between the two groups in the reduction of ucOC. Menatetrenone significantly reduced serum ucOC as well as beta-CTX and P1NP from the baseline. *GGCX* polymorphism appeared to have an influence over the reduction of ucOC especially in older women (age $\geq$65). Furthermore, the groups which have "A" allele trend to being more efficient in reducing the serum ucOC level than the group which does not have it.

Keywords *GGCX* gene · Under-carboxylated osteocalcin · Bone turnover markers · Menatetrenone · Postmenopausal Thai women

T. Songpatanasilp (✉)
Orthopaedic Oncology and Bone Metabolism Unit, Department of Orthopaedics, Phramongkutklao Hospital and College of Medicine, 315 Rajvithi Rd., Rajthevee, Bangkok 10400, Thailand
e-mail: thaweesps@yahoo.com

L. Chailurkit · S. Chantprasertyothin
Endocrine Laboratory, Department of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

B. Ongphiphadhanakul
Division of Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

N. Taechakraichana
Department of Obstetric and Gynaecology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Introduction

Menatetrenone or vitamin K2, a dual action drug, has been shown to promote osteoblastogenesis and inhibit osteoclastogenesis by several in vitro and in vivo studies [1–3]. Many clinical studies demonstrated the effects of menatetrenone on bone formation and bone resorption, nonetheless the results are inconclusive particularly the treatment effects in postmenopausal women. Vitamin K, a coenzyme for glutamate carboxylase, mediates the conversion of glutamate (Glu), an undercarboxylated form to gamma-carboxyglutamate (Gla), a carboxylated one which is essential for the proteins to attract Ca^{2+} and to incorporate these into hydroxyapatite crystals. Osteocalcin (OC), one of the three bone-related Gla proteins, is a vitamin K dependent (VKD) protein. It contains three Gla-residues and is indigenous to the organic matrix of bone, dentin, and possibly other mineralized tissues. Although the precise role of OC in bone metabolism is not fully understood, a number of findings suggest its importance in regulating bone mineralization, maturation, and remodeling [4, 5].

To measure serum vitamin K level is impracticable in clinical practice, but serum undercarboxylated osteocalcin (ucOC) is deemed to be a good indicator of vitamin K insufficiency. A high ucOC level indicates that there is poor carboxylation status of OC, which reflects the vitamin K insufficiency state. The carboxylation status of OC is considered to be an independent risk factor for fractures. ucOC levels are found to be significantly increased in postmenopausal women, especially in the elderly [6]. Serum levels return to normal values for young adults after they are treated with low doses of vitamin K1 [7]. Many studies showed that ucOC negatively correlated with hip bone mineral density (BMD) [8] and that the fraction of ucOC is a powerful predictor of hip fracture [9, 10]. However, there is no consensus about the cutoff value of ucOC level in serum for vitamin K insufficiency. Bunyaratavej et al. [11] studied the level of ucOC in 357 premenopausal Thai women and found that the average of serum ucOC was 2.69 ng/ml (95% CI 2.48–2.90), and concluded that the serum level at 2.69 ng/ml is an appropriate cutoff value. In contrast, the study of Plantalech et al. [6] found that the cutoff value for Caucasian women was 1.65 ng/ml. The dissimilarity between these results might come from the difference in the method used for measurement of ucOC, dietary habits or to genetic differences. Various studies revealed variable effects of vitamin K on bone metabolism, which may be due to the type of vitamin K administration (K1 or K2), the difference of absorption and transport in the body, which depends on lipoproteins, the tissue specific differences in uptake of vitamin K (K2 is better than K1 in extra-hepatic tissue) [5], the influence of other VKD proteins in bone with osteocalcin and of potential important, the differences in activity of enzyme GGCX with genetic polymorphism.

GGCX is the gene that encodes a VKD enzyme, i.e., gamma-glutamyl carboxylase, which is essential in the conversion of glutamate (Glu) residues in the VKD proteins into gamma-carboxyglutamic acid (Gla) residues [12]. It is reported that the rare mutations of *GGCX* gene with amino acid substitution (Leu395Arg, Trp501Ser) cause consequential abnormal enzymatic activity, and these lead to VKD protein defects and severe bleeding disorders [13, 14]. However, among *GGCX* gene polymorphism, only one single nucleotide polymorphism (SNP) reported was found to correlate with BMD. A study by Kinoshita et al. [15] showed that the *GGCX* gene is on chromosome 2p12 and the mutation in exon 8 in the position *rs699664* (Arg325Gln) is correlated with BMD. In order to be carboxylated, VKD proteins are assumed to be bound specifically to 343–355 residues of GGCX with high affinity. Among these residues, 343 (Cys) and 345 (Tyr) were suggested to be located near the catalytic center [16]. Moreover, it was also reported that chemical modification of 323-Cys and 343-Cys decreased its carboxylase activity [17]. Considering a study of human GGCX membrane topology, human GGCX span the endoplasmic reticulum membrane five times and the interval of fourth and fifth transmembrane region may be composed of amino acids 313–361 [15]. Since amino acids 323-Cys, 325-Arg/Gln, 343-Cys, 345-Tyr and 343–355 are involved in the interval of fourth and fifth transmembrane regions (313–361), the amino acid substitution of 325 residue (Arg/Gln) may affect enzymatic activity directly or indirectly through influencing the function of these residues. People with 325-Gln might have a higher activity of carboxylation of these proteins with the given status of vitamin K, considering that carboxylase activity of 325-Gln was higher than that of 325-Arg in vitro. Nevertheless, the study of Kinoshita et al. [15] did not examine the response of ucOC and the BTMs. Therefore, we aimed to look into the effect of *GGCX* gene polymorphism on responses of serum ucOC and BTMs after treatment with the standard dose of menatetrenone in postmenopausal Thai women.

Materials and methods

Subjects enrollment, blood testing and treatment allocation

From July to December 2009, 140 postmenopausal women 40 years of age and older and menopausal for at least 2 years were enrolled into this study. Patients with chronic medical conditions or metabolic bone diseases that may affect bone metabolism, e.g., patients with major gastrointestinal surgery, steroid therapy, rheumatoid arthritis, renal dysfunction, liver dysfunction and thyroid dysfunction, were excluded. Patients with history of cancer within 5 years, recently consumed an excess alcohol (>4 drinks per day) or abused drugs, were also excluded. They must be able to walk independently and none of them engaged in regular physical exercise programs. All of the subjects were never users of bisphosphonate, strontium ranelate, teriparatide or warfarin and had stopped postmenopausal hormone therapy, SERMs or any calcium and/or vitamin D supplement for over 1 month. Biochemistry, bone turnover markers, ucOC level and *GGCX* (*rs699664*, *A/G*) (Arg325Gln) genotyping were done in all patients at baseline by masked technicians. Genotyping of *GGCX* gene was analyzed and were named as follows: GG, GA and AA according to the amino acid substitution of 325 residue (Arg/Gln) (GG = 325Arg, GA = 325Arg/Gln and AA = 325Gln). After baseline blood collection, all of them received menatetrenone 45 mg per day in three divided doses and calcium carbonate 1.2 g plus vitamin D 400 IU per day in two divided doses. Subjects were followed for the other two visits at 1 month (visit 2) and 3 months (visit 3). At visit 2, blood tests for serum beta-CTx and P1NP were repeated. At visit 3, blood tests for ucOC and serum beta-CTx and P1NP were repeated. We also check the subject's compliance by returning the product packages on each follow-up visit before prescribing the next intervention.

Measurement of undercarboxylated osteocalcin and bone turnover markers

Undercarboxylated osteocalcin is the fraction of the osteocalcin in the circulation which is not fully carboxylated. ucOC levels were measured using Glu OC-EIA kit, which was assessed with enzyme-link immunoassay (EIA) technique (Takara Bio Inc., Tokyo, Japan, CV 4.6–6.7%) [18, 19]. For bone resorption markers, we used serum C-telopeptide cross-linked of collagen type-I (beta-CTx) or beta-Crosslaps assay, which was assessed with the electrochemiluminescence immunoassays (ECLIA) technique (Elecsys, Roche Diagnostics, Mannheim, Germany, ng/ml; intra-assay CV 2.4–7.2%) [20]. For bone formation markers, we used serum pro-collagen type-I N-terminal propeptide (P1NP), which was assessed with the ECLIA technique (Elecsys, Roche Diagnostics, Mannheim, Germany, ng/ml; intra-assay CV 2.3–2.8%) [21]. Due to the diurnal variation, sera for ucOC, beta-CTx and P1NP assessment were collected in the morning at around 8.00 a.m., after an overnight 12 h fast and were frozen at −20°C until analysis. All specimens were sent to the central laboratory for test and quality control.

GGCX genotyping and real-time PCR system

All subjects' DNA was isolated from frozen buffy coats of whole blood (3 ml, collected into EDTA) by a non-enzymatic extraction method. Sequences of the *GGCX* (*rs699664*, *A/G*) gene were amplified, using the polymerase chain reaction (PCR) with primers designed as reported previously [15] (forward primer 5′-TCCTACTGCCCCC GAAGGTTGCAACAA-3′ and reverse primer 5′-TTGTT GCAACCTTCGGGGGCAGTAGGA-3′). PCR cycling conditions and genotyping were run by the standard procedure according to the endocrine laboratory in Ramathibodi Hospital as following. DNA 20 ng was add into the PCR reaction which consists of TaqMan® Universal Master Mix(1×), TaqMan® MGB probes(1x) in a total volume of 10 μl. The real-time PCR reaction protocol was 10 min 95°C, and 40 cycles of 15 s 92°C, 1 min 60°C using 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

Data analysis

All baseline demographic data and distribution frequency of *GGCX* (*rs699664*, *A/G*) gene polymorphism (AA, AG and GG genotype) were presented in percent distribution. Mean % change from baseline in ucOC level were calculated and compared before and after treatment within group using paired t test, and between group between GG-group of genotype and GA + AA group using independent t test. Mean % change from baseline in ucOC level in subgroup of patients with age of 65 years of age or more were also analyzed. Mean % change from baseline in bone turnover markers (both serum beta-CTx and P1NP) were calculated and compared before and after treatment within group, and between groups of AA, AG genotypes and GG genotype at 1 and 3 months using repeated measurements ANOVA.

Ethical consideration

The protocol was reviewed and approved by the Royal Thai Army Medical Department Institutional Review Committee. All eligible subjects were informed of all the details of the study about objective, method of study, treatment outcomes, potential adverse events and also the subjects' right to refuse to participate or withdraw from this study at any time without affecting their proper medical care. A signed informed consent was obtained from the subjects without enforcement prior to the study initiation.

Results

Subject characteristics

The demographic data and baseline characteristics of all these postmenopausal women are shown in Table 1. Five cases reported to have at least one previous vertebral compression fracture from lateral, plain thoraco-lumbar radiography. None of these subjects has previous hip fracture. The distribution frequency of *GGCX* gene in all the women is shown in Table 1 and Fig. 1.

Distribution frequency of *GGCX* genotypes

There were 69 cases (49.3%) having GG genotype (325-Arg), 66 cases (47.1%) having GA genotype (325-Gln/Arg) and five cases (3.6%) having AA genotype (325-Gln). Kinoshita et al. [15] found significant differences between theBMI-adjusted Z score BMD among the two groups (325-Gln compared to 325-Gln/Arg + 325Arg) in subjects over 75 years. We decided to compare postmenopausal Thai women by using two groups in which numbers per group are slightly equal for purpose of statistical

Table 1 Baseline characteristics and GGCX distribution frequency ($n = 140$)

	Mean	SD	Minimum–maximum
Age (years)	59.7	7.2	43–79
Weight (kg)	47.1	5.4	31.5–78.0
Year after menopause (years)	14.6	6.5	3–30
Serum iPTH (pg/ml)	47.78	4.80	16.28–100.90
Serum ucOC (ng/ml)	2.91	2.07	0.11–9.86
Serum bCTx (ng/ml)	0.47	0.20	0.06–1.02
Serum P1NP (ng/ml)	52.75	21.88	18.32–186.90
GGCX genotype [*N* (%)]	GG	69 (49.3)	
	GA	66 (47.1)	
	AA	5 (3.6)	

GGCX = gamma-glutamyl carboxylase gene, GG, GA and AA is a SNP of *GGCX* gene according to the amino acid substitution of 325 residue (Arg/Gln) (GG = 325Gln, GA = 325Arg/Gln and AA = 325Arg)

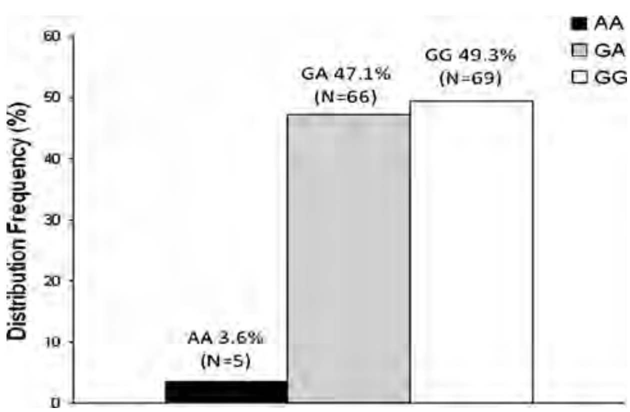

Fig. 1 Distribution frequency of *GGCX* genotypes in this study

calculation, i.e., the GG-group (69 cases) and GA + AA-group (71 cases). The demographic data and baseline characteristics were compared between the two groups as shown in Table 2. There was no significant difference between these two groups in demographic data and baseline biochemical values.

Change in undercarboxylated osteocalcin

There were 14 subjects lost to follow up by the third visit due to loss of contact, which left 126 cases for the ucOC analysis. The baseline value of ucOC in GG-group was 2.92 ± 2.02 ng/ml (mean ± SD), and in GA + AA-group was 2.89 ± 2.15 ng/ml (mean ± SD). ucOC levels significantly decreased from baseline in both groups ($p < 0.001$) (Fig. 2). The overall reduction in ucOC levels in both groups was 61.7% from baseline and mean % changes from baseline in each group were −54.2 and −69.9% in GG-group and GA + AA-group, respectively (Table 3). To compare between the % changes for baseline between GG-group and GA + AA-group at 3 months using independent *t* test, there are no statistical significant difference between GG-group and GA + AA-group ($p = 0.69$). But there is a trend to more reduction of ucOC level in GA + AA group.

Subgroup analysis of mean % change from baseline of serum ucOC in patients aged 65 years or over

There were 37 patients who were 65 years of age or over and two of them were lost to follow-up in last visit which remains 35 cases for subgroup analysis. There were 22 cases who had GG genotype and 15 cases who were GA + AA group of genotypes. The analysis revealed that there are statistical significant difference in mean % change from baseline between GG-group and GA + AA group ($p = 0.025$) in flavor of GA + AA group as shown in Table 4.

Change in bone turnover markers

There were eight cases of subjects that dropped out from the study after the first month. Fourteen cases dropped out from the study before the final visit then left 126 subjects for the final analysis. Mean baseline levels of beta-CTx and P1NP are shown in Table 5. There were a significant reduction in serum beta-CTx and P1NP levels from baseline at 1 and 3 months after the treatment (one-way repeated ANOVA) ($p < 0.001$). The overall mean % reductions in serum beta-CTx and serum P1NP from baseline were −13.8 ± 47.1 and −14.1 ± 20.6%, respectively. However, there is no statistical significant difference in mean % reduction between GG-group and GA + AA-group in both serum beta-CTx and serum P1NP at 3 months as shown by Fig. 3 ($p = 0.176$ and $p = 0.429$, respectively, by two-way repeated ANOVA). Again, there are some trends to more reduction of serum beta-CTx and serum P1NP in GA + AA group.

Analysis of AA genotype group

There are only 5 cases in AA group, which account for 3.6% of all this SNP. One in these five cases lost to contact on visit 3. All baseline characteristic and % changes from baseline of serum ucOC, beta-CTx and P1NP at 3 months of these subjects are shown in Table 6.

Adverse events

There was very good compliance by mos of our subjects, with no any serious adverse events found in this study. The

Table 2 Baseline characteristic expressed as mean ± SD between the two groups ($N = 140$)

	GG genotype ($N = 69$)	GA + AA genotype ($N = 71$)	p value
Age (years)	60.55 ± 7.51	58.92 ± 6.85	0.18
Weight (kg)	46.98 ± 5.96	47.29 ± 4.86	0.74
Year after menopause (years)	15.19 ± 6.54	13.99 ± 6.42	0.27
Previous vertebral fractures	2	3	–
Serum iPTH (pg/ml)	46.24 ± 15.22	49.27 ± 14.33	0.23
Serum ucOC (ng/ml)	2.92 ± 2.02	2.89 ± 2.14	0.95
Serum beta-CTx (ng/ml)	0.49 ± 0.21	0.44 ± 0.19	0.17
Serum P1NP (ng/ml)	54.49 ± 25.29	51.06 ± 17.98	0.35

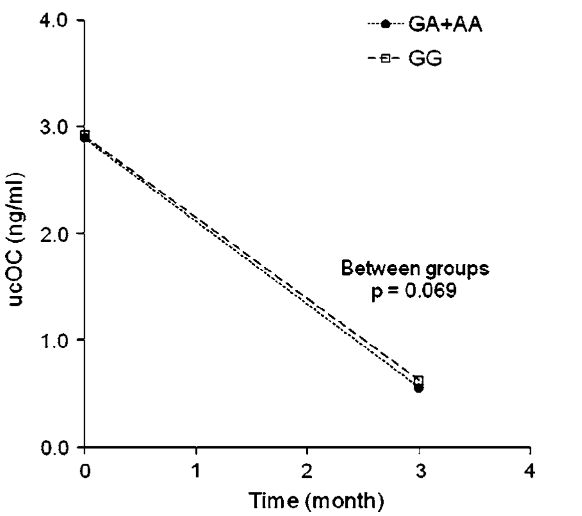

Fig. 2 Reduction in serum ucOC level from baseline at 3 months by different genotypes

Table 3 Mean % changes from baseline of serum ucOC at 3 months in all patients

Genotype groups	N	Mean (%)	SD	p value (difference from baseline)
Total	126	−61.6967	49.77296	<0.001
GG	66	−54.2244	62.24363	<0.001
GA + AA	60	−69.9163	29.14080	<0.001

Table 4 Mean % changes from baseline of serum ucOC at 3 months of patients aged 65 years or over

Genotype groups	N	Mean (%)	SD	p value (between groups)
Total	35	−54.2581	63.15529	
GG	22	−39.5151	75.83853	
GA + AA	13	−79.2076	12.77014	0.025

incidence of non-serious adverse events was 2.8% (4/140 patients). One subject had an erythematous skin rash on her extremities during the first week of drug administration, which subsided following anti-histamine treatment. Three other subjects had mild symptoms of dyspepsia during the first week after drug administration with spontaneous subsided. All patients with these non-serious adverse events did not drop out from the study. One subject sustained a right ankle fracture 3 weeks after enrollment. By the final visit, 14 subjects—four cases in the GG-group and 10 cases in the GA + AA-group—had dropped out. The overall dropout rate was 10% (14/140) in this study.

Discussion

Vitamin K, once viewed as having a single physiologic function in blood clotting, is now receiving substantial attention for several biological effects beyond hemostasis. New research indicates that vitamin K plays a role in bone metabolism and is potentially protective against osteoporosis [22, 23]. The effects of vitamin K in bone metabolism involve a similar mechanism of action, that of posttranslational modifying certain VKD proteins for their full biologic activity. Remarkable for bone, it is a protein call osteocalcin (OC) which needs the posttranslational modification to a fully gamma-carboxylated form before completely its function in bone matrix. Osteocalcin was produced by osteoblast under the stimulation of $1{,}25(OH)_2D_3$ [24], which is one of non-calcemic action of vitamin D. Although the precise role of OC in bone metabolism is not fully understood, a number of findings suggest its importance in regulating bone mineralization, maturation, and remodeling [5]. So one of the functions of all analogs of vitamin K (both vitamin K1 and K2) is to enhance bone matrix formation by osteoblast and further improved the quality of bone. In addition to the role of vitamin K in the synthesis of OC, recent research suggests that certain forms of vitamin K may have an effect on bone

Table 5 Changes in serum beta-CTx and P1NP from baseline at 1 and 3 months, in different groups of genotypes (mean, *N* and SD)

Genotype	Baseline beta-CTx	Beta-CTx at 1 month	Beta-CTx at 3 months	*p* value	Baseline P1NP	P1NP at 1 month	P1NP at 3 months	*p* value
GG								
Mean	0.4908	0.3950	0.4147	<0.001	54.4964	50.5953	45.2670	<0.001
N	69	68	66		69	68	66	
SD	0.2119	0.1720	0.2232		25.2883	24.1211	19.4222	
GA + AA								
Mean	0.4449	0.3669	0.3421	<0.001	51.0551	47.2789	43.2862	<0.001
N	71	64	60		71	64	60	
SD	0.1878	0.1720	0.1485		17.9785	18.4427	16.6427	
Total								
Mean	0.4675	0.3814	0.3798	<0.001	52.7511	48.9873	44.3237	<0.001
N	140	132	126		140	132	126	
SD	0.2006	0.1720	0.1937		21.8771	21.5388	18.1073	

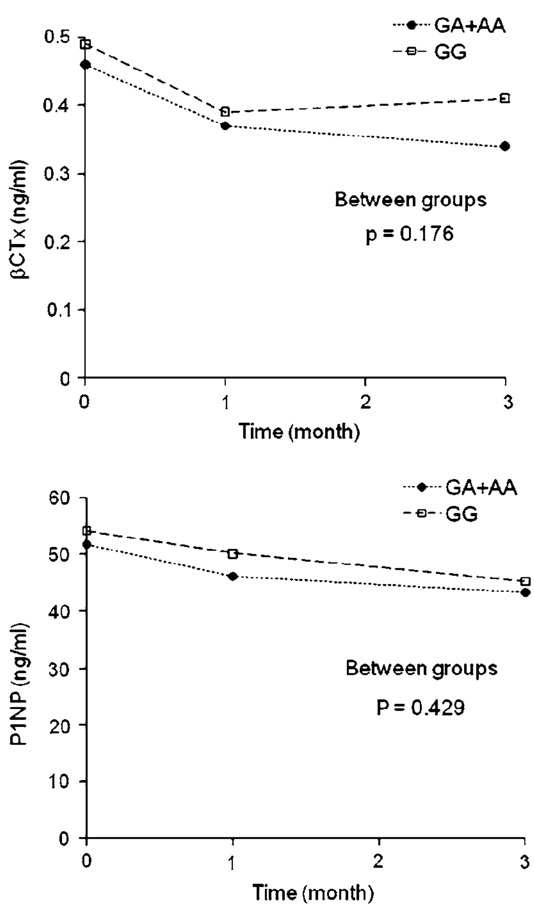

Fig. 3 Reduction in serum beta-CTx and P1NP levels from baseline at 1 and 3 months by different genotypes

metabolism unrelated to OC. In vitro studies demonstrated that menatetrenone or menaquinone-4 (MK-4), a form of vitamin K2, can inhibit bone resorption through inhibition of osteoclast formation and activity [1, 2] and induction of apoptosis in osteoclasts [25], whereas vitamin K1 did not. It has also been suggested that inhibitory effect of MK-4 on bone resorption may be independent of the gamma-carboxylation system, but related to its side chain [26], which may be interfere with generylgeneration process in prenylation of small G-protein system. Hence, in preclinical studies, the only vitamin K that might have both functions on improved bone formation and inhibition of bone resorption is menatetrenone (MK-4).

Unfortunately, clinical studies to elucidate the main action of menatetrenone are still complicated. Although several clinical trials of menatetrenone have shown that it prevents fractures in patients with osteoporosis [27–29], the exact mechanisms are not fully understood. Since menatetrenone has a minimal influence on BMD [27], explanations for its preventive effect on fracture have focused on improvement of bone quality. The quality of bone is determined by several factors, including bone architecture and bone turnover, which can be measured in the clinical setting. Recently, Knapen et al. [30] reported that menatetrenone inhibited deterioration of bone architecture in the femoral neck of postmenopausal women. The other bone quality factors that cannot be measured easily in clinical studies are the mineralization condition and quality of bone matrix proteins. As we know, matrix proteins also influence bone properties. Several bone matrix proteins such as osteocalcin probably have an important role in determining bone strength. For example, a rapid decrease of bone stiffness after oophorectomy has been observed in osteocalcin knockout mice in one study [31].

Detection of ucOC in the serum is evidence of skeletal vitamin K insufficiency or deficiency [5], with further result in a decrease of osteocalcin in the bone matrix. A high serum ucOC level or low vitamin K level has been

Table 6 Baseline characteristics and % changes from baseline of serum ucOC, beta-CTx and P1NP at 3 months of all subjects who have AA genotype

	Age	Year after menopause	Weight (kg)	PTH	Baseline			% change from baseline		
					ucOC	Beta-CTx	P1NP	ucOC	bCTx	P1NP
Case 1	55	10	57.0	48.30	3.606	0.424	62.25	−89.21	8.02	−23.94
Case 2	55	8	43.0	41.90	0.719	0.392	46.84	−33.10	−47.19	−12.40
Case 3	58	13	43.0	43.52	2.477	0.445	70.77	−43.68	−14.83	−11.46
Case 4	59	14	45.5	68.99	3.313	0.371	44.84	−70.87	9.97	1.54
Case 5	78	30	42.0	47.78	1.664	0.357	48.32	[a]	[a]	[a]
Mean	61	15	46.1	50.10	2.356	0.398	54.60	−59.22	−11.01	−11.57
SD	9.67	8.72	6.23	10.91	1.189	0.037	11.35	25.55	26.62	10.42

[a] Dropped out at 3 months due to loss to contact

reported to be associated with an increased incidence of femoral neck fracture [6, 9]. In this recent study, mean baseline level of ucOC before initiated MK-4 treatment was 2.91 ± 2.07 ng/ml (mean ± SD) and range from 0.11 to 9.86 ng/ml (Table 1), which is higher than the cutoff value of Thai premenopausal women purpose by Bunyaratavej et al. [11]. In Japanese study, Shiraki et al. [32] also purpose that the level of 2.6 ng/ml or more of serum ucOC is the high ucOC cutoff value for patients treated with bisphosphonates. Therefore, in average of postmenopausal Thai women may have some inadequacy of vitamin K intake. However, in comparisons between Japanese postmenopausal women [34] and postmenopausal Thai women, the level of ucOC in postmenopausal Thai women was still lower. Unfortunately, there have been neither studies about the direct measurement of serum vitamin K1 or K2 level in the Thai population nor any studies about vitamin K content in Thai food. Previous studies have shown that menatetrenone treatment reduces the serum level of ucOC [8, 27, 33, 34]. A study by Tsugawa et al. [35] revealed that the vitamin K requirement for gamma-carboxylation of osteocalcin was greater in older postmenopausal women than in younger women. In this present study, we not only evaluated the effect of menatetrenone on ucOC, we also evaluated the effect of gene *GGCX*, which encodes for the enzyme gamma-glutamyl carboxylase (GGCX) in response to menatetrenone. This enzyme is essential for conversion of glutamate (Glu) residues in ucOC into gamma-carboxyglutamic acid (Gla) residues, and become fully carboxylated osteocalcin or mature osteocalcin (OC). This enzyme is VKD which needs vitamin K as a co-factor in its metabolism. One of the essential SNP of this *GGCX* gene has been previously elucidated to correlate with BMD [15]. The mutation in exon 8 in the position *rs699664* (Arg325Gln) is our interested outcome. Amino acid substitution of 325 residue (Arg/Gln) may affect enzymatic activity directly. People with 325-Gln (AA) may have higher activity of carboxylation of these VKD proteins with the given status of vitamin K [15], considering that carboxylase activity of 325-Gln (AA) enzyme was higher than that of 325-Arg (GG) in vitro.

For the primary outcome of this study, we have demonstrated that treatment with a combination of menatetrenone (MK-4) together with calcium and vitamin D3 supplement can dramatically reduce serum level of ucOC in the overall groups to about 61.7% from baseline and to about 54.2 and 69.9% in GG and GA + AA genotypes, respectively. In this recent study, there was no control group since this was addressed in a previous study of postmenopausal Thai women by Bunyaratavej et al. [36], which showed that postmenopausal Thai women who took MK-4, have reduction in serum ucOC level dramatically compared to the control group which did not take MK-4. There is no statistically significant difference in percent reduction between the GG-group and the GA + AA group. But there is some trend in more reduction in the GA + AA group than in the GG group, as seen in the study by Kinoshita et al. [15]. They revealed that the kinetic study and reaction rate for carboxylation of the 325-Arg enzyme (GG) was about 1.2-fold slower than that of 325-Gln (AA). It turns out that it can be explained that the 325-Gln (AA) enzyme has higher carboxylase activity than 325-Arg (GG) [15].

The distribution of *GGCX* gene is found to be 49.3% for GG genotype (325-Arg), 47.1% for GA genotype (325-Gln/Arg) and only 3.6% for AA genotype (325-Gln). We did a subgroup analysis for those who were 65 years of age or older ($N = 37$). Two patients in this subgroup could not complete the 3-month follow-up due to loss to contact, so only 35 patients were recruited for the analysis. The exploratory analysis showed a statistically significant difference between GG-group and GA + AA-group which unveiled a high activity in the GA + AA group ($p = 0.025$). This is confirming the finding on BMD study by Kinoshita et al. [15]. We need a further study to enroll a population with a higher number of AA genotype to have enough statistical power to demonstrate the difference between the GG group and AA groups. Other effects that may also influence this study are the dietary factors [5]. In this study, we could not control for the diets of our ambulatory patients. There may be some differences in habitual food around different regions in Thailand. The dissimilarities in natural vitamin K content in those foods can affect our results. The variable gastrointestinal absorption capability on vitamin K may also have some influence on the result of the study [5].

When looking into the effect of menatetrenone on bone turnover, we found a significant reduction in serum beta-CTx and serum P1NP from baseline in both groups of genotypes (GG-group and GA + AA-group). These findings were detectable as early as 1 month post-administration though mean percent changes from baseline are not large (14% reduction in both serum beta-CTx and serum P1NP at 3 months) when compared to a previous study of the Thai population [36]. This is not considered to have clinical significance when compared with treatment effect from other osteoporotic medications such as bisphosphonates. To compare the reductions between the two groups of genotypes, there is no significantly difference between the GG-group and the GA + AA group in terms of both serum beta-CTx and serum P1NP. Although menatetrenone is considered to be a dual action drug, the decrease in bone resorption marker serum beta-CTx is the hallmark of anti-resorptive agent, as also demonstrated in previous in vitro studies [1, 2]. The reductions in serum beta-CTx and P1NP did differ from the variation of *GGCX* polymorphism. It is possible that GGCX enzyme does not influence the mechanism of anti-resorption, and one of the bone formation improving mechanisms of menatetrenone is not affected by GGCX enzyme.

Conclusion

Treatment with menatetrenone concurrently with calcium and vitamin D promotes gamma-carboxylation of osteocalcin was followed by a decrease of ucOC. There was no significant difference between groups of the GGCX genotypes in reduction of serum ucOC levels. However in the subgroup analysis of those subjects who are 65 years of age or older, there was a significant difference between the groups that have the “A” allele and the group that do not have it. This study confirmed the findings discussed by Kinoshita et al. [15] on the hypothesis that different activities of GGCX enzyme can cause polymorphism of the GGCX gene, which will further influence the clinical outcomes. It is reported that dietary vitamin K intake decreases with age, and elevated levels of ucOC may result from subclinical vitamin K deficiency and are frequently observed in the elderly [37]. We have no study about the dietary vitamin K intake in the Thai population; thus, we do not know whether there is some difference in vitamin K intake between the younger and the elder age group or not, this is one of our limitations. There are no differences between the decrease of serum beta-CTx and serum P1NP between the genotypes, which may be due to the inadequacy of sample size. This is the other limitation of our study because our sample size is calculated from the ucOC data. However, the evidence still supports the anti-resorptive effect of menatetrenone from the previous studies. The effect of menatetrenone on bone quality is indirectly demonstrated by a decrease in serum ucOC; in other words, increasing mature osteocalcin is believed to be associated with improving quality of bone matrix. Further investigations are needed to clarify the mechanism of actions of menatetrenone on bone remodeling as well as its action under the influence of genetic polymorphism, which may the first step towards pharmacogenomic development.

Acknowledgments We express our deep gratitude to the Thai Osteoporosis Foundation (TOPF) for their strong support and partial funding. We also thank Eisai (Thailand) Marketing for their support on the ucOC kit. Special thanks also go to all the patients who participated in this study to completion.

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Clinical Endocrinology (2012) **77**, 42–46 doi: 10.1111/j.1365-2265.2011.04215.x

ORIGINAL ARTICLE

A reduced serum level of total osteocalcin in men predicts the development of diabetes in a long-term follow-up cohort

Chardpraorn Ngarmukos*, La-or Chailurkit*, Suwanee Chanprasertyothin†, Bunlue Hengprasith‡, Piyamitr Sritara§ and Boonsong Ongphiphadhanakul*

**Division of Endocrinology and Metabolism, Department of Medicine, †Research Center, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, ‡Medical and Health Office, Electricity Generating Authority of Thailand, Nonthaburi and §Division of Cardiology, Department of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand*

Summary

Background Osteocalcin (OC), an osteoblast-specific protein, has been demonstrated to affect glucose metabolism in both animals and humans. Studies in animals have shown an effect of undercarboxylated OC (ucOC) on beta-cell proliferation and insulin resistance. It remains unclear whether OC is associated with the future development of diabetes in humans, as well as the relative importance of ucOC *vs* OC.

Objective The aim of this study was to examine serum OC and its post-translational forms as potential biomarkers for future the development of type 2 diabetes.

Methods This was a nested case–control study using data from the Electricity Generating Authority of Thailand (EGAT). We identified 63 men without diabetes in the exploratory cohort at baseline who developed type 2 diabetes (DM) during the 10-year follow-up period from 1998–2008, and also 63 men age- and BMI-matched for a non-diabetes control group (non-DM). Serum N-mid OC and ucOC were measured in baseline blood samples. Logistic regression models were used to explore and identify baseline factors, including OC and ucOC, that predicted the subsequent development of diabetes.

Results The mean age and BMI were similar in both non-DM and DM groups ($47{\cdot}2 \pm 0{\cdot}5$ *vs* $47{\cdot}8 \pm 0{\cdot}8$ years and $25{\cdot}2 \pm 0{\cdot}5$ *vs* $25{\cdot}9 \pm 0{\cdot}5$ kg/m^2, respectively). Only baseline mean serum N-mid OC ($15{\cdot}2 \pm 0{\cdot}5$ *vs* $13{\cdot}0 \pm 0{\cdot}5$ μg/l, $P < 0{\cdot}05$) and fasting plasma glucose ($4{\cdot}92 \pm 0{\cdot}04$ *vs* $5{\cdot}28 \pm 0{\cdot}07$ mmol/l, $P < 0{\cdot}05$) were significantly different between the two groups. Multiple logistic regression analysis showed that baseline serum N-mid OC and glucose, but not ucOC, were independent risk factors for the development of diabetes in this long-term study cohort.

Conclusions Circulating total OC is associated with incident diabetes in men. Further studies to evaluate the potential utility of OC as a biomarker to predict the development of type 2 diabetes are warranted.

(Received 29 July 2010; returned for revision 3 September 2010; finally revised 26 July 2011; accepted 25 August 2011)

Introduction

Osteocalcin (OC), an osteoblast-specific protein, has been demonstrated recently to have a role in glucose and energy metabolism. It has been shown in experimental animals with OC gene knockout that there is a decrease in pancreatic beta-cell proliferation and an increase in insulin resistance.[1] On the other hand, in a gain of function OC mouse model, an increase in insulin secretion and beta-cell proliferation has been described. The findings suggest an endocrine function of OC, as well as the well-described pro-osteoblastic function in bone.[2] Human studies thus far have supported a similar role of OC in glucose metabolism. For example, cross-sectional studies have demonstrated the association of circulating OC levels with components of the metabolic phenotype, such as fasting plasma glucose (FPG), insulin, homeostasis model assessment (HOMA)-IR and body mass index.[3,4] In patients with type 2 diabetes, OC was inversely related to FPG and haemoglobin A1c levels.[5] In addition, OC was inversely related to parameters reflecting atherosclerosis, including brachial-ankle pulse wave velocity and intima-media thickness in diabetic men (but not in diabetic women).[5]

Although most studies in humans to date have examined the relationship between total OC and the regulation of glucose metabolism, studies in experimental animals have established that it is the secreted form, undercarboxylated OC (ucOC), which is of importance in regulating glucose metabolism.[1,6]

In this longitudinal study, we have followed a cohort of normal men over 10 years and have identified a subgroup who have developed type 2 diabetes. We have compared serum levels of ucOc and total OC at the time of enrolment in the study to address the question of the usefulness of serum total OC and ucOC as potential biomarkers for the future development of diabetes.

Correspondence: Chardpraorn Ngarmukos, Division of Endocrinology and Metabolism, Department of Medicine, Ramathibodi Hospital, Rama 6 Rd., Rajthevi 10400, Bangkok, Thailand. Tel.: +66 8 9776 3062; Fax: +66 2 201 1647; E-mail: tecng@mahidol.ac.th

Materials and methods

This study was a nested case–control study using data from the Electricity Generating Authority of Thailand (EGAT) cohort. The EGAT study is a cohort study that has followed and collected health data of full-time EGAT staff members, aged 35–55 years, since 1985. The main objectives of the EGAT study were to describe the prevalence of major cardiovascular risk factors, the association between cardiovascular risk factors and mortality and changes in cardiovascular risk factors over time in urban Thais. Details and analyses of data from the original cohort have been described.[7,8] Data from the second phase of the EGAT study (EGAT-2), which was conducted between 1998 until 2008, were used in this study. Data and blood samples were collected in 1998 and again in 2008. We identified 63 men without diabetes at baseline who later developed type 2 diabetes during the 10-year follow-up (DM group). For control subjects, data and blood samples of 63 age- and BMI-matched men who did not develop diabetes during the period were retrieved (non-DM group). Subjects were asked whether they regularly exercise or not, but this was not otherwise quantified. The diagnosis of diabetes was based on fasting glucose >7·0 mmol/l as well as diagnosis and/or receipt of diabetes medication during the follow-up period. The study was approved by the Institutional Review Board of Ramathibodi Hospital, Mahidol University.

Plasma glucose levels were analysed by glucose oxidase method (Peridochrome, Boehringer Mannheim, Mannheim, Germany). Serum N-mid OC, which represents both carboxylated and ucOC, were measured in baseline blood samples, which were stored at −80 °C until the time of measurement. Serum OC levels were determined by electrochemiluminescence immunoassay on an Elecsys 2010 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Serum ucOC levels were measured by enzyme immunoassay (Takara Bio Inc., Shiga, Japan). The intra-assay coefficients of variation for OC and ucOC were 1·4% and 5·2%, respectively, for samples within the measured range.

Normality of data was analysed using the Kolmogorov–Smirnov test. Non-normally distributed variables were log-transformed before a parametric test. Comparison between the two groups was made by Student's *t*-test. Pearson's correlation coefficient was used to assess the relationship between two variables. Multiple logistic regression analysis was performed to determine independent factors associated with incident diabetes. *P* value <0·05 was considered statistically significant. All statistical analyses were performed using SPSS Statistics version 18·0. Data were presented as mean ± SEM.

Results

Baseline characteristics and OC levels are shown in Table 1. The mean age and BMI were similar among the non-DM and DM groups (47·2 ± 0·5 *vs* 47·8 ± 0·8 years and 25·2 ± 0·5 *vs* 25·9 ± 0·5 kg/m^2, respectively). Subjects in the non-DM group tended to exercise more, but this did not reach statistical significance. Baseline serum OC (15·2 ± 0·5 *vs* 13·0 ± 0·5 μg/l) and FPG levels (4·92 ± 0·04 *vs* 5·28 ± 0·07 mmol/l) differed between the two groups. No difference in ucOC or the ucOC to OC ratio was found.

Table 1. Characteristics of the subjects at baseline (mean ± SE)

	Non-diabetes (n = 63)	Diabetes* (n = 63)	P-value
Age (year)	47·2 ± 0·5	47·8 ± 0·8	NS
BMI (kg/m^2)	25·2 ± 0·5	25·9 ± 0·5	NS
Waist circ. (cm)	87·4 ± 1·9	90·5 ± 1·9	NS
Regular exercise	29 (46·0%)	20 (31·7%)	NS
FPG (mmol/l)	4·92 ± 0·04	5·28 ± 0·07	<0·05
OC (μg/l)	15·16 ± 0·49	13·04 ± 0·48	<0·01
ucOC (μg/l)	1·51 ± 0·14	1·10 ± 0·11	NS
ucOC to OC ratio	0·10 ± 0·01	0·09 ± 0·01	NS

FPG, fasting plasma glucose; OC, osteocalcin; ucOC, undercarboxylated OC.
*Subjects who did not have diabetes but developed diabetes during the follow-up period.

As ucOC was not normally distributed, ucOC was log-transformed before correlation analyses. There was a positive correlation between OC and ucOC (r = 0·43, P < 0·001) (Fig. 1). For the relationship of OC, ucOC or ucOC/OC ratio with regard to FPG, it was found that only OC was negatively correlated with baseline FPG (r = −0·27, P < 0·001) (Fig. 2). Neither ucOC nor the ucOC/OC ratio was related to FPG.

Multiple logistic regression models – which included OC, ucOC or ucOC/OC ratio adjusted for baseline FPG, age, body mass index and exercise – were used to identify independent risk factors for the future development of diabetes. As shown in Table 2, baseline serum OC and FPG, but not ucOC or the ucOC/OC ratio, were independent risk factors for the development of diabetes in this long-term study cohort.

Discussion

This study has established that total serum OC, but not ucOC, is significantly lower at baseline in men who subsequently develop

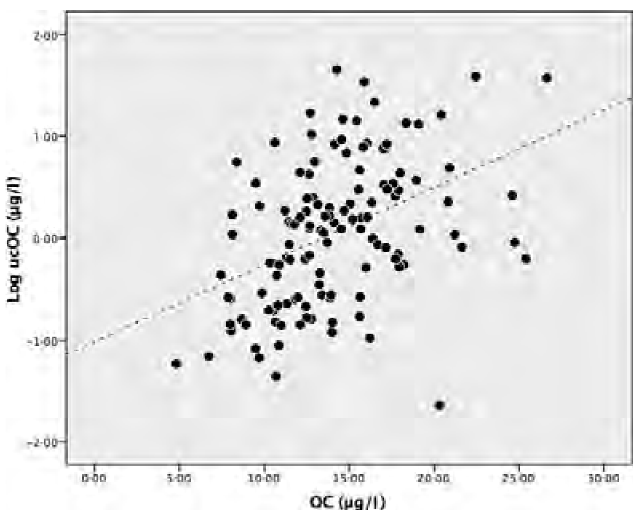

Fig. 1 Relationship between osteocalcin (OC) and undercarboxylated OC (ucOC) by Pearson's correlation. OC was positively correlated with ucOC (r = 0·4, P < 0·001).

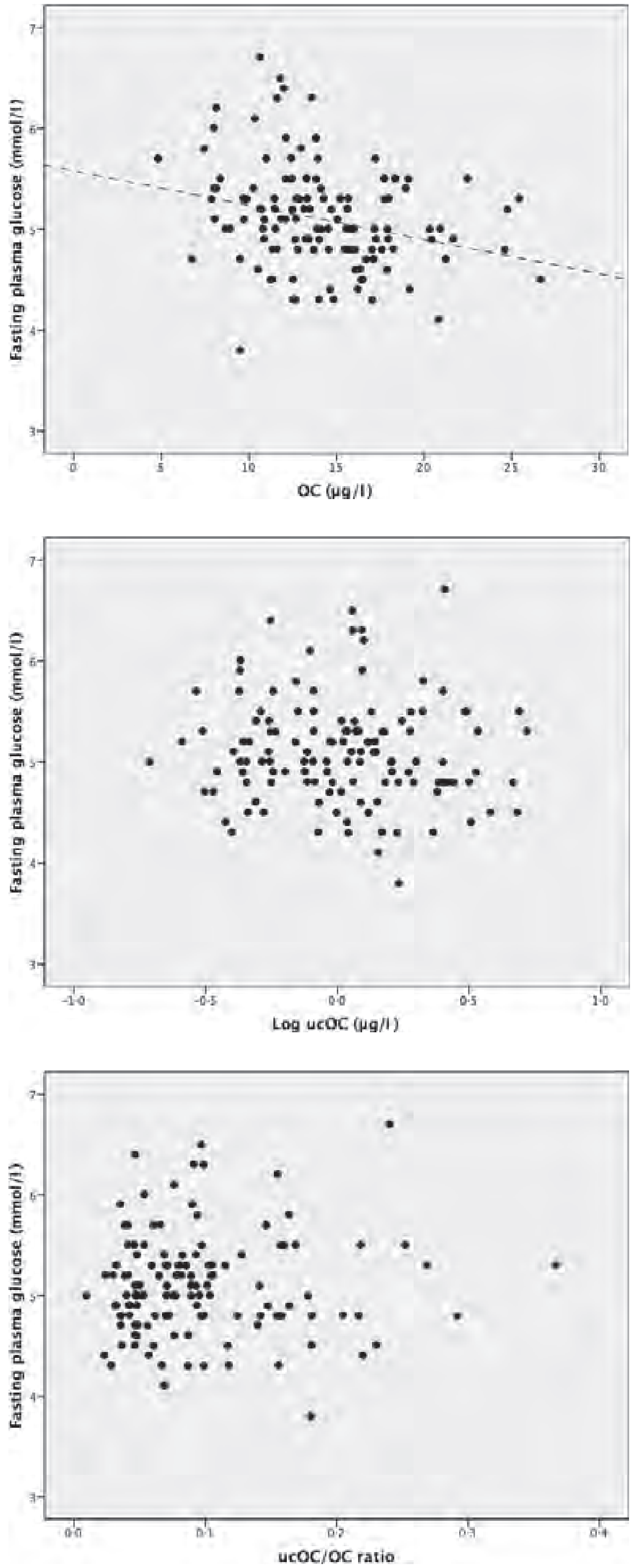

Fig. 2 Relationship between osteocalcin (OC), log undercarboxylated OC (ucOC), ucOC/OC ratio and baseline fasting plasma glucose (FPG) by Pearson's correlation. OC was negatively related to baseline FPG ($r = -0{\cdot}27$, $P < 0{\cdot}001$). No correlation between log ucOC ($r = -0{\cdot}07$, NS) or ucOC/OC ratio to FPG ($r = 0{\cdot}04$, NS) was found.

type 2 diabetes. This is in keeping with a shorter longitudinal study by Pittas *et al.*[3] found that serum OC predicted changes in FPG after 3 years and with a number of cross-sectional studies that have shown an inverse relationship between serum OC and serum glucose levels and various metabolic parameters in elderly, children, men, postmenopausal women and people with diabetes.[3,4,9–13]

Whether the relationship between lower OC and the development of diabetes is causal remains unclear as the relationship between OC and diabetes tends to be reciprocal in nature. A number of studies in patients with diabetes have shown that lower serum OC increases after the amelioration of hyperglycaemia.[14,15] Furthermore, OC and reduced diabetes risk are covariates that are correlated with protective factors for diabetes in these patients. In a study of women with gestational diabetes, serum OC was found to be associated with an increase in insulin secretion.[16] Exercise and weight loss, which help prevent diabetes, also increased serum OC, further implicating a pathogenic role.[17] In experimental studies, administration of OC to animals improves glucose tolerance.[1,6] Although similar studies in humans are lacking, administration of calcitriol, which increases OC, improves glucose intolerance and dyslipidaemia in patients receiving dialysis.[18]

It is of note that studies with regard to OC and energy metabolism in experimental animals have demonstrated an effect with ucOC but not with total OC, suggesting that ucOC is more likely to be the pathogenic molecule. In keeping with this, a recent cross-sectional study by Kanazawa *et al.*[19] showed that ucOC was inversely associated with plasma glucose and fat mass in type 2 diabetes. Similarly, in a study of middle-aged male subjects, ucOC in the upper tertile was associated with improved glucose tolerance and enhanced beta-cell function as assessed by the HOMA index.[20] A higher ucOc to OC ratio was also found to be associated with higher HOMA-beta in lean children, and a higher adiponectin level in heavier healthy children.[11]

Despite this, in this study, we could not demonstrate a relationship of ucOC, only of total OC, on subsequent development of diabetes. This is consistent with a similar study of 348 non-diabetic subjects by Shea *et al.*, who found an association of total OC concentration, but not ucOC, with insulin resistance at baseline and at 3 year follow-up.[10] The reason for the discrepancy is unclear, but it could have arisen from the difference in study design, small number of subjects in our study as well as the systematic error associated with the ucOC assay. In our study, we did not use a hydroxyapatite binding assay that might have avoided the tendency to overestimate fragments of OC.[21] As this was a nested case–control study, we only selected subjects from the cohort with complete information and who were matched for age and BMI. However, the small number of subjects in each group might not have provided enough analytical power to reveal a clear association between OC, and especially ucOC, and the development of diabetes. Furthermore, factors that can influence bone metabolism or OC levels – such as vitamins K and D, use of bisphosphonates or glucocorticoids, history of fracture and information regarding physical activity and exercise which can increase OC levels[22] – were not included in our study. These important variables unfortunately were not completely collected in our initial survey or in our follow-up survey where the main objective was to examine the prevalence of cardiovascular risk factors.

Table 2. Odds ratio (OR) and 95% confidence interval (CI) of incident diabetes per one unit change in OC, ucOC or ucOC/OC ratio adjusted for baseline FPG, age and BMI from multiple logistic regression analysis

	Model 1		Model 2		Model 3	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Baseline FPG (mmol/l)	1·09 (1·04–1·15)	<0·01	1·10 (1·04–1·16)	<0·001	1·10 (1·05–1·16)	<0·001
Age (year)	0·99 (0·91–1·07)	NS	0·99 (0·91–1·07)	NS	0·99 (0·91–1·07)	NS
BMI (kg/m^2)	1·01 (0·91–1·13)	NS	1·03 (0·92–1·15)	NS	1·02 (0·91–1·13)	NS
Regular exercise	0·46 (0·20–1·04)	NS	0·46 (0·21–1·04)	NS	0·46 (0·21–1·03)	NS
OC (μg/l)	0·90 (0·81–0·99)	<0·05	–	–	–	–
ucOC (μg/l)	–	–	0·71 (0·47–1·06)	NS	–	–
ucOC/OC ratio	–	–	–	–	0·07 (0–34·4)	NS

FPG, fasting plasma glucose; OC, osteocalcin; ucOC, undercarboxylated OC.
Model 1, OC adjusted for baseline FPG, age, BMI and exercise.
Model 2, ucOC adjusted for baseline FPG, age, BMI and exercise.
Model 3, ucOC to OC ratio adjusted for baseline FPG, age, BMI and exercise.

In addition to the proposed effect of OC on beta-cell proliferation and insulin secretion, animal studies have also suggested that OC deficiency is associated with visceral obesity, which might also increase the risk of diabetes.[1] Within the limits of the size of our study, we did not find any relationship between BMI or waist diameter and serum OC and ucOC.

In conclusion, this study has indicated that a reduced circulating total serum OC, but not serum ucOC, is associated with the subsequent development of diabetes in men. We suggest that this is a further indication of the important role of OC in the regulation of glucose metabolism and that further studies are warranted to evaluate the potential utility of OC as a biomarker to predict the development of diabetes.

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Vitamin D Insufficiency and Deficiency among HIV-1-Infected Patients in a Tropical Setting

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Journal of the International Association of Physicians in AIDS Care (JIAPAC 2012 11: 305 originally published online 13 January 2012

DOI: 10.1177/1545109711432142

Journal of the International Association of Physicians in AIDS Care
11(5) 305-310

DOI: 10.1177/1545109711432142
http://jiapac.sagepub.com
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Vitamin D Insufficiency and Deficiency among HIV-1-Infected Patients in a Tropical Setting

Surasak Wiboonchutikul, MD[1], Somnuek Sungkanuparph, MD[1], Sasisopin Kiertiburanakul, MD, MHS[1], La-or Chailurkit, PhD[1], Angkana Charoenyingwattana, PharmD[2], Wittaya Wangsomboonsiri, MD[3], Wasun Chantratita, PhD[1], and Boonsong Ongphiphadhanakul, MD[1]

Abstract

Vitamin D plays role in bone health and the regulation of the immune system. A cross-sectional study of serum 25-hydroxyvitamin D (25[OH]D) levels was conducted among HIV-1-infected Thai patients to determine the prevalence and associated factors of low vitamin D levels (25[OH]D <30 ng/mL) in tropical setting. 25-Hydroxyvitamin D was measured by liquid chromatography/tandem mass spectrometry. Of 178 patients, 58% received antiretroviral therapy at median (interquartile range [IQR]) duration of 7.4 (5.9-8.5) years. The prevalence of 25(OH)D deficiency (<20 ng/mL) and insufficiency (20-29.9 ng/mL) was 26.8% and 44.9%, respectively. Multivariate analysis showed that receiving efavirenz (EFV) was significantly associated with low vitamin D status (odds ratio = 3.60; 95% confidence interval, 1.06-12.15, $P < .05$). The mean ($\pm$standard deviation) level of 25(OH)D in patients receiving and not receiving EFV was 22.9 (6.6) and 28.6 (10.7) ng/mL, respectively, ($P < .05$). Low vitamin D status is common and needs to be assessed among HIV-infected patients including tropical residents especially when EFV is used.

Keywords

HIV-1, vitamin D, deficiency, insufficiency, tropical setting

Introduction

Vitamin D is essential for calcium and bone homeostasis. The discovery of vitamin D receptors in many organs of the body has led to a new perspective of vitamin D function.[1–3] In the past decade, increasing evidences suggest that vitamin D plays an important role in regulating the immune system.[4–7] In vitro data demonstrated that HIV-1 replication is inhibited by cathelicidin, an antimicrobial peptide that can be activated in macrophages and monocyte by active vitamin D.[8,9] Vitamin D can also regulate the cytokine release from adaptive immunity that involves tropism-specific HIV-1 co-receptor expression.[10] Recent data have demonstrated both higher risk of HIV-1 disease progression and higher mortality in HIV-1-infected pregnant women with low vitamin D status.[11]

25-Hydroxyvitamin D (25[OH]D) is currently considered the best indicator of overall vitamin D status.[1,12,13] Parathyroid hormone levels tend to rise when 25(OH)D is less than 30 ng/mL and active calcium absorption is optimal when the level of 25(OH)D is at least 30 ng/mL.[14,15] Therefore, the level of 30 ng/mL or greater is currently considered as the optimal value for calcium homeostasis and healthy bone metabolism. Nowadays, the level of 25(OH)D less than 20 ng/mL is considered deficiency of vitamin D, and the level of 20 to 29 ng/mL indicates a relative insufficiency of vitamin D. The level of 30 ng/mL or greater indicates sufficient vitamin D.[1]

Low vitamin D status in HIV-1-infected patients has been described in many studies.[16–21] However, most of them were conducted in European countries or the United States. Although ethnicity and sun exposure are important factors associated with vitamin D status,[22,23] limited data with regard to vitamin D status in HIV-1-infected patients are available in Asians and populations residing in the tropics. It is therefore the purpose of the present study to assess vitamin D status among HIV-1-infected patients in a tropical setting and to determine factors associated with low vitamin D levels.

[1] Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
[2] Pharmacogenomics Project Under Collaboration Between Thailand Center of Excellence for Life Sciences, Mahidol University, Bangkok, Thailand
[3] Sawanpracharak Hospital, Nakornsawan, Thailand

Corresponding Author:
Somnuek Sungkanuparph, Division of Infectious Diseases, Department of Medicine Ramathibodi Hospital, 270 Rama 6 Road, Bangkok, 10400, Thailand
Email: somnuek.sun@mahidol.ac.th

Methods

Patients

A cross-sectional study was conducted among HIV-1-infected patients visiting HIV clinic at a university hospital in Bangkok, Thailand. Participants were HIV-1-infected patients aged more than 15 years. For naive patients, we enrolled consecutive cases during the study period. Patients were excluded if they had (1) active granulomatous diseases, (2) liver cirrhosis, (3) chronic kidney disease stages 4 and 5, (4) malignancies, (5) pregnancy, (6) vitamin D supplement, or (7) treatment of low bone mineral density (BMD). Demographic, clinical, history of HIV-1 infection, treatment, and laboratory data were retrieved from medical records. Behavioral data were retrieved from questionnaire. Bone mineral density assessment was performed, and blood samples were collected for measurement of 25(OH)D at the time of enrollment. All participants provided written informed consent. The study was reviewed and approved by the local Institution Review Board.

Measurement of 25(OH)D

Serum 25(OH)D was analyzed by liquid chromatography/tandem mass spectrometry (LC-MS)/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP, 5500 tandem mass spectrometer (AB SCIEX, Foster City, California) using a MassChrom, 25-OH-Vitamin D3/D2 diagnostics kit (Chromsystems, Munich, Germany). The inter-assay and intra-assay coefficients of variation of total serum 25(OH)D level were 6.3% and 5.0%, respectively.

A level of less than 20 ng/mL was defined as vitamin D deficiency. Levels between 20 and 29.9 ng/mL were defined as vitamin D insufficiency. Low vitamin D status referred to a level less than 30 ng/mL (vitamin D deficiency or vitamin D insufficiency).

Bone Mineral Density Assessment

Whole-body dual-energy X-ray absorptiometry (DEXA) scans (Hologic Discovery A, version 12.6.1; Hologic) were conducted by radiologists. Scans were performed to assess BMD using T score of lumbar spine and neck of femur. The World Health Organization classification was used for diagnosis purposes. A T score between -1 and -2.5 standard deviation (SD) were defined as osteopenia, and a T score less than -2.5 SD was defined as osteoporosis. Low BMD referred to osteopenia or osteoporosis.

Statistics

Mean ($\pm$SD), median (interquartile range [IQR]), and frequencies (percentage) were used to show the patients' characteristics. Study patients were categorized into 2 groups based on vitamin D status: low vitamin D status group and normal vitamin D status group. The mean values of continuous variables with normal distribution between the 2 groups were compared by Student *t* test. Mann-Whitney *U* test was used to compare the median values of continuous variables with non-normal distribution. Categorical variables were compared by the chi-square test and Fisher exact test as appropriate. Univariate analysis was used to define the factors associated with low vitamin D status. Variables with a $P < .2$ in univariate analysis were included in the multiple logistic regression model. We used linear regression analysis to define the factors associated with 25(OH)D level. All statistical analyses were performed using SPSS software, version 14.0 (SPSS Inc, Chicago, Illinois). $P < .05$ was considered statistically significant.

Results

There were 178 patients with mean ($\pm$SD) age of 42.1 ($\pm$8.5) years and 47.2% of patients were males. Median (IQR) CD4 was 445 (333-524) cells/mm^3. Median (IQR) duration of known HIV-1 infection was 6.3 (1.3-9.4) years. Of all, 58% of patients had received antiretroviral therapy (ART) at a median (IQR) duration of 7.4 (5.9-8.5) years and nonnucleoside reverse transcriptase inhibitor (NNRTI)-based ART was the most common regimen (87.5%; Table 1). The prevalence of vitamin D deficiency (25[OH]D <20 ng/mL), insufficiency (25[OH]D = 20-29.9 ng/mL), and low vitamin D status (25[OH]D <30 ng/mL) were 26.8%, 44.9%, and 70.7%, respectively. The mean ($\pm$SD) level of 25(OH)D of patients who were naive to ART was 25.0 ($\pm$7.8) ng/mL, whereas level of those receiving ART was 26.9 ($\pm$9.9) ng/mL ($P = .154$). There was no statistically significant difference ($P > .05$) in the prevalence of vitamin D deficiency, insufficiency, and low vitamin D status between the HIV-1-infected patients who were naive or receiving ART.

When the patients were categorized into 2 groups, 126 patients had low vitamin D status and 52 patients had normal vitamin D status. No statistically significant difference ($P > .05$) with regard to age, gender, body mass index (BMI), CD4 count, duration of known HIV-1 infection, duration of receiving ART, backbone ART regimens, BMD status, creatinine clearance, lipodystrophy, hepatitis coinfection, and behavioral data at the time of enrollment between the 2 groups (Table 1). In univariate analysis, taking efavirenz (EFV)-based regimen, duration of known HIV-1 infection, BMD at lumbar spine, creatinine clearance, and lipodystrophy were the factors that had $P < .2$ (Table 2). These factors together with the traditional factors associated with vitamin D level (age, gender, and BMI) were considered candidates for the multivariate model to determine the factors associated with low vitamin D status. In multivariate analysis, only taking EFV-based regimen was significantly associated with the occurrence of low vitamin D status (odds ratio 3.60; 95% confidence interval, 1.06-12.15; $P = .040$; Table 2).

When we used multiple linear regression model with the same candidate factors as in multiple logistic regression analysis, we found that only taking EFV-based regimen was

Table 1. Baseline Characteristics of 178 HIV-Infected Participants

Variables	Low Vitamin D Status (n = 126)	Normal Vitamin D Status (n = 52)	*P* Value
Gender, number (%)			.417
Male	57 (45.2)	21 (51.9)	
Female	69 (54.8)	25 (48.6)	
Age, years, mean ±SD	41.6 ± 8.6	43.4 ± 8.3	.207
BMI, kg/m^2, mean ±SD	22.2 ± 3.9	21.7 ± 3.6	.444
Diabetes mellitus, number (%)	4 (3.2)	3 (5.8)	.418
Hypertension, number (%)	7 (5.6)	3 (5.8)	.955
Dyslipidemia, number (%)	22 (17.5)	12 (23.1)	.386
Current smoking, number (%)	26 (19.8)	9 (17.3)	.696
Current alcohol drinking, number (%)	25 (19.8)	12 (23.1)	.626
HBV coinfection, number (%)	10 (9.2)	4 (8.9)	.955
HCV coinfection, number (%)	8 (9.6)	5 (13.5)	.528
Duration of known HIV infection, years, median (IQR)	6.0 (0.7-8.9)	7.7 (4.7-10.3)	.069
Receiving ART, number (%)	72 (57.1)	32 (61.5)	.588
ART regimens, number (%)[a]			
NVP-based	40 (55.6)	21 (65.6)	.336
EFV-based	26 (36.1)	4 (12.5)	.014
PI-based	5 (6.9)	7 (21.9)	.028
ART backbone, number (%)[a]			
ZDV/3TC	31 (43.1)	15 (46.9)	.717
d4T/3TC	11 (15.3)	6 (18.8)	.659
TDF/3TC	27 (37.5)	11 (34.4)	.760
Duration of ART, years, median (IQR)[a]	7.3 (5.9-8.3)	7.8 (6.0-8.8)	.211
Undetectable HIV RNA, number (%)[a]	70 (97.2)	31 (96.9)	.371
CD4 count, cell/mm^3, median (IQR)	446 (287-643)	465 (338-411)	.789
Lipodystrophy, number (%)[a]	45 (62.5)	26 (81.2)	.058
BMD, T score, mean ±SD			
Lumbar spine	–0.2 ± 1.0	–0.5 ± 1.0	.105
Neck of femur	–0.5 ± 1.0	–0.6 ± 1.0	.458
Low BMD (T score <−1), number (%)	47 (39.2)	24 (46.2)	.393
Osteoporosis (T score <−2.5), number (%)	3 (2.5)	3 (5.8)	.282
Creatinine clearance, mL/min, mean ±SD[b]	87.8 ± 24.0	82.3 ± 25.8	.203

Abbreviations: BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; IQR, interquartile range; ART, antiretroviral therapy; NVP, nevirapine; EFV, efavirenz; PI, protease inhibitor; ZDV, zidovudine; 3TC, lamivudine; d4T, stavudine; TDF, tenofovir; BMD, bone mineral density; SD, standard deviation.
[a]Only ART-receiving patients.
[b]By Cockcroft-Gault formula.

independently associated with lower levels of 25(OH)D (coefficient = −5.36; standard error 2.19; *P* = .015; Table 3). The mean (±SD) level of 25(OH)D in patients taking EFV-based regimen was 22.9 (±6.6) ng/mL, while in those not taking EFV-based regimen, it was 28.6 (±10.7) ng/mL (*P* = .007; Figure 1). There was no correlation between vitamin D level and BMD at lumbar spine and femoral neck (*P* = .142 and *P* = .539, respectively.

Discussion

The results from the present study have demonstrated that vitamin D deficiency or insufficiency was highly prevalent among HIV-1-infected adults even in a tropical country such as Thailand. Comparing the general population in Thailand,[24-27] our HIV-1-infected patients had a similar prevalence of vitamin D deficiency or insufficiency. Among our HIV-1-infected patients in the present study, no difference in the prevalence of low vitamin D status was observed between patient naive to ART or receiving ART. In a multivariate analysis, taking EFV-based regimen was found to be the only associated factor of low vitamin D status. In contrast, we did not find an association between taking nevirapine (NVP)-based regimen and low vitamin D status although NVP is also an NNRTI. For protease inhibitor (PI)-based regimen, we observed a lower risk of low vitamin D status only in univariate but not in multivariate analyses. The mean level of 25(OH)D was lower in the patients who took EFV-based regimen as compared to those who were on non-EFV-based regimen.

To our knowledge, the present study is the first report evaluating prevalence and associated factors of low vitamin D status in HIV-1-infected men or women, naive or previously exposed to ART in a tropical setting. We found that the prevalence of vitamin D deficiency or insufficiency in the present study is much higher than a recent report from Brazil[28] but comparable to a study from the United States[21] when using the same definition (25[OH]D <30 ng/mL). A number of studies from European countries have also demonstrated high

Table 2. Univariate and Multivariate Analyses of Factors Associated with Low Vitamin D Status

Factors	Crude OR (95% CI)	*P* Value	Adjusted OR (95% CI)	*P* Value
Female gender	1.31 (0.68-2.50)	.417	1.68 (0.63-4.53)	.303
Age	0.98 (0.94-1.01)	.211	0.99 (0.92-1.06)	.730
BMI	1.03 (0.95-1.13)	.453	1.09 (0.91-1.30)	.329
Diabetes mellitus	0.54 (0.17-2.48)	.425	–	–
Hypertension	0.96 (0.24-3.87)	.955	–	–
Dyslipidemia	0.71 (0.32-1.56)	.387	–	–
Current smoking	1.18 (0.51-2.74)	.696	–	–
Current alcohol drinking	0.83 (0.38-1.70)	.629	–	–
HBV coinfection	1.04 (0.31-3.49)	.955	–	–
HCV coinfection	0.68 (0.21-2.25)	.530	–	–
Duration of known HIV infection	0.94 (0.87-1.01)	.067	0.96 (0.89-1.03)	.291
Receiving ART	0.83 (0.43-1.61)	.589	–	–
ART regimens[a]				
NVP-based	0.66 (0.28-1.56)	.337	–	–
EFV-based	3.96 (1.25-12.53)	.019	3.60 (1.06-12.15)	.040
PI-based	0.27 (0.08-0.92)	.036	–	–
ART backbone[a], number (%)				
ZDV/3TC	0.86 (0.37-1.98)	.717	–	–
d4T/3TC	0.78 (0.26-1.34)	.659	–	–
TDF/3TC	1.15 (0.48-2.74)	.760	–	–
Duration of ART[a]	0.93 (0.76-1.15)	.502	–	–
Undetectable HIV RNA[a]	1.13 (0.10-12.92)	.922	–	–
CD4 count	1.00 (0.99-1.01)	.892	–	–
Lipodystrophy[a]	0.39 (0.64-1.05)	.063	0.56 (0.18-1.73)	.313
BMD				
Lumbar spine	1.29 (0.94-1.76)	0.110	1.24 (0.73-2.10)	.432
Neck of femur	1.14 (0.82-1.58)	0.440	–	–
Low BMD (T score <−1)	0.75 (0.39-1.45)	0.393	–	–
Osteoporosis (T score <−2.5)	0.42 (0.08-2.15)	0.297	–	–
Creatinine clearance	1.01 (0.99-1.02)	0.190	1.01 (0.98-1.04)	.575

Abbreviations: BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; ART, antiretroviral therapy; NVP, nevirapine; EFV, efavirenz; PI, protease inhibitor; ZDV, zidovudine; d4T, stavudine; TDF, tenofovir; BMD, bone mineral density.
[a] Only ART-receiving patients.

Table 3. Linear Regression Analysis of Factors Associated with 25(OH)D Levels

	Simple Linear Regression			Multiple Linear Regression		
Factors	Coefficient	Standard Error	*P* Value	Coefficient	Standard Error	*P* Value
Female gender	–0.49	1.38	.725	–2.07	1.99	.302
Age	0.11	0.08	.171	0.13	0.15	.356
Body mass index	0.02	0.18	.895	–0.31	0.27	.244
Duration of known HIV infection	0.18	0.11	.082	0.03	0.15	.836
Receiving EFV	–5.72	2.10	.007	–5.36	2.19	.016
BMD at lumbar spine	–0.97	0.66	.142	–0.45	1.05	.667

Abbreviations: BMD, bone mineral density; EFV, efavirenz.

prevalence of vitamin D deficiency or insufficiency.[16,18,20] These results suggest that inadequate vitamin D status in HIV-1-infected patients is likely to be common regardless of geographic locations or ethnicity. Awareness and monitoring of vitamin D deficiency are warranted when providing long-term care for HIV-1-infected patients.

We found no difference in vitamin D status between HIV-1-infected patients who were naive or exposed to ART. This finding is consistent with studies from Bang et al[18] and Van Den Bout-Van Den Beukel et al.[16] In contrast, some cross-sectional studies have found ART to be associated with lower vitamin D level.[19,21] Efavirenz (EFV) use was found to be the only factor associated with low vitamin D status. It is currently believed that EFV causes lower 25(OH)D by inducing 24 hydroxylase which hydrolyzes 25(OH)D and $1,25(OH)_2D$ to their inactivate form.[29,30] We did not observe this association in NVP users. This finding is consistent with 2 previous reports from Western countries.[19,21] Taken together, this

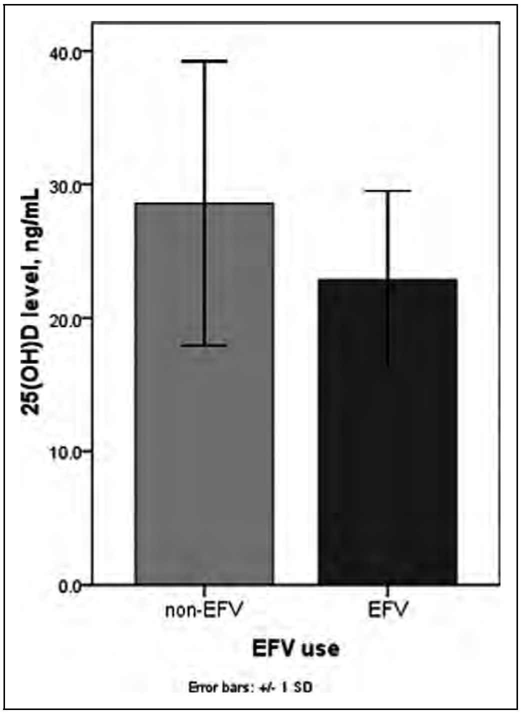

Figure 1. Mean (±standard deviation) level of 25(OH)D between patients taking efavirenz (EFV) and those not taking EFV.

suggests that low vitamin D is not an NNRTI class phenomenon but more likely a specific effect of EFV.

There are some limitations in this study. Due to the cross-sectional nature of the study, we cannot elucidate the causal relationship between HIV-1 infection and vitamin D levels or vice versa. The small number of patients taking PI-based regimen may not have enough statistical power to demonstrate the association of PI use and low vitamin D status. We did not have information on occupation or daily activity related to sun exposure. Since our hospital is located in the middle part of Bangkok, a metropolitan city, our patients can represent tropical HIV-1-infected patients in urban setting.

In conclusion, vitamin D deficiency and insufficiency are highly prevalent among HIV-1-infected patients even in a tropical country such as Thailand. There was no difference in low vitamin D status between patients who were naive or exposed to ART. Use of EFV, which can induce CYP450 enzymes involved in vitamin D metabolism, is significantly associated with low vitamin D status. This suggests that vitamin D status may need to be assessed in HIV-1-infected patients including tropical residents especially when EFV is used. Further studies to define the effect of ART on the metabolism of vitamin D, impact of vitamin D on HIV-1 as well as benefits of vitamin D supplement in HIV-infected patients are needed.

Authors' Note

Abstract of this study was presented in the Sixth IAS Conference on the HIV Pathogenesis, Treatment, and Prevention, Rome, July 17-20, 2011.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Research grant of Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

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The Journal of Nutrition, Health & Aging©
Volume 16, Number 3, 2012

MALIC ENZYME GENE POLYMORPHISM IS ASSOCIATED WITH RESPONSIVENESS IN CIRCULATING PARATHYROID HORMONE AFTER LONG-TERM CALCIUM SUPPLEMENTATION

L. CHAILURKIT[1], S. CHANPRASERTYOTHIN[2], S. CHAROENKIATKUL[3], N. KRISNAMARA[4], R. RAJATANAVIN[1], B. ONGPHIPHADHANAKUL[1]

1. Department of Mecidine, Ramathibodi Hospital, Mahidol University, Thailand; 2. Research Center, Ramathibodi Hospital, Mahidol University, Thailand; 3. Institute of Nutrition, Mahidol University, Nakhon Pathom, Thailand; 4. Department of Physiology, Faculty of science, Mahidol University, Thailand. Correspondence author: La-or Chailurkit, Ph.D. Division of Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine, Ramathibiodi Hospital, Rama 6 Rd., Rajthevi, Bangkok 10400, Thailand, Phone: 66-2-2012810, Fax: 66-2-2011715 Email: ralcl@mahidol.ac.th

Abstract: *Objective:* To identify genetic variations associated with parathyroid hormone (PTH) suppression after long-term calcium supplementation. *Design and Participants:* For high throughput SNP screening, subjects consisted of 171 postmenopausal women without osteoporosis at the lumbar spine. A separate group of 19 premenpausal women were recruited for calcium absorption study. Postmenopausal women in the screening group were given 500 mg/day calcium supplementation. *Setting:* Bangkok, Thailand. *Measurements:* Parathyroid hormone (PTH) and bone mineral density (BMD) were measured at baseline and 2 years after calcium supplementation. High throughput single-nucleotide polymorphism (SNP) screening was performed by comparing estimated allele frequencies derived from hybridization signal intensities of pooled DNA samples on Affymetrix's 10K SNP genotyping microarrays based responsiveness in PTH after calcium supplementation. Genotyping of SNP rs1112482 in malic enzyme gene (ME1) gene, a SNP among those with highest odds ratio of being related to PTH suppression after calcium, was performed in all postmenopausal subjects in the screening group and premenopausal women in the calcium absorption study group in which fractional calcium absorption was assessed by stable isotope dilution. Data were expressed as mean +/- SEM. *Results:* PTH significantly decreased after 2 years of calcium supplementation (4.7 ± 1.9 vs. 4.4 ± 1.6 pmol/L, $P < 0.01$). There was a significant increase in lumbar spine BMD (1.03 ± 0.01 vs. 1.01 ± 0.01 g/cm^2, $P < 0.001$) but not femoral neck BMD. In 108 subjects whose PTH levels decreased after calcium, the suppression of PTH was higher in those with at least one C allele in rs1112482 of ME1 gene (-26.3 ± 2.1 vs. -16.9 ± 1.4%, $P < 0.001$). Fractional calcium absorption also tends to the higher in subjects in the calcium absorption study group with at least one C allele (n = 6) compared to those without the C allele (n = 13) (58.0 ± 4.9 vs. 49.3 ± 2.8%, $P = 0.054$). *Conclusion:* Cytosolic malic enzyme 1 gene polymorphism is associated with the degree of suppression of parathyroid hormone after long-term calcium supplementation. The effect is probably mediated through an increase in intestinal calcium absorption.

Key words: Malic enzyme gene, calcium supplementation, calcium absorption, bone mineral density, parathyroid hormone.

Introduction

It is well established that osteoporosis is partially genetically determined (1). Since bone strength is the result of multiple interacting biological pathways, genes encoding proteins along these pathways all carry potentials as genetic determinants of osteoporosis. Candidate gene approach has at least revealed genes involved in collagen biosynthesis, estrogen action and Wnt signaling as susceptibility genes for osteoporosis (2). The results, however, are still conflicting among different populations. Nevertheless, meta-analyses have confirmed the role of some of these genes although the effect of each gene is relatively minute (3-5)

Calcium is essential for bone health. The beneficial effect of calcium probably results from the attenuation of secondary hyperparathyroidism associated with aging. The effect of calcium supplementation on bone mineral density as demonstrated in clinical trial is minute and can be transient (6, 7). However meta-analysis has shown that calcium supplementation reduces fracture risk only in those with very good adherence (8). It is unclear at present whether genetic factors influence skeletal responsiveness to calcium. It has been shown that genetic variation in vitamin D receptor (VDR) is associated with intestinal calcium absorption (9). However, whether such association will result in improved bone mass or bone strength is unknown. Besides VDR, study of other genes pertaining to skeletal responsiveness to calcium is scarce. It is therefore the purpose of the present study to identify genetic variations associated with the responsiveness to calcium supplementation by using a genome-wide screening of pooled DNA samples.

Received January 26, 2010
Accepted for publication February 2, 2011

Participants and Methods

Microarray screening of SNP associated with responsive to calcium

Subjects

Physically active healthy postmenopausal women over the age of 60 years were recruited by public advertisements. Demographic data such as age, sex, occupation and clinical variables including reproductive history were assessed. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Each woman was screened by medical history and physical examination. Exclusion criteria included history of metastatic or non-osteoporotic metabolic disease, history of kidney stone within previous five years, vertebral fractures as confirmed by radiographs of the thoracolumbar spines at the beginning of the study, thyroid or parathyroid diseases, the use of calcium or vitamin D supplementation within the previous two months, the use of hormone replacement therapy including estrogens, estrogen receptor modulator or testosterone within the previous six months. Subjects taking glucocoticoids, anticonvulsants or fluoride within the previous year and those with lumbar spine (L2-L4) bone mineral density (BMD) T-score less than or equal -2.5 were also excluded. On the basis of these criteria, 197 women were enrolled in the study. They were assigned to receive 500 mg of elemental calcium in the form of calcium carbonate (British dispensary (L.P.) Co., Ltd., Thailand). Subjects were told to avoid other calcium supplementation during the study period. Compliance to calcium supplementation was assessed by tablet counting every 6 months. Fasting blood samples were obtained at baseline and after 2 years of treatment.

Fractional calcium absorption was measured in a separate group of 19 healthy premenopausal women aged 22-42 years recruited from Mahidol University community through interviews and physical examination by a physician. Exclusion criteria included pregnant or lactating, anemia, body mass index (BMI) <18 or >24 Kg/m2 and a history of disorders or medications known to affect calcium absorption. The study protocol was approved by the Institutional Review Board of Mahidol University.

Bone mineral density (BMD)

BMD of lumbar spine (L2 –L4) and femoral neck was measured by dual energy x-ray absorptiometry (Lunar Corp, Madison, Wisconsin) at baseline and 2 years after treatment. Quality assurance was maintained by daily calibration and use of one phantom.

Biochemical Measurement

Serum and plasma samples were kept frozen at –80°C until analysis. Serum calcium, phosphorus and creatinine were analyzed on an automated biochemical analyzer (Dimension RxL, Dadebehring Co Ltd, USA). Plasma intact parathyroid hormone (PTH) was determined by electrochemiluminescence immunoassay on an Elecsys 2010 analyzer (Roche Diagnostic GmbH, Mannheim, Germany) with an intra-assay precision of 3.6 %. Serum 25(OH)D level was measured by liquid chromatography coupled with mass spectrometry (LC-MS/MS) using MassChrom® 25-OH-Vitamin D3/D2 in serum/plasma reagent kit (Chromsystems Instrument & Chemicals GmbH, Munich, Germany) with intra- and inter-assay precision of 5.0 % and 6.3 %, respectively. Estimated glomerular filtration rate (GFR) was calculated as (140-age) x weight x 1.23 x 0.85 divided by serum creatinine in mmol/L.

DNA microarrays analysis

Genomic DNA was extracted from whole blood using the phenol-chloroform method. DNA was then titrated and quantified by fluorimetry (PicoGreen®, Cambridge Bioscience, UK) to a concentration of 50 ng/μL. Subjects were separated into tertiles based on changes in serum PTH after 2 years of calcium administration. Pooled DNA was then separately constructed for samples in tertiles 1 and 3 by mixing equal amounts of DNA from 50 individuals of each group and then purified prior to hybridization on microarrays (Quick PCR purification kit®, Qiagen,USA). Single-nucleotide polymorphism (SNP) genotyping of pooled DNA was performed using hybridization reaction with single probe technique on Affymatrix GeneChip® Mapping 10K Xba 142 2.0 Arrays following the manufacturer's protocol (10). Two hundred and fifty nanograms of pooled genomic DNA were digested by XbaI, ligated to adaptor, amplified by PCR by using 3 min 95°C hot start; 35 cycles of 20 sec, 95°C; 15 sec, 59°C; 15 sec, 72°C; and a final 7 min 72°C extension. PCR products were purified (MinElute 96 UF kits, Qiagen, Valencia, CA), digested for 30 min with 0.04 unit/μL DNase I to produce 30- to 200-bp fragments, end-labeled by using terminal deoxynucleotidyl transferase and biotinylated dideoxynucleotides, and hybridized to 10K GeneChip® arrays (Affymetrix), which were stained and washed as described by using immunopure streptavidin (Pierce), biotinylated antistreptavidin antibody (Vector Laboratories), and R-phycoerythrin streptavidin (Molecular Probes). Arrays were scanned and fluorescence intensities were quantitated using an Affymetrix array scanner, as described. We duplicated 10K GeneChip genotyping for each group. GeneChip® DNA Analysis Software (GDAS) program version 3.0 was used for analyzing the GeneChips.

Estimated allele frequency for each SNP in each DNA pool was assessed based on averaging hybridization intensity signals from two arrays. Allele frequency estimates were derived from relative allele signals (RAS) for the sense strand (RAS1) and the antisense strand of SNPs (RAS2). RAS scores should vary between 0 (for a BB homozygote) and 1.0 (for an AA homozygote) and heterozygotes should generate a relative allele signal of about 0.5. As previously reported (11, 12), the average RAS scores derived from RAS1 and RAS2 were employed to estimate the SNP allele frequencies in each pool. Estimated odds ratios were then calculated from the estimated allele frequencies and SNPs ranked according to their estimated odds ratios.

Fractional calcium absorption assessment

Milk was extrinsically labeled as previously described (13) by adding 100 mg ^{44}Ca as a $^{44}CaCl_2$ solution to 92.4 g milk containing 100 mg Ca. The $^{44}CaCl_2$ solution was prepared by dissolving $^{44}CaCO_3$ (enriched to 95.9%, Trace Science International) in concentrated HCl, adjusting the pH to 5 with NaOH, and diluting with deionized water to a final concentration of 5 mg ^{44}Ca/mL. The labeled milk was allowed to equilibrate overnight in the refrigerator.

All subjects were studied for fractional calcium absorption during day 4-11 of the follicular phase of their menstrual cycle. The test foods which were vegetable soup, one was ivy gourd and the other was winged bean were served as part of breakfast after an overnight fast. The calcium content of each test food is set at 100 mg per serving. The test foods were served with 100 g cooked rice. All containers were rinsed 3-4 times with deionized water and the rinsing consumed. Subjects' weight and height were measured at the test meal. Blood samples were collected at baseline immediately prior to consuming the test meal and precisely 5 h after consumption of the labeled test meals. Subjects were not allowed to have food or drink except deionized water between consuming the labeled test meal and the second blood draw.

Calcium isotope ratios at baseline and 5 h were measured by ICP-MS (ELEMENT-2 inductively coupled plasma mass spectrometer, ThermoFinnigan) as previously described (14). Fractional calcium absorption was calculated according to the methods described elsewhere (15, 16). Briefly, absorption fraction is given by:

Calcium fractional absorption = 0.3537*(SA5 0.92373) *(Ht 0.52847)*(Wt 0.37213)

where SA5 = fraction of ^{44}Ca dose in serum at 5 h, Ht= height in meters and Wt = weight in Kg.

Statistical Analysis

Data were expressed as mean +/- SEM unless stated otherwise. The unpaired Student's t test was used to analyze differences between groups. A p value of < 0.05 was considered statistically significant.

Results

After 2 years, there were 171 postmenopausal women completed the calcium supplementation study. The mean (± SD) rate of compliance during the two-year study period was 89.3 ± 4.9 percent.

The demographic characteristics, biochemical parameters and BMD of the subjects are shown in Table 1.

Table 1
Baseline characteristic of postmenopausal subjects in the calcium supplementation study (n = 171)

Characteristics	Mean + SD
Age (year)	66.0 ± 4.5
Weight (kg)	59.3 ± 8.8
Height (cm)	153.0 ± 5.0
BMI (kg/m^2)	25.3 ± 3.5
Serum calcium (mmol/L)	2.37 ± 0.11
Serum phosphorus (mmol/L)	1.23 ± 0.15
Glomerular filtration rate	66.2 ± 17.5
Plasma PTH (pmol/L)	4.7 ± 1.9
Serum 25(OH)D (nmol/L)	62.0 ± 13.6
Lumbar spine L2-L4 BMD (g/cm^2)	1.01 ± 0.12
Femoral neck BMD (g/cm^2)	0.79 ± 0.10

PTH significantly decreased after 2 years of calcium supplementation (baseline, 4.7 ± 1.9 pmol/L vs. year 2, 4.4 ± 1.6 pmol/L; P < 0.01). There were significant increases in lumbar spine BMD (baseline, 1.01 ± 0.01 g/cm^2 vs. year 2, 1.03 ± 0.01 g/cm^2; P < 0.001) but not femoral neck BMD (baseline, 0.79 ± 0.01 g/cm^2 vs. year 2, 0.80 ± 0.01 g/cm^2; NS). Despite the nearly 90% reported compliance rate, there were only 63.2 % of subjects (108 of 171) whose PTH levels decreased after 2 years of calcium supplementation. In addition, the mean levels of 25(OH)D and GFR in both the suppressed and non-suppressed PTH groups were not statistically significantly different (61.9 ± 12.3 nmol/L vs. 62.1 ± 15.6 nmol/L and 66.5 ± 19.2 vs. 65.6 ± 14, respectively).

Table 2
Top 10 SNPs with highest estimated odds ratios with regard to PTH responsiveness after calcium supplementation. Group 1: subjects with change in serum PTH after 2 years of calcium supplementation in the lower tertile. Group 2: subjects with change in serum PTH after 2 years of calcium supplementation in the higher tertile

SNP ID	Gene symbol	Estimated allele frequency in group 1	Estimated allele frequency in group 2	P
SNP_A-1515078	ME1	0.40	0.10	< 0.01
SNP_A-1511076	ORF1-FL49	0.28	0.54	< 0.01
SNP_A-1508082	STXBP6	0.49	0.75	< 0.01
SNP_A-1518582	EGFR	0.49	0.25	< 0.01
SNP_A-1515236	c14orf39	0.50	0.26	< 0.01
SNP_A-1516674	RGC32	0.66	0.41	< 0.01
SNP_A-1509080	LPPR4	0.32	0.56	< 0.01
SNP_A-1512864	STK38	0.15	0.36	< 0.01
SNP_A-1514809	FLJ39155	0.48	0.26	< 0.01
SNP_A-1508405	ANGPT1	0.33	0.15	< 0.01
SNP_A-1516437	SNTG1	0.32	0.53	< 0.01

Table 2 demonstrates the top 10 SNPs with highest estimated odds ratios. SNP rs1112482 in cytosolic malic enzyme 1 (ME1) gene was chosen for validation in the present study. When subjects were grouped according to whether PTH was suppressed and the presence of at least one C allele in rs1112482 of ME1 gene it was found that all baseline

Table 3
Baseline characteristics according to PTH suppression after 2 years of calcium supplementation and the presence of C allele in SNP rs1112482 of ME1 gene. Values are mean ± SE

	Suppressed PTH (n =108)			Non-suppressed PTH (n = 63)		
Characteristics	C allele absent (n = 77)	C allele present (n= 31)	P	C allele absent (n = 45)	C allele present (n = 18)	P
Age (year)	66.4 ± 0.53	66.2 ± 0.74	NS	65.5 ± 0.67	65.3 ± 1.08	NS
Weight (kg)	59.1 ± 1.09	61.9 ± 1.63	NS	58.2 ± 1.12	58.3 ± 1.63	NS
Height (cm)	152.8 ± 0.56	152.5 ± 1.00	NS	153.5 ± 0.73	153.6 ± 1.04	NS
BMI (kg/m2)	25.3 ± 0.46	26.5 ± 0.53	NS	24.7 ± 0.44	24.7 ± 0.66	NS
Serum calcium (mmol/L)	2.36 ± 0.01	2.35 ± 0.02	NS	2.40 ± 0.02	2.38 ± 0.01	NS
Serum Phosphorus (mmol/L)	1.21 ± 0.14	1.19 ± 0.15	NS	1.27 ± 0.14	1.29 ± 0.18	NS
Glomerular filtration rate	66.0 ± 18.5	67.7 ± 21.3	NS	64.9 ± 14.2	67.5 ± 13.7	NS
Plasma PTH (pmol/L)	4.81 ± 1.74	5.61 ± 2.45	NS	4.12 ± 1.45	3.93 ± 1.32	NS
Seum 25(OH)D (nmol/L)	61.8 ± 12.4	62.3 ± 12.5	NS	64.1 ± 16.0	57.2 ± 13.5	NS
Lumbar spine L2-L4 BMD (g/cm^2)	0.99 ± 0.01	1.06 ± 0.03	< 0.05	1.01 ± 0.02	0.99 ± 0.03	NS
Femoral neck BMD (g/cm2)	0.78 ± 0.01	0.80 ± 0.02	NS	0.81 ± 0.01	0.78 ± 0.02	NS

characteristics did not differ between subjects with and without the C allele except for lumbar spine BMD in the suppressed PTH group. In this group, subjects with the C allele had significantly higher baseline lumbar spine BMD than those of subjects without the C allele (Table 3). Since the reliability of estimated compliance rate was in question, we chose only subjects with decreased PTH concentrations at the end of the study for further analyses. The degree of suppression of PTH after calcium was higher in those with at least one C allele in SNP rs1112482 of ME1 gene (-26.3 ± 2.1 % vs. -16.9 ± 1.4%, P < 0.001, Fig 1). In the separate group of premenopausal women for calcium absorption study, fractional calcium absorption also tends to be higher in subjects with at least one C allele (n = 6) compared to those without the C allele (n = 13) (58.0 ± 4.9 % vs. 49.3 ± 2.8%, P = 0.054, Fig 2).

Figure 1
Percentage change from baseline in serum PTH in subjects whose PTH levels were suppressed (n = 108) after calcium supplementation grouped according to the present of C allele in rs1112482 of ME1 gene. Values are mean ± SE

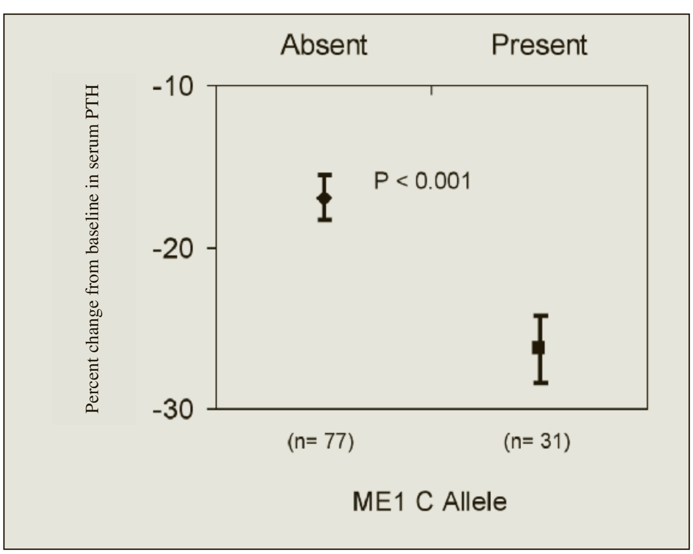
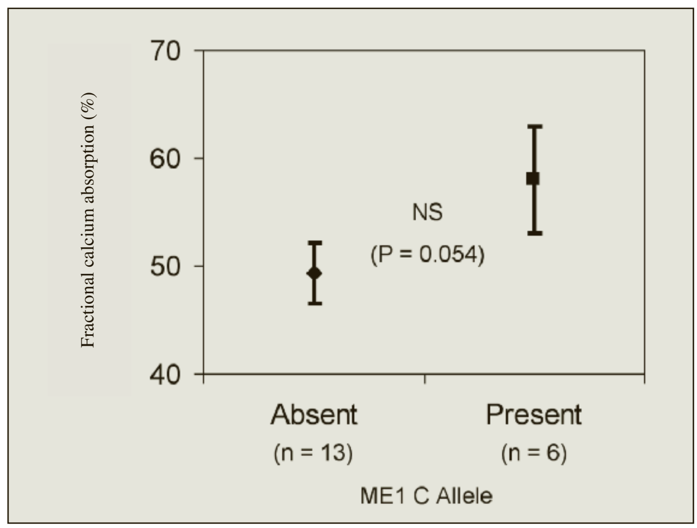


Figure 2
Fractional calcium absorption grouped according to the present of C allele in rs1112482 of ME1 gene. Values are mean ± SE



Discussion

Calcium absorption depends on both modifiable and non-modifiable factors such as age, gender and race. Although genetic factor is likely to play role in intestinal calcium absorption, studies on the issue are surprisingly scarce. In the present study, we utilized SNP genotyping microarray to screen for genetic variations associated with the suppression of PTH after calcium supplementation. We demonstrated that an ME1 gene variant is related to PTH suppression after calcium and the effect is likely to be due to the variation in intestinal calcium absorption. It is unknown previously that intestinal calcium absorption and ME1 are related. Intestinal calcium absorption is an active transport process utilizing ATP generated from the mitochondria as the energy source. ME1 is an important cytosolic enzyme playing role in the regeneration of pyruvate from malate exported from the mitochondira. It is thus likely that alteration in the activity of ME1 can influence the intestinal calcium active transport process. Direct investigation of the influence of ME1 in intestinal calcium absorption is scarce. A number of enzymes involved in malate metabolism including ME1 are altered by menadione (17). There is at least a study looking at the effect of menadione on intestinal calcium absorption. The study found that menadione affects intestinal calcium by inducing mitochondrial dysfunction (18). However, whether the alteration in ME1 mediates the effect of menadione on calcium absorption or just represents a confounder is as yet unclear. Our finding regarding the association of ME1 gene variant with PTH suppression and probably with intestinal calcium absorption suggests the potential role of ME1 in the process of calcium absorption or more distal biological processes.

There have been many studies looking at the skeletal effect of calcium supplementation. The results, however, are not without dispute. The RECORD trial could not demonstrate beneficial effect of calcium with or without vitamin D on fracture risk in elderly women with osteoporotic fractures. However, the WHI study as well as a recent meta-analysis (8) suggests that calcium supplementation can reduce fractures but only in women with good compliance. Non-compliance is a common problem and can limit the benefit of anti-fracture therapy (19). Strategies have been proposed to enhance compliance as well as persistence of anti-fracture therapy (20-22). It is of note that the compliance rate in our study as assessed by calcium tablet counting was high. Nevertheless, PTH suppression was demonstrated in only 62% of subjects. It is likely that other factors as well as genetic variation may be accountable for the responsiveness to calcium besides compliance rate. The notion needs to be addressed in a study specifically initiated to examine the issue.

One of the common problems of genetic association study is the high rate of false association. Validation in a separate population or study indicating functional significance is therefore necessary. Although separate validation was not performed in the present study, we have demonstrated that the ME1 gene variant tended to affect intestinal calcium absorption. However, although the ME1 polymorphism is associated with variation in the suppression of PTH after calcium supplementation, no effect on the change in BMD after calcium was demonstrated. Nevertheless, baseline BMD was different among ME1 genotypes. It is therefore likely that the effect of the ME1 gene variant on BMD is accumulative and the differential influence exerted by various genotypes may only be apparent after prolonged exposure to calcium. The long term influence of the ME1 gene variant on fractures is, however, unknown. Nevertheless, although calcium supplementation results in a marginal increase in BMD, epidemiological studies have demonstrated reduced fracture risks in population with higher prolonged calcium intake (23).

It is of note that although PTH suppression is generally believed to be the main basis for the benefit of calcium on bone due to the concurrent changes in PTH, bone resorption and bone mass after calcium (24, 25). It is also likely that the effect of calcium on bone may not be entirely mediated through PTH. Osteoblast function has been demonstrated to be affected by change in extracellular calcium (26) and a number of osteoblast differentiation markers increases after small elevation of extracellular calcium. The effect is independent of calciotropic hormones and likely mediated through calcium-sensing receptors. Moreover, extracellular calcium also appears to enhance expression of PTH-related peptide which plays role in bone formation (27). Taken together, it likely that calcium can directly influence bone cells. One of the limitation of our study is choosing suppression of PTH as the phenotype studied after calcium supplementation which may not capture the total influence of calcium on bone.

In conclusion, we demonstrated for the first time that cytosolic malic enzyme 1 gene polymorphism is associated with the degree of suppression of parathyroid hormone after long-term calcium supplementation. The effect is probably mediated through increased intestinal calcium absorption.

Acknowledgments: This study was supported by the Thailand Research Fund, National Health Foundation and National Science and Technology Development Agency.

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Chailurkit et al. BMC Public Health 2011, 11:853
http://www.biomedcentral.com/1471-2458/11/853

BMC Public Health

RESEARCH ARTICLE Open Access

Regional variation and determinants of vitamin D status in sunshine-abundant Thailand

La-or Chailurkit[1,3*], Wichai Aekplakorn[2] and Boonsong Ongphiphadhanakul[1]

Abstract

Background: Vitamin D insufficiency is highly prevalent. Most of the studies concerning vitamin D status were generated from countries situated at temperate latitudes. It is less clear what the extent of vitamin D insufficiency is in countries situated in the tropics and how geographical regions within country would affect vitamin D status. In the present study, we investigated vitamin D status in Thais according to geographical regions and other risk factors.

Methods: Subjects consisted of 2,641 adults, aged 15 - 98 years, randomly selected from the Thai 4th National Health Examination Survey (2008-9) cohort. Serum 25 hydroxyvitamin D were measured by liquid chromatography/ tandem mass spectrometry. Data were expressed as mean ± SE.

Results: Subjects residing in Bangkok, the capital city of Thailand, had lower 25(OH)D levels than other parts of the country (Bangkok, central, northern, northeastern and southern regions: 64.8 ± 0.7, 79.5 ± 1.1, 81.7 ± 1.2, 82.2 ± 0.8 and 78.3 ± 1.3 nmol/L, respectively; $p < 0.001$). Within each region, except for the northeastern part of the country, subjects living inside municipal areas had lower circulating 25(OH)D (central, 77.0 ± 20.9 nmol/L vs 85.0 ± 22.1 nmol/L, $p < 0.001$; north 79.3 ± 22.1 nmol/L vs 86.8 ± 21.8 nmol/L, $p < 0.001$; northeast 84.1 ± 23.3 nmol/L vs 87.3 ± 20.9 nmol/L, $p = 0.001$; south, 76.6 ± 20.5 nmol/L vs 85.2 ± 24.7 nmol/L, $p < 0.001$). Overall, the prevalence of vitamin D insufficiency was 64.6%, 46.7%, and 33.5% in Bangkok, municipal areas except Bangkok, and outside municipal area in other parts of the country, respectively. In addition, the prevalence of vitamin D insufficiency according to geographical regions was 43.1%, 39.1%, 34.2% and 43.8% in the central, north, northeast and south, respectively. After controlling for covariates in multiple linear regression analysis, the results showed that low serum 25(OH)D levels were associated with being female, younger age, living in urban and Bangkok.

Conclusions: Vitamin D insufficiency is common and varies across geographical regions in Thailand.

Background

Vitamin D is produced endogenously when the skin is exposed to sunlight, or obtained exogenously from nutrients or supplements. The major role of vitamin D is to maintain calcium homeostasis and bone health. In addition, recent research has revealed that vitamin D may play an important role in a variety of non-skeletal health activities, such as modulation of neuromuscular and immune function, reduction of inflammation, and regulation of cell proliferation, differentiation and apoptosis. Vitamin D insufficiency is highly prevalent and is now recognized as a worldwide health problem [1-3]. It has been observed to varying degrees in many different countries, regardless of geographical location [4]. However, most of the studies concerning vitamin D status have focused on countries situated at temperate latitudes. It is less clear what the extent of vitamin D insufficiency is in countries situated in the tropics, and how geographical regions within a country could affect vitamin D status.

Thailand, a Southeast Asian country, is located at latitudes between 5°30’ N and 20°30’ N. Vitamin D intake among Thais is generally low because few natural vitamin D-rich food sources are found in Thailand, and foods are not fortified with vitamin D. Up to now, there has been a lack of reliable epidemiological data concerning vitamin D status in Thais. Therefore, the purpose of this study is to investigate vitamin D status in Thais

* Correspondence: ralcl@mahidol.ac.th
[1]Department of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand
Full list of author information is available at the end of the article

according to geographical region by assessing levels of serum 25-hydroxyvitamin D (which is the major metabolite and represents the stored form of vitamin D) by a reference method, liquid chromatography/tandem mass spectrometry (LC-MS/MS).

Subjects and methods

Population

This study used data from the Thai 4th National Health Examination Survey (NHESIV) conducted between August 2008 to March 2009 by the National Health Examination Survey Office, Health System Research Institute. Subjects aged 15-98 years were randomly selected from 21 provinces in four geographical regions of Thailand as well as the capital city, Bangkok (Figure 1) using stratified, multistage probability sampling of the population aged ≥15 years with a sample size of 21,960 individuals. The skin color of Thais are categorized into skin types 4 (Brown)-5(Dark Brown) by the Fitzpatrick Classification Scale, both of which possess the high capability to produce melanin. Demographic data such as age, sex, and religion were included. Body weight and height were measured using standard procedure. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Fasting blood samples were obtained and transferred to a freeze at a central laboratory in Ramathibodi Hospital, a university hospital Bangkok, where they were kept at -80°C. The present study used a subsample of the NHES-IV serum samples to measure serum levels of 25-hydroxyvitamin D (25(OH)D). The subsamples were randomly selected according to age group (15-29, 30-44, 45-59, 60-69, 70-79, and ≥80 years), sex, urban/rural and region. In each stratum, 25 individuals were randomly selected using statistical software. A total of 2,700 were sampled of which 2,641 serum samples were available. The study was approved by the ethics committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Serum 25-hydroxyvitamin D (25(OH)D) measurement

Serum 25(OH)D2 and 25(OH)D3 were analyzed by LC-MS/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) using a MassChrom® 25-OH-Vitamin D3/D2 diagnostics kit (Chromsystems, Munich, Germany). The summation of serum 25(OH)D2 and 25(OH)D3 was used to reflect vitamin D status. The inter-assay and intra-assay coefficients of variation of total serum 25(OH)D level were 6.3% and 5.0%, respectively.

Statistical analysis

Data were expressed as mean ± SE. Differences between two groups were assessed by Student's *t*-test. Comparisons among three or more groups were analyzed by analysis of variance followed by Scheffé's test. Stepwise multiple linear regression analysis was used to examine the independent determinants of variables. A p value less than 0.05 was considered statistically significant. All analyses were performed using Stata version 10.1 (StataCorp LP, Texas, USA) and SPSS statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA). All the data analyses were weighted to the probability of sampling to take into account for complex survey design.

Results

The Baseline characteristics of the population study were shown in Table 1. The frequency distribution of serum 25(OH)D levels was shown in Figure 2. Data on average duration of sunlight (Table 2) and temperature in Thailand were obtained from the Thai Meteorological Department, for year 2008 and 2009. The minimum and maximum temperature ranged from 6.0°C to 42.4°C. Mean serum 25(OH)D levels in Thais according to geographical region are shown in Table 2. Subjects residing in Bangkok had lower 25(OH)D levels than those in other parts of the country, and the mean value was also below the sufficient level (75 nmol/L); whereas subjects residing in the northeastern region had the highest mean serum 25(OH)D level. Within each region, except for the northeastern part of the country, subjects living inside municipal areas had lower circulating 25(OH)D: central, 73.5 ± 1.2 nmol/L vs 82.5 ± 1.7 nmol/L, $p < 0.001$; north 75.6 ± 1.9 nmol/L vs 83.3 ± 1.1 nmol/L, $p < 0.001$; northeast 81.3 ± 1.4 nmol/L vs 82.4 ± 0.9 nmol/L, $p = 0.001$; south, 71.9 ± 1.1 nmol/L vs 80.1 ± 1.3 nmol/L, $p < 0.001$ (Figure 3). When only municipal areas were analyzed, subjects in Bangkok still had significant lower 25(OH) D levels than the rest of the country ($p < 0.01$) (Figure 3). In addition to geographical region, there were significant differences in mean serum 25(OH)D levels by gender, age, living in municipal areas, BMI status and religion (Table 2 and Table 3). Moreover, lower serum 25(OH)D levels was observed in younger age (Table 3). Overall, the prevalence of vitamin D insufficiency, as defined by 25(OH)D levels less than 75 nmol/L, in Bangkok was 64.6%; in municipal areas other than Bangkok, 55.1 % and outside municipal areas in other parts of the country, 39.1%. Table 4 shows the prevalence of vitamin D insufficiency at 25(OH)D < 75 nmol/L and 25(OH) < 50 nmol/L by geographical regions and ge Individuals lived in Bangkok had the highest prevalence of vitamin D insufficiency, whereas people in the northeast had the lowest prevalence. After controlling for covariates in multiple linear regression analysis, the results showed that low serum

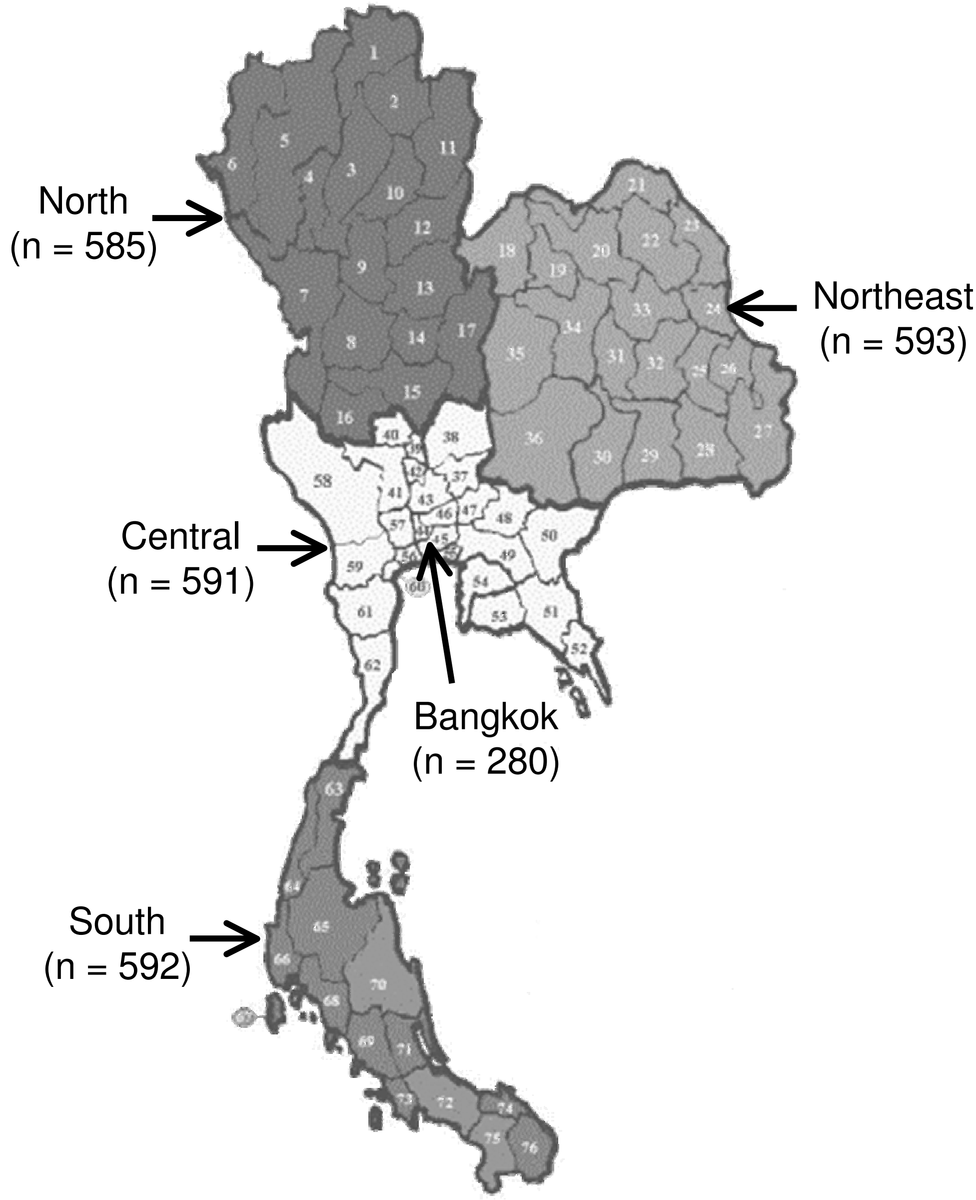

Figure 1 Geographical regions of the study.

25(OH)D levels were associated with being female, younger age, urban and Bangkok (Table 5).

Discussion

Our study represents the first large-scale examination of vitamin D status in the Thai population. Despite the fact that Thailand is located near the equator, a sizable proportion of Thais have inadequate vitamin D status. When using a 25(OH)D threshold of 75 nmol/L, nearly half of Thais are vitamin D insufficient. When a lower threshold of 50 nmol/L was used, the prevalence of vitamin D insufficiency was found to be more than 10% in Bangkok, which is as high as the prevalence of diabetes in Thailand [5]. Studies examining vitamin D status in the tropics are scarce, but have mostly demonstrated similarly low vitamin D status. For example, even in the sunniest areas like Saudi Arabia, the United Arab Emirates, Australia, Turkey, India and Lebanon, a high prevalence of vitamin D insufficiency has been reported in 30 to 50% of children and adults, with 25(OH)D levels under 50 nmol/L [6-10]. On the other hand, people living near the equator who are exposed to sunlight without sun protection have robust levels of 25(OH)D, well above 75 nmol/L [11]. Taken together, this suggests that

Table 1 Baseline characteristics of the population studies

Characteristics	Men	Women	Total
	(*n* = 1,321)	(*n* = 1,320)	
Age (years)	39.6 ± 0.5	41.0 ± 0.4	40.3 ± 0.3
BMI (kg/m^2)	22.7 ± 0.2	24.4 ± 0.2*	23.6 ± 0.1
rural	71.5%	70.2%	70.8%
urban	28.5%	29.8%	29.2%
Religion:			
Muslim	2.5%	3.9%	3.2%
non Muslim	97.5%	96.1%	96.8%
Region:			
Bangkok	8.8%	9.4%	9.1%
Central	24.4%	24.9%	24.6%
North	18.3%	18.4%	18.3%
Northeast	34.9%	33.5%	34.2%
South	13.6%	13.8%	13.7%

Values are mean ± SE or percentage
* Significantly different from the men ($p < 0.001$)

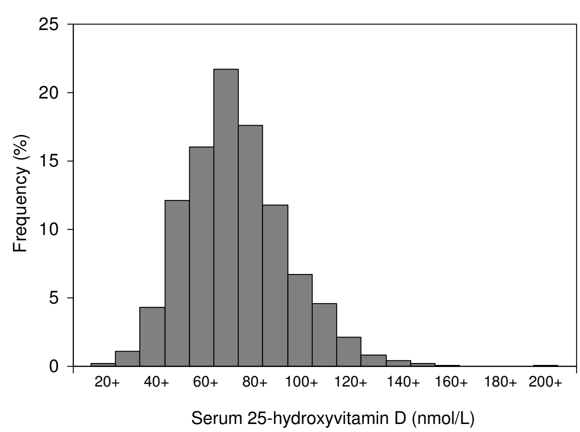

Figure 2 Frequency distribution of 25-hydroxyvitamin D levels in Thais.

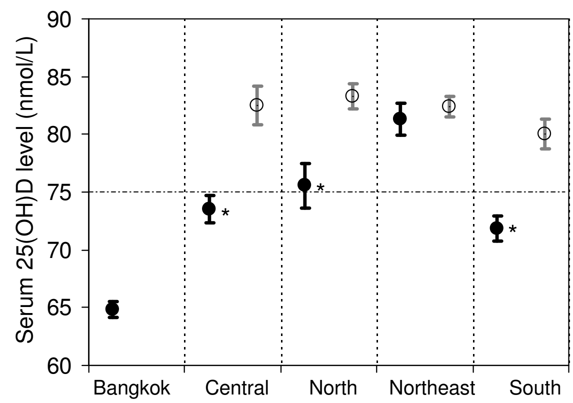

Figure 3 Vitamin D status inside (closed circle) and outside (open circle) the municipal area in each region. Values are mean ± SE. * = significant compared to outside the municipal area within region

low vitamin D status is not an uncommon problem even in countries that receive abundant sunshine. Despite this, outdoor sun exposure can be limited and is likely to be the main contributing factor.

The mean serum 25(OH)D levels in Thais seem relatively high when compared to those reported in various countries in the West [3,12], Middle East [13] and Asia [14]. This might be caused by a higher exposure to sunshine all year round, since the latitude of Thailand is more southerly (closer to the equator) than the countries studied. Nevertheless, differences in vitamin D status were found between regions in Thailand; subjects residing in the southern parts of the country, women in particular, generally had lower serum 25(OH)D concentrations than those residing in the northern region. This finding conflicts with the belief that vitamin D status decreases with increasing latitude. However, our results are in agreement with a European study which showed a positive relationship between serum 25(OH)D and

Table 2 Duration of sunshine and mean serum vitamin D levels according to geographical region and gender

Regions	Duration of sunshine (hours/day)	25(OH)D (nmol/L)		
		Men	Women	Total
Bangkok	4.7 - 9.1	69.0 ± 0.6	61.1 ± 1.3*	64.8 ± 0.7***
Central	4.1 -8.5	86.5 ± 1.7**	73.0 ± 1.1*, **, ***	79.5 ± 1.1**, ***
North	3.6 - 8.1	88.5 ± 1.7**	75.1 ± 1.8*, **	81.7 ± 1.2**
Northeast	3.5 -8.0	87.8 ± 1.3**	*76.7* ± 1.1*, **	82.2 ± 0.8**
South	2.0 - 8.8	87.7 ± 2.9**	69.5 ± 0.8*, **, ***	78.3 ± 1.3**, ***
Total	2.0 - 9.1	85.9 ± 1.1	73.0 ± 0.8*	79.3 ± 0.8

Values are range or mean ± SE
*Significantly different from men ($p < 0.001$), ** significantly different from Bangkok ($p < 0.001$), *** significantly different from Northeast ($p < 0.05$)

Table 3 Mean serum 25(OH)D levels between gender by age, municipal area, BMI and religion

Variables		25(OH)D (nmol/L)			
		Men	**Women**	***p***	**Total**
Age (years)	15 - 29	79.3 ± 1.3	69.3 ± 1.1	<0.001	74.4 ± 0.9
	30 - 44	89.1 ± 1.7*	70.7 ± 1.2 [a]	<0.001	79.9 ± 1.1*„ a
	45 - 59	86.5 ± 1.5*,	75.3 ± 1.2*,	<0.001	80.6 ± 1.0*
	60 - 69	90.7 ± 1.3*	80.2 ± 1.1*	<0.001	85.1 ± 1.0*
	70 - 79	95.0 ± 1.4*	83.8 ± 1.7*	<0.001	88.6 ± 1.2* [a]
	> 80	96.9 ± 1.5*	80.7 ± 1.7*	<0.001	88.2 ± 1.4* [a]
Municipal area	rural	88.9 ± 1.1	75.8 ± 0.8	<0.001	82.3 ± 0.6
	urban	78.4 ± 1.6**	66.6 ± 1.1**	<0.001	72.3 ± 1.3**
BMI (kg/m2)	≥ 25	84.1 ± 1.9	73.7 ± 1.1	<0.001	77.5 ± 1.1
	< 25	86.5 ± 1.1	72.6 ± 0.9	<0.001	80.3 ± 0.8***
Religion	Muslim	81.9 ± 5.2	61.1 ± 4.2	= 0.001	69.0 ± 4.4
	non Muslim	86.0 ± 10****	73.5 ± 0.7****	<0.001	79.7 ± 0.7****

*Significantly different from age 15 -29 years ($p < 0.001$), **significantly different from rural ($p < 0.001$), *** significantly different from BMI ≥ 25 ($p < 0.001$), **** significantly different from Muslim ($p < 0.05$) [a]significantly different from age > 80 years

northern latitude [4]. This finding could be explained by the common use of cod liver oil and vitamin supplements in many northern European countries; while people in southern Europe typically have more skin pigmentation (with consequently less vitamin D production) and may prefer shade instead of sunshine. An explanation for this observation in Thailand might be regional differences in religion. Southern Thailand has a much higher percentage of Muslims, and the clothing style of Muslim women generally allows for greater body coverage. In addition, most northern Thai people are agricultural. Working in the fields and spending more time outdoors in the sunshine probably accounts for much of this difference.

Both lifestyle and environmental factors are important determinants of serum 25(OH)D concentration because of their relationship to ultraviolet exposure. In the present study, a difference in vitamin D status between populations in rural and urban areas was clearly demonstrated. Lower vitamin D levels in the urban populations were evident in almost all geographical regions of Thailand. Although a number of studies have investigated the vitamin D status of urban or rural residents, the disparity in vitamin D status between rural and urban populations has been investigated less often; but existing studies have generally shown lower vitamin D reserves among urban populations [15-17]. A number of factors may be causally related to lower vitamin D status associated with urbanization. Besides lifestyle factors, which may preclude adequate outdoor sun exposure, it is also likely that air pollution may have a contributory role. Tropospheric ozone is a common urban air pollutant and an efficient absorber of ultraviolet radiation [18]. The phenomenon is likely to be more marked in big cities, and may partially explain why residents of Bangkok, the largest city in Thailand, had the lowest 25(OH)D concentrations.

Lower vitamin D status has been demonstrated to be more prevalent with advancing age in most studies [19-22]; this may be caused by less sun exposure and the decreased ability of the skin to produce vitamin D [23]. In contrast, we demonstrated in the present study that vitamin D levels unexpectedly became higher with increasing age. Younger age, rather than older, was an

Table 4 Prevalence of vitamin D insufficiency by geographical region and gender

Regions	Age, yrs (range)	Serum 25(OH)D levels					
		< 75 nmol/L			**< 50 nmol/L**		
		Men	**Women**	**Total**	**Men**	**Women**	**Total**
Bangkok	15 - 93	66.7%	75.5%	64.6%	10.8%	24.2%	14.3%
Central	15 - 91	36.2%	59.2%	43.1%	2.1%	11.4%	6.5%
North	15 - 98	27.9%	50.8%	39.1%	0.9%	6.5%	4.3%
Northeast	15 - 91	25.1%	51.0%	34.2%	0.1%	3.7%	2.8%
South	15 - 92	29.4%	65.8%	43.8%	1.5%	12.9%	6.3%
Total	15 - 98	32.6%	57.3%	45.2%	1.9%	9.3%	5.7%

Table 5 Independent variables for serum 25(OH)D levels by multiple regression analysis

Independent variables	Regression coefficient	SE	*p*
gender	12.97	0.957	< 0.001
Age	0.28	0.019	< 0.001
Urban	-6.47	1.04	< 0.001
BMI	-0.08	0.094	0.422
Muslim	-0.82	2.358	0.733
Region[a]:			
Bangkok	-12.01	1.519	<0.001
Central	-1.73	1.543	0.279
North	-0.69	1.575	0.664
South	-3.32	1.648	0.060

[a]reference group: Northeast

independent risk factor for inadequate vitamin D status. The phenomenon of higher vitamin D levels with advancing age was also observed in both sexes, making it less likely that the observation was simply a chance finding. Although most studies have demonstrated lower vitamin D levels with advancing age, such findings have been predominantly generated from studies of populations residing in temperate geographical locations. There are only limited data on this issue for countries in the tropics. It was found in a study of postmenopausal women in Malaysia that vitamin D levels do not decrease with age [24]. Likewise, 25(OH)D levels remain more or less constant from age 20 to more than age 60 in Iranian men [25]. It is therefore conceivable that despite the decreased dermal synthesis of vitamin D in the elderly, the abundant sunlight may overcome this disadvantage, given that sun exposure is not limited. The elderly in Thailand, after retirement, may have more leisure time and spend more time in the sun. On the other hand, it is also likely that the increased use of sunblock by younger people may be partly accountable for their lower vitamin D status compared to the older population.

Conclusion

Vitamin D insufficiency is highly prevalent in the general adult population in Thailand. Vitamin D status is better in northern than in southern regions of the country. Low serum 25(OH)D levels were associated with being female, younger age, living in urban and Bangkok.

Acknowledgements

This study was supported by the Thailand Research Fund and the National Health Examination Survey Office, Health Systems Research Institute, Thailand.

NHESIV was conducted by the National Health Examination Survey Office, Health Systems Research Institute, Thailand. The NHESIV study group includes: National Health Examination Survey Office: Wichai Aekplakorn, Rungkarn Inthawong, Jiraluck Nonthaluck, Supornsak Tipsukum, Yawarat Porrapakkham; Northern region : Suwat Chariyalertsak, Kanittha Thaikla (Chiang Mai University), Wongsa Laohasiriwong, Wanlop Jaidee, Sutthinan Srathonghon, Ratana Phanphanit, Jiraporn Suwanteerangkul, Kriangkai Srithanaviboonchai; Northeastern region: Pattapong Kessomboon, Somdej Pinitsoontorn, Piyathida Kuhirunyaratn, Sauwanan Bumrerraj, Amornrat Rattanasiri, Suchada Paileeklee, Bangornsri Jindawong, Napaporn Krusun, Weerapong Seeuppalat (Khon Kaen University); Southern region: Virasakdi Chongsuvivatwong, Rassamee Sangthong, Mafausis Dueravee (Prince of Songkla University); Central region: Surasak Taneepanichskul, Somrat Lertmaharit, Vilai Chinveschakitvanich, Onuma Zongram, Nuchanad Hounnaklang, Sukarin Wimuktayon(Chulalongkorn University); Bangkok region: Panwadee Putwatana, Chalermsri Nuntawan, Karn Chaladthanyagid (Mahidol University). The Thai National Health Examination Survey IV was supported financially by the Health System Research Institute; Bureau of Policy and Strategy, Ministry of Public Health; Thai Health Promotion Foundation; National Health Security Office, Thailand.

Author details

[1]Department of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand. [2]Department of Community Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand. [3]Division of Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Rama 6th Road, Bangkok 10400, Thailand.

Authors' contributions

BO and WA designed research. LC and WA conducted research and performed the statistical analysis. LC and BO drafted the manuscript. All authors read and approved the final manuscript

Competing interests

The authors declare that they have no competing interests.

Received: 16 June 2011 Accepted: 10 November 2011
Published: 10 November 2011

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Pre-publication history

The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2458/11/853/prepub

doi:10.1186/1471-2458-11-853
Cite this article as: Chailurkit *et al.*: Regional variation and determinants of vitamin D status in sunshine-abundant Thailand. *BMC Public Health* 2011 **11**:853.

Clinical Endocrinology (2012) **77**, 658–664 doi: 10.1111/j.1365-2265.2012.04422.x

ORIGINAL ARTICLE

The association between vitamin D status and type 2 diabetes in a Thai population, a cross-sectional study

La-or Chailurkit*, Wichai Aekplakorn† and Boonsong Ongphiphadhanakul*

*Department of Medicine, and †Department of Community Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Summary

Objective To explore vitamin D status in relation to diabetes, based on data from a national health examination survey in Thailand.

Design and Methods A total of 2641 adults, aged 15–98 years, were randomly selected according to geographical region from the Thai 4th National Health Examination Survey sample. Logistic regressions were used to examine the cross-sectional association between diabetes status and level of 25(OH)D separately by age groups and areas of residence.

Results Fifty per cent of the subjects were men and 5·8% had diabetes. The mean level of 25(OH)D was 79·3 ± 0·8 nM. Based on cut-off values of 50 and 75 nM, six per cent and 45% had vitamin D insufficiency, respectively. In a regression model, it was found that 25(OH)D3 and total 25(OH)D were positively associated with diabetes. In addition, logistic regression analysis showed that low circulating 25(OH)D3, but not 25(OH)D2, levels was significantly associated with an increased odds of diabetes in older persons (aged $\geq$70 years) in urban areas. However, for subjects residing in rural areas, no association between serum 25(OH)D3 or total 25(OH)D and diabetes was found. Furthermore, vitamin D insufficiency was associated with a higher risk of diabetes (OR, 1·56; 95% CI, 1·10–1·12) only in the urban elderly.

Conclusion Low vitamin D status is modestly associated with a small increase in the risk of diabetes in the urban Thai elderly. The observation that higher vitamin D status is associated with increased diabetic risk in young adults needs to be further explored and confirmed.

(Received 21 December 2011; returned for revision 29 January 2012; finally revised 12 April 2012; accepted 19 April 2012)

Correspondence: Wichai Aekplakorn, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Rama 6th Road, Bangkok 10400, Thailand. Tel.: +66 2 201 2810; Fax: +66 2 201 1715;
E-mail: rawap@mahidol.ac.th

Introduction

Vitamin D possesses myriad biological functions. Observational studies have demonstrated the association of vitamin D status and a number of chronic disorders besides osteoporosis, including cardiovascular diseases, certain cancers and diabetes mellitus. Both type 1 and type 2 diabetes have been found to be related to vitamin D status.[1,2] For type 1 diabetes, vitamin D modulates immunity, and increased vitamin D intake in children results in a decreased risk of type 1 diabetes.[1,3] Although a number of studies have found an association between circulating 25-hydroxyvitamin D (25(OH)D) levels and type 2 diabetes,[4] interventional studies looking at the effect of the repletion of vitamin D on incident type 2 diabetes have provided less clear results.[5] The causal role of vitamin D in type 2 diabetes is still in question.

Epidemiological data regarding vitamin D and type 2 diabetes have so far been generated mostly from Caucasian populations. It is unclear if there is a relationship between vitamin D status and type 2 diabetes for populations residing close to the equator. Although vitamin D insufficiency is highly prevalent, geographical location and distance from the equator can partly influence the extent of the problem. Moreover, type 2 diabetes is pathophysiologically heterogeneous. While heredity influences the risk of type 2 diabetes, environmental factors such as urbanization, modern lifestyle, with a high-fat diet and little exercise can also influence its risk. Towards that end, the purpose of the present study is to explore vitamin D status in relation to diabetes in the Thai population, based on the results of a recent national health examination survey.

Patient and methods

Population

This study used data from the Thai 4th National Health Examination Survey (NHES-IV) conducted in 2008–2009 by the National Health Examination Survey Office, Health Systems Research Institute.[6] Subjects aged 15–98 years were randomly selected from 21 provinces in four geographical regions of Thailand as well as the capital city, Bangkok, using stratified, multistage probability sampling of the population aged $\geq$15 years, with a sample size of 21 960 individuals. Demographic data such as age, gender and religion were included. Urban and rural area were defined according to the administrative area, as the urban areas are municipal areas where rural are nonmunicipal areas. In general, municipal areas were more likely to be economically developed than rural areas. Body mass index (BMI) was measured using standard procedure. Fasting blood samples were obtained and transferred to a freezer at a central laboratory in Ramathibodi Hospital, a university hospital in Bangkok, where they were kept at −80° C. The present study used a subsample of the NHES-IV serum samples to measure serum levels of 25 (OH)D. The subjects were randomly selected according to age group (15–29, 30–44, 45–59, 60–69, 70–79 and ≥80 years), gender, region and area of residence. In each stratum, 25 individuals were randomly selected using statistical software. A total of 2700 were sampled, of which 2641 serum samples were available. The study was approved by the ethics committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Serum 25-hydroxyvitamin D (25(OH)D) measurement

Serum 25(OH)D2 and 25(OH)D3 were analysed by LC-MS/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) using a MassChrom® 25-OH-Vitamin D3/D2 diagnostics kit (ChromSystems, Munich, Germany). The summation of serum 25(OH)D2 and 25(OH)D3 was used to reflect vitamin D status. The interassay and intraassay coefficients of variation of serum total 25(OH)D level were 6·3% and 5·0%, respectively.

Assessment of diabetes mellitus

"Individuals with diabetes" was defined as (i) individuals with a previous diagnosis of diabetes by physician and intake of hypoglycaemic drug during 2 weeks prior to the study or (ii) individuals who had a fasting plasma glucose concentration of ≥7·0 mM at the time of the present study.[7]

Statistical analysis

Levels of 25(OH)D2 and 25(OH)D3 were expressed as mean (SE) and compared between those having diabetes and those without diabetes according to other independent variables including gender, age groups (15–44, 45–69 and ≥70 years), area of residence, BMI categories (<25 and ≥25 kg/m^2), smoking status (current smoker *vs* nonsmoker), alcohol drinking (assessed by graduated frequency questionnaire and categorized into two groups at cut-off points: ≥41 g/day for men and ≥21 g/day for women) and leisure time physical activity status (assessed using global physical activity questionnaire), categorized into two groups as those having moderate or higher ≥150 min/week and those <150 min/week using *t*-test statistics. Logistic regression analyses were conducted to examine the association of levels of vitamin D (25(OH)D2 and 25(OH)D3), with diabetes status controlling for other covariates including age, gender, BMI, area of residence, smoking, alcohol drinking and physical activity. Initially, the regression model included independent variables and several product terms including between 25(OH)D3 and gender; 25(OH)D3 and age; 25(OH)D3 and BMI; 25(OH)D3 and smoking; 25(OH)D3 and alcohol drinking; age and area of residence as well as between area of residence and gender to test for interactions. The results indicated that there was an interaction between area of residence and age group, but not for the others. Subsequently, the regression models were separately analysed for area of residence and the three age groups. There was no correlation between serum 25(OH)D2 and 25(OH)D3 (r = 0·004), but the serum 25(OH)D3 was highly correlated with serum total 25(OH)D (r = 0·99), and hence, we include 25(OH)D2 and 25(OH)D3 in the same model and run a separate model for total 25(OH)D. In the logistic regression model, the odds ratios for serum 25(OH)D2, 25(OH) D3 and total 25(OH)D were calculated as per one unit change of their corresponding standard deviation (SD): 3, 22·3 and 22·5 nM, respectively. Additional logistic regression analysis was also performed using total 25(OH)D as a dichotomous variable with a cut-off point at 75 nM (25(OH)D <75 nM coded as 1 and ≥75 nM as 0. A *P*-value <0·05 was considered statistically significant, and a *P*-value <0·10 was considered significance for interaction. All analyses were weighted to the probability of sampling using Stata version 10.1 (StataCorp LP, College Station, TX, USA), with a svy command to take into account the complex survey design.

Results

The mean age of the study population was 40·3 ± 0·3 years. Fifty per cent of the subjects were men and 5·8% had diabetes. The mean level of 25(OH)D was 79·3 ± 0·8 nM. Six per cent and 45% were considered to have vitamin D insufficiency based on the cut-off values of 50 and 75 nM, respectively.

Table 1 shows mean serum 25(OH)D2, 25(OH)D3 and total 25(OH)D in subjects with and without diabetes according to gender, age, area of residence, BMI, smoking, alcohol drinking and physical activity**.** In general, it was found that most subjects with diabetes had higher serum 25(OH)D2, 25(OH)D3 and total 25(OH)D than those without diabetes except that subjects aged ≥70 years with diabetes had lower 25(OH)D3 and total 25 (OH)D levels compared to those without diabetes. In a regression model investigating variables associated with diabetes adjusted for potential confounding factors, it was found that 25 (OH)D3 and total 25(OH)D were positively associated with diabetes (Table 2). In addition, it was found that age interacted with area of residence, and (age also interacted) with 25(OH)D status on the risk of diabetes. Therefore, subjects were separately analysed by area of residence and age groups. Table 3 shows mean serum 25(OH)D2, 25(OH)D3 and total 25(OH)D in subjects with and without diabetes by area of residence. There were no significant differences in 25(OH)D2 between individuals with diabetes and those without diabetes in both urban and rural areas except in the urban 15–44 age group. With regard to vitamin D3 and total vitamin D, subjects in the 15–44 age group who had diabetes had higher levels of 25(OH)D3 and total 25 (OH)D than those without diabetes in both urban and rural areas. In contrast, diabetic subjects aged ≥ 70 years residing in urban areas had a significant lower level of serum 25(OH)D3 and total 25(OH)D compared to those without diabetes. In addition, subjects without diabetes who resided in urban areas also had lower serum 25(OH)D2, 25(OH)D3 and total 25(OH) D levels compared to those without diabetes in rural areas.

Table 4 shows the levels of 25(OH)D2, 25(OH)D3 and variables associated with diabetes according to age groups and areas of residence. Higher BMI was associated with an increased risk of diabetes in all age categories. The association of 25(OH)D3 with diabetes varied by age group and area of residence. Circulating 25(OH)D3 and total 25(OH)D levels was negatively associated with diabetes only in subjects aged ≥ 70 years in urban areas. However, for subjects residing in rural areas, no association between serum 25(OH)D3 or total 25(OH)D and diabetes was found. Table 5 shows a consistent result of an additional analysis for logistic regression using total 25(OH)D as a dichotomous variable (<75 nM indicating vitamin D insufficiency) indicating that vitamin D insufficiency in subjects aged 15–44 years residing in the urban or rural areas vitamin D insufficiency was associated with a lower risk of diabetes. In contrast, vitamin D insufficiency was associated with a higher risk of diabetes (OR, 1·56; 95% CI, 1·10–1·12) only in the urban elderly (Table 5).

Discussion

In this study, we found that low vitamin D status was modestly associated with a small increase in the risk of diabetes in urban Thai elderly. Most of the existing data regarding vitamin D status in relation to diabetes have been generated in the Western countries; studies investigating the role of vitamin D in the modulation of the risk of diabetes in other populations are scarce[8,9] and based on limited sample sizes.[10–12] To our knowledge, our study is the first population-based study to demonstrate the association of vitamin D status, as directly assessed from circulating 25(OH)D, and diabetes in the tropical region. Thailand is situated in a geographical area (latitudes between 5° 30′ N and 20°30′ N.) where sun always shines brightly all year round causing little of the seasonal fluctuations seen when there is in winter (the sunshine length during daytime in summer and winter is between 4·0–8·1 h and 3·1–8·2 h). Because of the cross-sectional nature of the study, our findings cannot delineate the causal role of vitamin D in the development of diabetes. However, some of the findings, particularly in the urban elderly, are in line with a number of previous studies demonstrating the association of vitamin D status, assessed by various methods, with incident type 2 diabetes.[2,13,14] This is not without dispute, as small interventional studies administering vitamin D supplements to individuals at risk in order to infer causality have provided controversial results.[15–17] If a causal role of vitamin D does exist, the discrepancy may be due in part to the lack of power, inadequate doses of vitamin D and possibly the inclusion of subgroups of subjects in which vitamin D plays only a small pathophysiological role with regard to glucose metabolism. In the light of the health and economic burdens of diabetes, further appropriately designed clinical studies to address the issue are very much warranted.

Multiple interacting factors play roles in the pathogenesis of type 2 diabetes. It is therefore conceivable that vitamin D can interact with other risk factors to alter type 2 diabetes susceptibility. In the present study, a statistical interaction among vitamin D levels, age and area of residence for the diabetes sta-

Table 1. Mean (SE) serum 25(OH)D2, 25(OH)D3 and total 25(OH)D in subjects with (n = 281) and without (n = 2360) diabetes according to gender, age, area of residence, BMI, smoking, alcohol drinking and physical activity

Variables		Serum 25(OH)D2 (nM)			Serum 25(OH)D3 (nM)			Serum total 25(OH)D (nM)		
		Diabetes	No Diabetes	*P*-value	Diabetes	No Diabetes	*P*-value	Diabetes	No Diabetes	*P*-value
Gender	Men	1·96 (0·09)	1·84 (0·04)	NS	83·89 (1·73)	84·10 (1·13)	NS	85·86 (1·77)	85·95 (1·13)	NS
	Women	2·48 (0·14)	2·07 (0·05)	0·01	77·64 (2·35)	70·49 (0·85)	<0·01	80·12 (2·32)	72·55 (0·87)	<0·01
Age (years)	15–44	1·78 (0·13)	1·79 (0·04)	NS	91·53 (2·86)	75·04 (0·88)	<0·001	93·31 (2·90)	76·84 (0·89)	0·001
	45–69	2·25 (1·0)	2·19 (0·07)	NS	75·52 (1·63)	80·02 (0·93)	<0·05	77·77 (1·68)	82·21 (0·94)	<0·05
	≥70	3·12 (0·5)	2·65 (0·17)	NS	80·16 (2·62)	86·71 (1·25)	<0·05	83·28 (2·44)	89·36 (1·23)	<0·05
Area of residence	Urban	2·26 (0·16)	1·64 (0·04)	<0·01	73·3 (1·30)	70·34 (1·36)	<0·05	75·56 (1·29)	71·98 (1·36)	<0·05
	Rural	2·26 (0·10)	2·08 (0·05)	NS	85·26 (1·98)	79·91 (0·63)	<0·05	87·52 (1·96)	81·99 (0·64)	<0·05
BMI (kg/m^2)	<25	2·48 (0·18)	2·00 (0·05)	<0·05	83·17 (1·92)	78·06 (0·83)	<0·05	85·65 (1·91)	80·06 (0·85)	<0·05
	≥25	2·10 (0·08)	1·87 (0·04)	<0·05	78·31 (2·32)	75·27 (1·18)	NS	80·41 (2·33)	77·14 (1·18)	NS
Smoking	No	2·26 (0·10)	1·97 (0·05)	<0·05	79·13 (1·72)	74·0 (0·87)	<0·01	81·39 (1·70)	75·97 (0·88)	<0·01
	Yes	2·26 (0·10)	1·92 (0·06)	<0·05	86·71 (1·89)	86·93 (1·15)	NS	88·97 (1·92)	88·85 (1·16)	NS
Alcohol drinking	Moderate	2·3 (0·10)	1·95 (0·04)	<0·01	79·32 (1·79)	76·58 (0·85)	NS	81·64 (1·78)	78·54 (0·85)	NS
	Heavy	1·79 (0·10)	1·82 (0·07)	NS	89·0 (2·01)	83·94 (1·65)	NS	90·78 (2·07)	85·76 (1·67)	NS
Physical activity	Inactive	2·25 (0·11)	2·04 (0·05)	NS	80·85 (1·91)	77·05 (0·85)	0·05	83·10 (1·89)	79·08 (0·87)	0·04
	Active	2·28 (0·14)	1·71 (0·05)	0·001	78·30 (1·81)	77·64 (1·26)	NS	80·58 (1·81)	79·35 (1·27)	NS

NS, non significance.

Table 2. Adjusted odds ratios of variables associated with diabetes

	Model 1		Model 2	
Variables	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age (years)	1·15 (1·10 1·20)	<0·01	1·12 (1·09, 1·16)	<0·01
Male	2·40 (0·36, 15·8)	NS	2·27 (0·37, 13·98)	NS
BMI (kg/m^2)	1·19 (1·03, 1·37)	<0·05	1·10 (0·98, 1·24)	NS
Urban residence	1·24 (0·35, 4·44)	NS	1·13 (0·33, 3·85)	NS
25(OH)D2 (nM)	1·02 (0·99, 1·05)	NS	–	
25(OH)D3 (nM)	1·11 (1·04, 1·18)	<0.01	–	
Total 25(OH)D (nM)	–	–	1·06 (1·02, 1·11)	0·01
Smoking	1·29 (0·27, 6·08)	NS	0·91 (0·21, 4·00)	NS
Alcohol drinking	0·96 (0·14, 6·5)	NS	0·75 (0·11, 5·15)	NS
Physical activity	2·18 (0·61, 7·73)	NS	2·12 (0·6, 7·44)	NS
Urban residence – age*	1·01 (1·0, 1·02)	<0·1	1·01 (1·0, 1·03)	<0·1
Male – 25(OH)D3*	0·98 (0·96, 1·0)	0·05	0·98 (0·96, 1·0)	<0·1
Age – 25(OH)D3*	1·00 (1·0,1·0)	<0·01	1·00 (1·0, 1·0)	<0·01

Model 1 is for 25(OH)D2 and 25(OH)3 and Model 2 for total vitamin D. Both models were adjusted for physical activity, current smoking (yes/no) and moderate-to-heavy alcohol drinking estimated as ≥41 g/day in men or ≥21 g/day in women (yes/no).
The odds ratios for 25(OH)D2 and 25(OH)D3 were calculated per one unit change of their corresponding standard deviation (SD): 3 and 22·3 nM, respectively.
*Interaction terms, NS, non significance.

tus was found. With regard to the interaction between age and vitamin D on the risk of type 2 diabetes, our results are in line with a recent cross-sectional study in the US population, in that the association between vitamin D and HbA1c was observed only in certain age groups (35–74 years).[18]

Urbanization is well recognized to be associated with increased risk of type 2 diabetes.[19,20] The relationship is generally believed to be attributable to lifestyle changes associated with improved socio-economic status and modern living conditions. Urban residents have less sun exposure, which can predispose individuals to lower vitamin D status. Besides lower vitamin D status, urbanization is associated with a number of shared risk factors for certain noncommunicable age-related diseases such as lack of physical activity and increased exposure to air pollution. It is of note that in the present study, we demonstrated a statistical interaction between vitamin D insufficiency and urbanization on diabetes, in that the influence of vitamin D insufficiency was evident only in the older subjects residing in an urban environment.

In contrast to the association between lower vitamin D status and diabetes in older subjects residing in urban area, the direction of the relationship reversed in the younger age group. Although the strength of the statistical association was relatively weak and the underlying basis unclear, the finding is intriguing. Despite the fact that lower circulating vitamin D is generally associated with increased adiposity and calcitriol inhibits lipogenesis,[21] studies in vitamin D receptor (VDR) knockout animals have yield opposite results in that VDR knockout animals are lean,[22] and there is an increase in brown adipose tissue. Brown adipose tissue has recently been confirmed to exist and is functional in human adults.[23] It might be that vitamin D adversely influences glucose tolerance in some individuals through its potentially adverse effect on brown fat. Further study to explore the comparative effects of vitamin D on brown and white adipose tissues is warranted.

Although controversial, the biological effect of vitamin D2 *vs* D3 is generally considered to be equivalent in humans.[24,25] This notion, however, was mainly derived from a study regarding calcium and bone metabolism and has never been explored in the context of other biological functions of vitamin D. In the present study, we observed that the relationship between vitamin

Table 3. Mean (SE) of serum 25(OH)D2, 25(OH)D3 and total 25(OH)D levels according to diabetes status, age group and area of residence

		Urban (n = 1458)			Rural (n = 1183)		
	Age (years)	Diabetes (n = 193)	No diabetes (n = 1265)	*P*-value	Diabetes (n = 88)	No diabetes (n = 1095)	*P*-value
Serum 25(OH)D2 (nM)	15–44	1·6 (0·1)	1·5 (0·03)*	0·05	1.8 (0·2)	1.9 (0·1)	NS
	45–69	2·0 (0·1)	1·8 (0·1)*	NS	2.5 (0·1)	2.3 (0·1)	NS
	≥70	3·7 (0·9)	2·3 (0·2)	NS	2.5 (0·2)	2.8 (0·2)	NS
Serum 25(OH)D3 (nM)	15–44	81·3 (0·03)	67·7 (1·4)*	<0·05	94.4 (3.1)	77.9 (0·8)	<0·01
	45–69	72·6 (1·8)	74·0 (1·5)*	NS	78.1 (2.2)	82.6 (0·8)	NS
	≥70	69·9 (1·7)	79·9 (1·4)*	<0·01	91.4 (3.2)	89.2 (1·5)	NS
Serum total 25(OH)D (nM)	15–44	82·9 (4·9)	69·2 (1·5)*	<0·05	96.2 (3.2)	79.8 (0·8)	<0·01
	45–69	74·6 (1·8)	75·9 (1·5)*	NS	80·5 (2.2)	84.9 (0·8)	NS
	≥70	73·6 (1·6)	82·3 (1·5)*	<0·01	93.9 (3.2)	91.9 (1·5)	NS

NS, non significance.
*P < 0·01 compared between those without diabetes in urban and rural areas.

Table 4. Adjusted odds ratios (OR) for 25(OH)D2, 25(OH)D3 and variables associated with having diabetes according to three age groups and area of residence

	Adjusted OR (95% CI)					
Age (years)	15–44	*P*-value	45–69	*P*-value	≥70	*P*-value
Urban	*n* = 483		*n* = 498		*n* = 477	
Age (years)	1·11 (1·05, 1·18)	0·001	1·07 (1·04, 1·11)	0·001	0·96 (0·92, 0·99)	0·01
Men	3·32 (1·15, 9·6)	<0·05	1·07 (0·65, 1·79)	NS	0·93 (0·63, 1·36)	NS
BMI (kg/m^2)	1·25 (1·12, 1·41)	0·001	1·19 (1·11, 1·28)	0·001	1·07 (1·03, 1·12)	<0·01
25(OH)D2 (nM)	0·79 (0·28, 2·20)	NS	1·13 (0·81, 1·57)	NS	1·12 (1·01, 1·24)	<0·05
25(OH)D3 (nM)	2·49 (1·15, 5·37)	<0·05	1·0 (0·82, 1·23)	NS	0·69 (0·55, 0·87)	<0·01
Rural	*n* = 399		*n* = 386		*n* = 398	
Age (years)	1·07 (0·97, 1·17)	NS	1·07 (1·03, 1·13)	0·005	0·97 (0·92, 1·03)	NS
Men	0·43 (0·08, 2·24)	NS	0·71 (0·39, 1·29)	NS	1·43 (0·76, 2·70)	NS
BMI (kg/m^2)	1·09 (1·02, 1·16)	<0·05	1·08 (1·02, 1·15)	0·01	1·05 (1·0, 1·09)	<0·05
25(OH)D2 (nM)	0·27 (0·05, 1·52)	NS	1·27 (0·81, 1·99)	NS	0·98 (0·87, 1·09)	NS
25(OH)D3 (nM)	2·05 (1·24, 3·39)	<0·01	0·68 (0·45, 1·01)	NS	1·06 (0·74, 1·52)	NS

NS, non significance.
Adjusted for physical activity, current smoking (yes/no), and moderate-to-heavy alcohol drinking estimated as ≥41 g/day in men or ≥21 g/day in women (yes/no).
The odds ratios for 25(OH)D2 and 25(OH)D3 were calculated per one unit change of their corresponding standard deviation (SD): 3 and 22·3 nM, respectively.

Table 5. Adjusted odds ratios (OR) for vitamin D insufficiency (total 25(OH)D <75 nM) and variables associated with having diabetes according to three age groups and area of residence

Age (years)	15–44	*P*-value	45–69	*P*-value	≥70	*P*-value
Urban	*n* = 483		*n* = 498		*n* = 477	
Age (years)	1·10 (1·03, 1·17)	<0·01	1·07 (1·04, 1·11)	<0·01	0·95 (0·92, 0·98)	<0·01
Men	3·82 (1·26, 11·58)	<0·05	1·08 (0·65, 1·78)	NS	0·86 (0·59, 1·24)	NS
BMI (kg/m^2)	1·24 (1·12, 1·37)	<0·01	1·19 (1·11, 1·28)	<0·01	1·07 (1·01, 1·12)	0·01
Total 25(OH)D <75 nM	0·26 (0·09, 0·75)	<0·05	0·84 (0·56, 1·25)	NS	1·56 (1·10, 2·20)	0·01
Rural	*n* = 399		*n* = 386		*n* = 398	
Age (years)	1·08 (0·99, 1·17)	NS	1·07 (1·02, 1·12)	<0·01	0·98 (0·93, 1·04)	NS
Men	0·69 (0·19, 2·49)	NS	0·63 (0·35, 1·11)	0·10	1·27 (0·66, 2·42)	NS
BMI (kg/m^2)	1·08 (1·02, 1·14)	0·01	1·08 (1·02, 1·15)	0·01	1·05 (1.01, 1·10)	<0·05
Total 25(OH)D <75 nM	0·12 (0·03, 0·51)	<0·01	1·33 (0·78, 2·26)	NS	0·50 (0·16, 1·55)	NS

NS, non significance.
Adjusted for physical activity, current smoking (yes/no), and moderate-to heavy alcohol drinking estimated as ≥41 g/day in men or ≥21 g/day in women (yes/no).
The odds ratios for total 25(OH)D were calculated per one unit change of their corresponding standard deviation (SD): 22·5 nM.

D status and diabetes existed in the case of 25(OH)D3 but not for 25(OH)D2. A number of possibilities may account for this finding. It is likely that adding 25(OH)D2 to 25(OH)D3 for the derivation of total 25(OH)D introduces an additional source of systematic error and may render the result less accurate for reflecting vitamin D status. On the other hand, this probably suggests that 25(OH)D3 and 25(OH)D2 are not equivalent in terms of their biological effects. In fact, vitamin D3 has been shown to differ from vitamin D2 in terms of metabolism, affinity to vitamin D binding protein and affinity of their active metabolites to VDRs.[26] Nevertheless, our study was not specifically designed to address the issue, and further more appropriate studies to better answer the question should be initiated. Furthermore, it is conceivable that the discrepancy in the results of the vitamin D intervention study may partly be due to the type of vitamin supplement administered.

There are a number of limitations in the present study. Oral glucose tolerance test was not performed which may misclassify diabetes status in some subjects. Distinguishing between type 1 and type 2 diabetes, particularly in those younger than 35 years old, was not feasible in this large cross-sectional survey. However, type 1 diabetes is very rare in Thailand even in recent years[27] and it is very likely that almost all diabetic subjects in this age group had type 2 diabetes. Data regarding vitamin D supplementation as well as the detailed dietary records were not gathered at the time of the survey. Such parameters may influence diabetes status or confound the association between vitamin D status and diabetes.

We concluded that low vitamin D status is associated with a modest increase in the risk of diabetes in the urban Thai elderly. The observation that higher vitamin D status is associated with increased diabetic risk in young adults needs to be further explored and confirmed.

Acknowledgements

This study was supported by: the Thailand Research Fund; the Health Systems Research Institute; the Bureau of Policy and Strategy, Ministry of Public Health; the Thai Health Promotion Foundation and the National Health Security Office, Thailand.

Authors' contribution

B.O. and W.A. designed the research. L.C., B.O. and W.A. conducted the research and performed statistical analysis. L.C. and B.O. wrote the manuscript. All authors read and approved the final manuscript.

Conflict interest and financial disclosure

Nothing to declare.

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Appendix

NHES-IV was conducted by the National Health Examination Survey Office, Health Systems Research Institute, Thailand. The NHES-IV study group included the following offices/regions:

National Health Examination Survey Office: Wichai Aekplakorn, Rungkarn Inthawong, Jiraluck Nontaluck, Supornsak Tipsukum, Yawarat Porrapakkham.

Northern region: Suwat Chariyalertsak, Kanittha Thaikla (Chiang Mai University), Wongsa Laohasiriwong, Wanlop Jaidee, Sutthinan Srathonghon, Ratana Phanphanit, Jiraporn Suwanteerangkul, Kriangkai Srithanaviboonchai.

North-eastern region: Pattapong Kessomboon, Somdej Pinitsoontorn, Piyathida Kuhirunyaratn, Sauwanan Bumrerraj, Amornrat Rattanasiri, Suchada Paileeklee, Bangornsri Jindawong, Napaporn Krusun, Weerapong Seeuppalat (Khon Kaen University).

Southern region: Virasakdi Chongsuvivatwong, Rassamee Sangthong, Mafausis Dueravee (Prince of Songkla University).

Central region: Surasak Taneepanichskul, Somrat Lertmaharit, Vilai Chinveschakitvanich, Onuma Zongram, Nuchanad Hounnaklang, Sukarin Wimuktayon (Chulalongkorn University).

Bangkok region: Panwadee Putwatana, Chalermsri Nuntawan, Karn Chaladthanyagid (Mahidol University).

Journal of Clinical Densitometry: Assessment of Skeletal Health, vol. ■, no. ■, 1−5, 2012

1094-6950/■:1−5/$36.00
http://dx.doi.org/10.1016/j.jocd.2012.05.008

Original Article

Serum Uric Acid Levels in Relation to Bone-Related Phenotypes in Men and Women

Chanika Sritara,[1] Boonsong Ongphiphadhanakul,,[2] Laor Chailurkit,[2] Sukit Yamwong,[2] Wipa Ratanachaiwong,[3] and Piyamitr Sritara[2]*

[1]*Department of Radiology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand;* [2]*Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand; and* [3]*Medical and Health Office, Electricity Generating Authority of Thailand, Nonthaburi, Thailand*

Abstract

Serum uric acid levels have recently been found to be associated with bone mineral density (BMD) in elderly males. The purpose of the present study was to investigate the relationship between bone-related phenotypes and serum uric acid levels in young and middle-aged males and females. Subjects consisted of 1320 males and 485 females aged 25−54 yr. Bone densitometry and quantitative ultrasonometry (QUS) were performed on each subject. Serum uric acid and biochemical markers of bone turnover were measured in fasting serum samples. When adjusted for covariates including age, body weight, and serum creatinine in multiple linear regression models, it was found that there was a positive association between uric acid levels and BMD in males at the lumbar spine ($p < 0.05$). The association between uric acid levels and BMD was found in females after controlling for age, body weight, and serum creatinine at the femoral neck, but in the opposite direction ($p < 0.05$). Uric acid levels were related to the stiffness index (SI) as assessed by QUS in males, independent of age, body weight, and serum creatinine ($p < 0.05$). No association between uric acid and SI in females was found. The present study demonstrated a positive association in males between serum uric acid levels and BMD, and SI from QUS, suggesting a beneficial influence of uric acid on both the quantity and quality of bone in males.

Key Words: Bone markers; bone mineral density; stiffness index; uric acid.

Introduction

Uric acid is traditionally believed to be a metabolic waste of purine metabolism, with no significant biological function in humans. However, it has been suggested that uric acid possesses antioxidant properties *(1,2)*. Oxidative stress plays an important role in a number of illnesses, including hypertension *(3)*, diabetes *(4)*, and chronic kidney disease *(5)*. A number of antioxidants, including vitamins C and E, have been shown to potentially affect such disorders favorably. Likewise, there is an evidence that uric acid also has a favorable effect in cases of thromboembolic stroke *(6,7)*.

Oxidative stress may increase the propensity for age-related bone loss *(8)*. In this regard, a recent study in elderly males demonstrated a positive association between serum uric acid levels and bone mineral density (BMD), independent of other associated factors including age and body mass index *(9)*. The finding is intriguing and suggests a probable role of uric acid in bone metabolism. Nevertheless, it is unclear at a present if there is an association between uric acid and bone-related phenotypes in younger age groups, and if there is any gender difference. Toward this end, the present study investigated the association between bone-related phenotypes and serum uric acid levels in young and middle-aged males and females.

Received 04/02/12; Revised 05/17/12; Accepted 05/18/12.
Conflicts of interest: None.
*Address correspondence to: Boonsong Ongphiphadhanakul, MD, Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand. E-mail: rabpk@mahidol.ac.th

Materials and Methods

The study was part of a health survey of employees of the Electricity Generating Authority of Thailand (EGAT). Institutional Review Board approval was obtained prior to commencement of the study, and all subjects gave informed consent. Survey data were collected by using a self-administered questionnaire, physical examination, electrocardiography, chest radiography, and blood analysis. Fasting blood samples were obtained and sent for uric acid analysis. Blood samples for procollagen I N-terminal propeptide (P1NP) and C-terminal telopeptide (CTX) were kept frozen until the time of analysis.

BMD Measurements

After changing into light clothing without dense objects, each volunteer underwent dual-energy X-ray absorptiometry (DXA) (QDR 4500W; Hologic, Bedford, MA) to obtain BMD values of the lumbar spine (L1−L4) and left proximal femur (femoral neck and total hip). The DXA procedure complied with the International Society for Clinical Densitometry (ISCD) Position Statement *(2)*. For lumbar spine DXA, each volunteer lay flat on the midline of the imaging table, with legs elevated by a supporting pillow so that the spine was straight. The scan included T12 and L5 vertebrae and both iliac crests. For the proximal femur DXA, the volunteer lay supine with the left foot fixed to a positioning device to keep the hip internally rotated and adducted so that the femoral shaft was straight, with minimal visualization of the lesser trochanter, while the ischium and the greater trochanter were included on the scan. The scans were then analyzed according to the manufacturer's recommendations. A daily quality control procedure was also performed every morning of the survey prior to taking measurements to ensure a machine accuracy of greater than 98.5%. The BMD coefficients of variation were 0.82%, 2.52%, and 1.51% for the lumbar spine, femoral neck, and total hip, respectively.

Quantitative Ultrasonometry

Each volunteer underwent quantitative ultrasonometry (QUS) measurement at the left calcaneus with a Lunar Achilles ultrasound machine. The stiffness index (SI), QUS T-score, and QUS Z-score were obtained. The SI describes bone quality by combining and standardizing broadband ultrasound attenuation (BUA) and speed of sound (SOS) into a single clinical quantity, using the formula:

$$SI = (0.67BUA + 0.28SOS) - 420.$$

The formula is derived so that half of the contribution is from BUA and the other half is from SOS over the adult age range, and where the young adult value is 100 *(3)*. Each morning of the survey, quality assurance procedure was carried out according to the manufacturer's recommendations to ensure machine deviation of less than 1.5%. The SI coefficient of variation was 1.33%.

Serum Uric Acid Measurement

Serum uric acid levels were determined using a uricase method (Siemens Healthcare Diagnostics Inc., Newark, DE). The assay range was 0−20 mg/dL with reference ranges of 2.6−6.0 mg/dL and 3.5−7.2 mg/dL for females and males, respectively.

Bone Marker Measurement

Serum CTX and P1NP levels were determined by electrochemiluminescence immunoassay on a Cobas e 411 analyzer (Roche Diagnostic GmbH, Mannheim, Germany). The assays had intra-assay precision of 5.4% and 3.8%, respectively.

Results

Table 1 demonstrates the clinical characteristics of the study population. Subjects were mostly males (73.1%), largely because of the demographic structure of the EGAT. When comparing males and females, it was found that males were slightly older, and had significantly higher BMD at the femoral neck and total femur but not at the lumbar spine. No difference was found in terms of SI as measured by QUS. Serum uric acid levels were significantly higher in males.

There were correlations between uric acid levels and BMD at various skeletal sites in males but not in females, as shown

Table 1
Clinical Characteristics of the Study Population

Characteristics	Males (n = 1320)	Females (n = 485)	*p* Value
Age (yr)	41.7 ± 0.2 (25−54)	40.2 ± 0.3 (25−54)	<0.001
Body weight (kg)	70.4 ± 0.3 (40.9−115.0)	55.1 ± 0.4 (37.3−95.4)	<0.001
Height (cm)	169.0 ± 0.2 (144.3−187.5)	157.7 ± 0.2 (145.0−180.0)	<0.001
Lumbar spine BMD (g/cm^2)	0.974 ± 0.003 (0.639−1.684)	0.970 ± 0.005 (0.616−1.298)	NS
Femoral neck BMD (g/cm^2)	0.817 ± 0.003 (0.472−1.30)	0.75 ± 0.005 (0.427−1.083)	<0.001
Total femur BMD (g/cm^2)	0.946 ± 0.003 (0.590−1.442)	0.856 ± 0.005 (0.477−1.318)	<0.001
SI	101.8 ± 0.5 (38−169)	102.7 ± 1.6 (23−172)	NS
Uric acid (mg/dL)	6.12 ± 0.03 (1.2−11.3)	4.07 ± 0.04 (1.7−7.6)	<0.001
Creatinine (mg/dL)	1.00 ± 0.01 (0.60−2.80)	0.69 ± 0.01 (0.40−1.10)	<0.001

Abbr: BMD, bone mineral density; SI, stiffness index.

Table 2
Correlation Coefficients (p Values) of BMD, Uric Acid Levels, and Covariates in Males and Females

	Uric acid	Age	Body weight	Lumbar BMD	Femoral neck BMD	Total femur BMD
Males						
Uric acid	1					
Age	0.03 (NS)	1				
Body weight	0.24 (<0.001)	−0.07 (<0.05)	1			
Lumbar BMD	0.12 (<0.001)	−0.08 (<0.01)	0.24 (<0.001)	1		
Femoral neck BMD	0.1178 (<0.001)	−0.21 (<0.001)	0.38 (<0.001)	0.62 (<0.001)	1	
Total femur BMD	0.14 (<0.001)	−0.1305 (<0.001)	0.3867 (<0.001)	0.6398 (<0.001)	0.8861 (<0.001)	1
Females						
Uric acid	1					
Age	0.11 (<0.05)	1				
Body weight	0.40 (<0.001)	0.14 (<0.01)	1			
Lumbar BMD	0.07 (NS)	−0.02 (NS)	0.30 (<0.001)	1		
Femoral neck BMD	0.09 (<0.05)	0.01 (NS)	0.43 (<0.001)	0.63 (<0.001)	1	
Total femur BMD	0.12 (<0.05)	0.07 (NS)	0.45 (<0.001)	0.64 (<0.001)	0.87 (<0.001)	1

Abbr: BMD, bone mineral density.

in Table 2. When adjusted for covariates including age, body weight, and serum creatinine in multiple linear regression models, it was found that the positive association between uric acid levels and BMD was still statistically significant in males at the lumbar spine. An association between uric acid levels and BMD was found in females after controlling for age, body weight, and serum creatinine only at the femoral neck, but in an opposite direction (Table 3). To explore the probable underlying mechanism of the observed relationship between BMD and uric acid, we further examined the association between uric acid levels and biochemical markers of bone turnover. As shown in Table 4, there was a significant relationship between serum CTX, but not P1NP, and uric acid in males. In females, the relationship between uric acid and serum CTX almost reached statistical significance. Similar to the finding in males, no association between uric acid and P1NP was found.

In the case of bone quality as assessed by QUS, the results were similar to the findings regarding BMD. Uric acid levels were related to SI in males, independent of age, body weight,

Table 3
Multivariate Analysis of the Relationship Between Uric Acid Levels and BMD in Males and Females

	Lumbar BMD		Femoral neck BMD		Total femur BMD	
	Beta	p Value	Beta	p Value	Beta	p Value
Males[a]						
Age (yr)	−0.07	<0.001	−0.19	<0.001	−0.11	<0.001
Body weight (kg)	0.22	<0.001	0.36	<0.001	0.37	<0.001
Uric acid (mg/dL)	0.06	<0.05	0.03	NS	0.04	NS
Creatinine (mg/dL)	0.33	NS	0.02	NS	0.03	NS
Females[b]						
Age (yr)	−0.06	NS	−0.05	<0.001	0.01	NS
Body weight (kg)	0.33	<0.001	0.48	<0.001	0.48	<0.001
Uric acid (mg/dL)	−0.07	NS	−0.10	<0.05	−0.10	NS
Creatinine (mg/dL)	0.04	NS	0.01	NS	0.06	NS

Abbr: BMD, bone mineral density.
[a]Uric acid levels were associated with BMD at the lumbar spine independent of age, body weight, and serum creatinine.
[b]Uric acid levels were associated with lower rather than higher BMD, independent of age, body weight, and serum creatinine at the femoral neck.

Table 4
The Relationship of Bone Markers to Uric Acid, Age, and Body Weight in Males and Females

	Males				Females			
	CTX		P1NP		CTX		P1NP	
	Beta	*p* Value	Beta	*p* Value	Beta	*p* Value	Beta	*p* Value
Uric acid (mg/dL)	−0.02	<0.001	−0.04	NS	0.09	0.06	0.04	NS
Age (yr)	−0.24	<0.001	−0.18	<0.001	0.07	NS	0.03	NS
Body weight (kg)	−0.08	<0.001	−0.04	NS	−0.09	NS	−0.05	NS
Creatinine (mg/dL)	0.16	<0.001	0.08	<0.01	0.07	NS	0.02	NS

Note: Uric acid was independently associated with CTX, a bone resorption marker, in males.
Abbr: CTX, C-terminal telopeptide; P1NP, procollagen I N-terminal propeptide.

and serum creatinine (Table 5). No association between uric acid and SI in females was found.

Discussion

In the present study, we demonstrated a relationship in males between serum uric acid levels and BMD at the lumbar spine, a skeletal site rich in trabecular bone. Although our study population was younger in age, our finding is in keeping with a previous study in elderly males *(9)*. This suggests that the influence of uric acid on bone, if causal in nature, is likely to begin at a younger age and could probably affect the attainment of peak bone mass. However, whether uric acid is causally related to bone mass is not entirely clear at present because studies so far have been cross-sectional in nature and causality cannot be readily established. Hyperuricemia is a common disorder, and uric acid—lowering agents are frequently used. To our knowledge, no study has been conducted examining the effects of uric acid on the reduction of bone mass. In addition, hypouricemia because of mutations in the renal uric acid transporters is not uncommon. Such individuals have very low uric acid levels, usually <2 mg/dL, and are mostly asymptomatic, but no bone-related phenotype has been described. However, monosodium urate monohydrate crystals can directly inhibit osteoblasts *(10)*, which may partly explain the bone erosion observed in chronic gout. Taken together, although it is biologically probable that uric acid may directly affect bone, more studies to delineate the causal role of uric acid in bone metabolism need to be performed.

The present study has also demonstrated that uric acid levels are not only related to BMD, but are also associated with SI derived from bone QUS. In men, SI was found to be positively related to serum uric acid. Discrepancies between results from QUS and bone densitometry are not infrequent, and do not necessary reflect technical errors *(11)*. QUS of bone may capture determinants of bone quality; and combining the results from QUS and densitometry has been shown to better predict fractures than either method alone *(12)*. It is believed that bone quality is a composite measure of multiple factors, including bone microarchitecture, bone quality and material properties, and bone turnover *(13)*. In this regard, our findings suggest that uric acid levels are related not only to bone quantity, as reflected in BMD, but likely bone quality as well because positive associations of serum uric acid with SI and a negative association with bone turnover (as assessed by serum CTX) were found.

In the present study, we demonstrated that although uric acid was related to BMD in males, its relationship to bone mass in females was not as apparent, being seen only at the femoral neck but in a reverse direction. What the underlying basis is for the observed difference in the association between bone mass and uric acid in females is unclear. Although bone mass decreases with age in both genders, and predisposes both men and women to osteoporosis, a number of differences exist and underlie the sexual dimorphism observed. For example, bone structures are different in elderly men when compared with women, and osteoporosis in men and women is likely to be pathophysiologically distinct *(14—16)*. Furthermore, although the established treatment for osteoporosis in women works as well in men *(17—19)*, some modalities show a gender-related difference in responsiveness. Vitamin K2, for example, was demonstrated to be mildly effective in increasing bone mass

Table 5
The Relationship Between Uric Acid and SI From QUS

	SI			
	Males		Females	
	Beta	*p* Value	Beta	*p* Value
Uric acid (mg/dL)	0.06	<0.05	0.04	NS
Age (yr)	−0.25	<0.001	−0.14	<0.001
Body weight (kg)	−0.10	<0.001	0.05	NS
Creatinine (mg/dL)	0.02	NS	0.02	NS

Note: Uric acid was independently related to SI in males but not in females.
Abbr: SI, stiffness index; QUS, quantitative ultrasonometry.

only in women but not in men in a recent meta-analysis (20). Uric acid levels in general are lower in premenopausal women when compared with men, but the degree of difference decreases after menopause. As the female population in our study was predominantly premenopausal, the degree of variability of uric acid levels may not be high enough for the association of uric acid and bone mass in women, if it exists, to be discerned. Moreover, estrogen reduces serum uric acid by enhancing renal clearance (21). In addition, it is well established that estrogen improves bone mass. The negative association of uric acid and BMD in females may therefore be partly explained by the confounding effect of estrogen on uric acid and BMD. There were, however, a number of limitations in this study. Although associations were found between uric acid levels and certain bone-related phenotypes, the causal role of uric acid in this regard is still unclear. Moreover, recruited subjects were not population based but from a single workplace, the generalization of the findings to other populations therefore cannot be readily made.

In conclusion, the present study demonstrated a positive association in males between serum uric acid levels and BMD, as well as the SI from QUS, suggesting a beneficial influence of uric acid on both the quantity and quality of bone in males.

Acknowledgments

This study was supported by the project for Higher Education Research Promotion and National Research University Development, Office of the Higher Education Commission, and the Thailand Research Fund. We thank Roche Diagnostics (Thailand) Ltd. for providing the serum CTX and serum total P1NP assays used in this study.

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THYROID
Volume 23, Number 1, 2013

DOI: 10.1089/thy.2012.0001

High Vitamin D Status in Younger Individuals Is Associated with Low Circulating Thyrotropin

La-or Chailurkit,[1] Wichai Aekplakorn,[2] and Boonsong Ongphiphadhanakul[1]

Background: Vitamin D is an immunomodulator and may affect autoimmune thyroid diseases. Vitamin D has also been shown to influence thyrocytes directly by attenuating thyrotropin (TSH)-stimulated iodide uptake and cell growth. However, it is unclear how vitamin D status is related to TSH at the population level. The goal of the present study was to investigate the relationship between vitamin D status and TSH levels according to thyroid autoantibodies in a population-based health survey in Thailand.
Methods: A total of 2582 adults, aged 15–98 years, were randomly selected according to the geographical region from the Thailand 4th National Health Examination Survey sample. By study design, the genders were equally represented. Serum levels of 25-hydroxyvitamin D (25(OH)D), TSH, the thyroid peroxidase antibody (TPOAb), and the thyroglobulin antibody (TgAb) were measured in all subjects.
Results: The mean age was 55.0 ± 0.4 (SE) years. In subjects positive for serum TgAb, serum TSH levels were higher, whereas total serum 25(OH)D levels were lower. In addition, the prevalence of vitamin D insufficiency in TgAb-positive subjects was significantly higher than that observed in TPOAb- and TgAb-negative subjects, whether based on cutoff values of 20 or 30 ng/mL: 8.3% versus 5.6%, $p < 0.05$; or 47.6% versus 42.0%, $p < 0.05$, respectively. However, vitamin D status was not associated with positive TPOAb and/or TgAb after controlling for gender and age. To explore the probable interaction between vitamin D status and age on serum TSH, analyses were performed according to age tertiles; it was found that higher 25(OH)D levels were independently associated with lower TSH, but only in subjects in the lowest age tertile.
Conclusions: This population-based study showed that high vitamin D status in younger individuals is associated with low circulating TSH.

Introduction

Vitamin D is produced endogenously when the skin is exposed to sunlight, or obtained exogenously from nutrients or supplements. Despite this ability of dermal production, less sunshine or the tendency to avoid sun exposure can potentially lead to vitamin D insufficiency. Vitamin D insufficiency is now recognized to be highly prevalent and is observed to varying degrees in many different countries, regardless of geographical location (1–3). Despite the fact that Thailand is located near the equator, the prevalence of vitamin D insufficiency was found to be at least 45% in a population survey when using a 25(OH)D threshold of 30 ng/mL (4). Vitamin D enhances intestinal calcium absorption and vitamin D deficiency can lead to osteomalacia as well as an increased propensity for osteoporosis when vitamin D is inadequate (5). Besides its role in calcium and bone metabolism, vitamin D may also affect nonskeletal functions. Observational studies have demonstrated the association of vitamin D status with a number of common disorders, including atherosclerosis (6), hypertension (7), and diabetes (8,9). Whether vitamin D insufficiency is causally related to these disorders is still unclear. Recent studies in Thais also demonstrated that vitamin D insufficiency is associated with diabetes in older individuals (10). However, no relationship between vitamin D status and high blood pressure was found (11).

Vitamin D is well recognized as an immunomodulator (12), and vitamin D insufficiency has been associated with autoimmune thyroid disease (13). Besides potentially affecting the thyroid gland through immune-mediated processes, vitamin D has also been shown to influence thyrocytes directly by attenuating the thyrotropin (TSH)-stimulated iodide uptake and cell growth of rat thyroid cells (14). On the other hand, it has been shown that vitamin D modulates TSH secretion of pituitary thyrotrophs by binding to specific binding sites (15). Moreover, an increased TSH level has been observed after acute administration of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (16). It is unclear, however, how vitamin D

Departments of [1]Medicine and [2]Community Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

status is related to TSH levels at the population level. Therefore, the purpose of the present study is to investigate whether vitamin D status is associated with circulating TSH according to thyroid autoantibodies in a population-based health survey in Thailand.

Materials and Methods

Population

This study used data and blood samples from the Thai 4th National Health Examination Survey (NHES-IV) conducted in 2008–2009 by the National Health Examination Survey Office, Health Systems Research Institute. Subjects aged 15–98 years were randomly selected from 21 provinces in four geographical regions of Thailand as well as the capital city, Bangkok, using stratified, multistage probability sampling of the population aged ≥15 years, with a sample size of 21,960 individuals. Demographic data, such as age, sex, and religion, were included. Body mass index (BMI) was measured using standard procedure. Fasting blood samples were obtained and transferred to a freezer at a central laboratory in Ramathibodi Hospital, a university hospital in Bangkok, where they were kept at −80°C.

The present study used a subsample of the NHES-IV serum samples to measure serum levels of 25-hydroxyvitamin D (25(OH)D). The subjects were randomly selected in 1:1 sex ratio according to the age group (15–29, 30–44, 45–59, 60–69, 70–79, and ≥80 years), region, and urban/rural place of residence from a sample size of 21,960. In each stratum, 25 individuals were randomly selected using statistical software. A total of 2700 were sampled, of which 2587 serum samples were available. Five subjects with marked hypothyroidism (serum TSH greater than 50 mIU/L) were excluded from the study. The study was approved by the ethics committee of Ramathibodi Hospital. Informed consent was obtained from all subjects.

Serum 25-hydroxyvitamin D (25(OH)D) measurement

Serum 25(OH)D_2 and 25(OH)D_3 were analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany), coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Foster City, CA) using a MassChrom® 25-OH-Vitamin D_3/D_2 diagnostics kit (ChromSystems, Munich, Germany). The 25(OH)D assay was performed according to the manufacturer's instructions. This method used a deuterated 25(OH)D3 as an internal standard to correct for sample and instrument variability. Samples were analyzed using an atmospheric pressure chemical ionization source for maximum sensitivity. The 25(OH)D separation was performed using a Chromsystems precipitation reagent and trap column in conjunction with an Agilent 1200 high-performance liquid chromatography system configured for online sample preparation according to the configuration included in the documentation with this method. Briefly, 25(OH)D3 and 25(OH)D2 were extracted by mixing 100 μL of serum sample with 25 μL precipitation reagent and 200 μL of the internal standard solution. The mixture was vortexed for 20 seconds and incubated for 10 minutes at 4°C. After centrifugation of the mixture for 5 minutes at 9000 *g*, the upper layer was transferred to an autosampler vial and 5 μL was injected to the LC-MS/MS. The summation of serum 25(OH)D_2 and 25(OH)D_3 was used to reflect vitamin D status. The interassay and intra-assay coefficients of variation of total serum 25(OH)D level were 6.3% and 5.0%, respectively.

Serum TSH, Thyroid peroxidase antibody, and antithyroglobulin antibody (TgAb) measurements

Serum TSH, the thyroid peroxidase antibody (TPOAb) and the thyroglobulin antibody (TgAb) were measured by electrochemiluminescence immunoassay on a Cobas e 411 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The assays have an intra-assay precision of 3.6%, 9.2%, and 6.1%, respectively. Positive TPOAb and TgAb were defined as a value greater than 34 IU/mL and 115 IU/mL, respectively.

Statistical analysis

Data are expressed as mean ± SE. Differences between two groups were assessed by the Student's *t*-test. Logistic regression analysis was performed to identify the predictive variables. Linear regression analysis was used to examine the relationship between vitamin D status and serum TSH. A *p*-value less than 0.05 was considered statistically significant. All analyses were performed using SPSS statistical software package, version 16.0 (SPSS Inc., Chicago IL).

Results

Table 1 demonstrates the clinical and laboratory characteristics of the remaining 2,582 subjects. The mean age was 55.0 ± 0.4 (SE) years. The mean level of total serum 25(OH)D (summation of 25(OH)D_2 and 25(OH)D_3) was 32.5 ± 0.18 ng/mL. Women had a higher BMI, serum TgAb and TPOAb levels, but lower serum 25(OH)D levels than men. Figure 1 shows the distribution of log-transformed TSH levels. Four percent had serum TSH levels above 5 mIU/L, while 19.8% had serum TSH levels above 2.5 mIU/L. Of the 2582 subjects, 425 (16.5%) and 315 (12.2%) subjects were positive for serum TPOAb and TgAb, respectively. When subjects with positive serum TPOAb and/or TgAb were excluded, the distribution of TSH did not change appreciably.

Table 2 compares the clinical and laboratory characteristics based on positive serum TPOAb, TgAb, and TPOAb and/or TgAb. Subjects with positive serum TPOAb and/or TgAb

Table 1. Baseline Characteristics of the Population Study

Characteristics	*Men* (n = *1290*)	*Women* (n = *1292*)	*Total* (n = *2582*)
Age (years)	54.7 ± 0.61	55.2 ± 0.60	55.0 ± 0.42
BMI (kg/m^2)	22.7 ± 0.11	23.9 ± 0.14[a]	23.3 ± 0.09
Serum 25(OH)D (ng/mL)	35.0 ± 0.26	30.0 ± 0.22[a]	32.5 ± 0.18
Serum TSH (mIU/L)	1.99 ± 0.05	2.18 ± 0.06	2.09 ± 0.04
Serum TPOAb (IU/mL)	39.4 ± 3.7	75.3 ± 9.3[a]	57.4 ± 5.0
Serum TgAb (IU/mL)	82.7 ± 11.4	143.3 ± 12.7[a]	113.0 ± 8.5

Values are mean ± SE.
[a]Significantly different from men ($p < 0.001$).
TSH, thyrotropin; BMI, body mass index; TPOAb, thyroid peroxidase antibody; TgAb, thyroglobulin antibody.

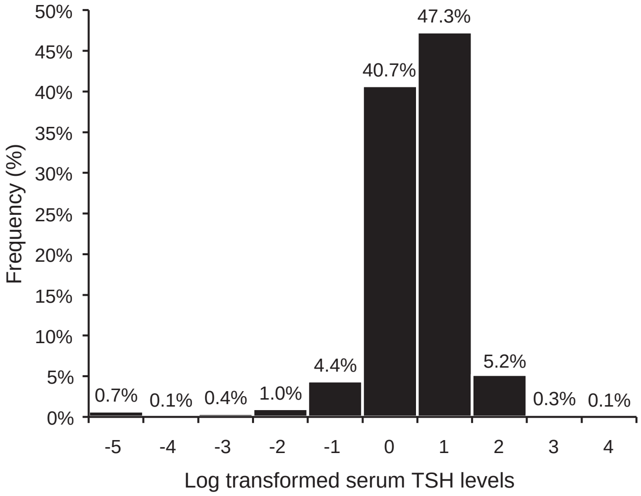

FIG. 1. Distribution of log-transformed serum thyrotropin (TSH) levels.

were older, had a higher serum TSH level, and included a lower proportion of males. After excluding subjects with positive TPOAb and TgAb, the mean serum TSH levels decreased from 2.09 ± 0.04 to 1.94 ± 0.03 mIU/L. In addition, the prevalence of vitamin D insufficiency [whether based on cutoff values of 20 or 30 ng/mL (17,18)] in TgAb-positive subjects was significantly higher than that observed in TgAb-negative subjects. Multivariate analysis using logistic regression revealed that independent determinants of positive TPOAb and/or TgAb were female gender and age. Serum 25(OH)D levels, however, were not associated with positive TPOAb and/or TgAb (Table 3), or positive TgAb alone (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/thy) after controlling for gender and age.

To account for the probable interaction between vitamin D status and age on serum TSH, further analyses were performed according to age tertiles. The mean age (±SD) of subjects in the first, second, and third tertiles were 29.2 ± 9.2., 57.6 ± 7.3, and 78.66 ± 5.4 years, respectively. Serum TSH levels were log-transformed before regression analysis. With regard to the relationship between vitamin D status and serum TSH, it was found that higher 25(OH)D levels were associated with lower TSH independent of age, gender, BMI, serum TPOAb, and/or TgAb only in subjects in the lowest age tertile (Table 4). Age, BMI, and thyroid autoantibodies, but not 25(OH) or gender, were associated with serum TSH in the upper age tertile.

Discussion

Similar to studies in other populations (19–21), the distribution of serum TSH in our study did not follow a true Gaussian distribution, but was skewed toward the higher values. In addition (also not unlike other previous studies), serum TSH levels were higher in subjects with evidence of autoimmune thyroid disease, as indicated by the presence of TPOAb and/or TgAb. After excluding subjects with positive TPOAb and/or TgAb, the mean serum TSH levels decreased, which is likely to more accurately reflect the mean reference value of serum TSH. It is now well established that serum TSH

Table 2. Comparison of Clinical and Laboratory Characteristics Based on the Positivity of Serum Antibodies

	Serum TPOAb			Serum TgAb			Serum TPOAb and/or TgAb		
	Positive (n = *426*)	*Negative* (n = *2156*)	p-*Value*	*Positive* (n = *315*)	*Negative* (n = *2267*)	p-*Value*	*Positive* (n = *564*)	*Negative* (n = *2018*)	p-*Value*
Age (years)	61.4 ± 0.93	53.7 ± 0.47	< 0.001	57.9 ± 1.12	54.6 ± 0.46	< 0.01	59.9 ± 0.84	53.6 ± 0.49	< 0.05
BMI (kg/m^2)	23.4 ± 0.23	23.3 ± 0.10	NS	23.9 ± 0.25	23.3 ± 0.10	NS	23.4 ± 0.19	23.31 ± 0.10	NS
Male gender [*n* (%)]	178 (41.9%)	1112 (51.6%)	< 0.001	87 (27.6%)	1203 (53.1%)	< 0.001	208 (36.9%)	1082 (53.6%)	< 0.001
Serum TSH levels (mIU/L)	2.7 ± 0.18	2.0 ± 0.03	< 0.001	2.9 ± 0.19	2.0 ± 0.04	< 0.001	2.6 ± 0.14	2.0 ± 0.03	< 0.001
Serum 25(OH)D (nM)	32.4 ± 0.45	32.5 ± 0.19	NS	31.5 ± 0.52	32.6 ± 0.19	NS	32.3 ± 0.39	32.54 ± 0.20	NS
Prevalence of vitamin D insufficiency [*n* (%)]									
25(OH)D < 20 nM	30 (7.1%)	124 (5.7%)	NS	26 (8.3%)	128 (5.6%)	< 0.05	39 (6.9%)	115 (5.7%)	NS
25(OH)D < 30 nM	183 (43.1%)	919 (42.6%)	NS	150 (47.6%)	952 (42.0%)	< 0.05	249 (44.1%)	853 (42.3%)	NS

Values are mean ± SE or number (percent). Cutoff values for serum TPOAb and serum TgAb were 34 IU/mL and 115 IU/mL, respectively.
NS, not significant.

Table 3. Determinants of Positive Serum Thyroid Peroxidase Antibody and/or Thyroglobulin Antibody

	Adjusted OR [95% CI]	*p-Value*
Age (years)	1.02 [1.01–1.02]	< 0.001
Male gender	0.51 [0.42–0.62]	< 0.001
BMI (kg/m^2)	1.00 [0.98–1.03]	NS
Serum 25(OH)D (ng/mL)	1.00 [0.99–1.01]	NS

OR, odds ratio; CI, confidence interval.

levels are partially defined by ethnicity; hence, the reference value of serum TSH should be determined specifically based on ethnicity. Besides thyroid autoimmunity, factors previously reported to be related to TSH levels or thyroid hormones include age, gender, smoke exposure, and ethnicity (19,20,22). The association of circulating TSH with age and gender was also reflected in the present study.

With regard to the immune system, it has been found that vitamin D regulates the differentiation and activation of CD4+ T lymphocytes and can prevent the development of certain autoimmune disorders (23). Moreover, 1,25-dihydroxyvitamin D (1,25(OH)$_2$D) reduces the number of antigen-presenting cells *in vitro* (24). *In vivo*, 1,25(OH)$_2$D has a direct immunosuppressive effect on dendritic cells and reduces the production of cytokines (25). Regarding autoimmune thyroid disease, previous studies have demonstrated a lower vitamin D status in Graves' disease (GD) (26,27), Hashimoto's thyroiditis (13), or thyroid autoimmunity (28). Moreover, an association between Graves' disease and vitamin D receptor (VDR) polymorphisms has been demonstrated in a number of studies (29). The effect of vitamin D on Graves' disease may not be mediated through the inhibition of antibody production, but by its effect on modulating autoimmune-induced thyroid hormone production (30).

In the present study, we could not demonstrate an independent relationship between vitamin D status and thyroid autoimmunity as assessed by the presence of TPOAb and/or TgAb. However, a number of limitations exist in the present study. First, TSH receptor (TSHR) stimulating antibodies were not measured in our study. Second, the study was cross-sectional in nature and the causative role of vitamin D, if any, on thyroid autoimmunity could not be readily determined. Lastly, in addition to environmental factors, genetics plays an important role in thyroid autoimmunity. Many genes have been proposed to be associated with GD (31), including genes encoding human leukocyte antigen (HLA) class II, cytotoxic T-lymphocyte antigen-4 (CTLA-4), protein tyrosine phosphatase-22 (PTPN22), TSHR, and more recently CD40 (32) and Fc receptor-like 3 (FCRL3) (33). It is possible that there might be an interaction between vitamin D status and genetic determinants of thyroid autoimmunity; this may render the effect of vitamin D less apparent if genetic variation in relation to thyroid autoimmunity is not concurrently determined.

We found in the present study that a higher circulating 25(OH)D was associated with lower TSH levels in younger individuals. Besides affecting the function of the thyroid gland through autoimmunity, it is likely that vitamin D may influence the thyroid gland through its action on the central nervous system and the thyrotrophs. There is evidence that vitamin D affects central nervous system function. Low vitamin D status may adversely affect brain development (34) and neurocognitive function (35). Moreover, vitamin D can enhance the responsiveness in TRH-induced TSH secretion of rat pituitary thyrotrophs (36). On the other hand, VDRs are present in thyrocytes and modulate their differentiation and function (15). It is therefore conceivable that the lower TSH in the presence of a higher vitamin D status observed in the present study could also be the result of increased thyroid hormones caused by the stimulatory effect of vitamin D on thyrocytes. (37). However, the underlying basis of the observed association of high vitamin D status with low serum TSH that is only present in the younger age group is unclear. Further clinical investigation of the effect of vitamin D supplementation on circulating TSH and thyroid hormones, particularly in younger individuals, is needed to elucidate an effect of vitamin D on either the thyrotrophs or the thyroid gland that might provide insight into this relationship.

Table 4. Standardized Regression Coefficients of Variables in Relation to Serum Thyrotropin According to Age Tertiles

	Age (years)					
	15–44 (n = *871*)		*45–69* (n = *864*)		*≥ 70* (n = *847*)	
	Beta	p	*Beta*	p	*Beta*	p
Age (years)	−0.05	NS	0.05	NS	0.13	< 0.001
Male gender	−0.02	NS	−0.03	NS	0.01	NS
BMI (kg/m^2)	0.04	NS	0.07	< 0.05	0.10	< 0.01
Presence of TPOAb and/or TgAb	−0.03	NS	−0.04	NS	0.09	< 0.01
Serum 25(OH)D (ng/mL)	−0.14	0.001	−0.05	NS	−0.01	NS

Acknowledgments

This study was supported by: the Thailand Research Fund; the Health Systems Research Institute; the Bureau of Policy and Strategy, Ministry of Public Health; the Thai Health Promotion Foundation; and the National Health Security Office, Thailand. NHES-IV was conducted by the National Health Examination Survey Office, Health Systems Research Institute, Thailand. The NHES-IV study group included the following offices/regions: National Health Examination Survey Office (Wichai Aekplakorn, Rungkarn Inthawong, Jiraluck Nontaluck, Supornsak Tipsukum, Yawarat Porrapakkham); northern region (Suwat Chariyalertsak, Kanittha Thaikla [Chiang Mai University], Wongsa Laohasiriwong, Wanlop Jaidee, Sutthinan Srathonghon, Ratana Phanphanit, Jiraporn Suwanteerangkul, Kriangkai Srithanaviboonchai); northeastern region (Pattapong Kessomboon, Somdej Pinitsoontorn, Piyathida Kuhirunyaratn, Sauwanan Bumrerraj, Amornrat Rattanasiri, Suchada Paileeklee, Bangornsri Jindawong, Napaporn Krusun, Weerapong Seeuppalat [Khon Kaen University]); southern region (Virasakdi Chongsuvivatwong, Rassamee

Sangthong, Mafausis Dueravee [Prince of Songkla University]); central region (Surasak Taneepanichskul, Somrat Lertmaharit, Vilai Chinveschakitvanich, Onuma Zongram, Nuchanad Hounnaklang, Sukarin Wimuktayon [Chulalongkorn University]); Bangkok region (Panwadee Putwatana, Chalermsri Nuntawan, Karn Chaladthanyagid [Mahidol University]). We thank Roche Diagnostics Thailand for kindly providing the TSH and TgAb assays and partial support TPOAb assay used in this study.

Disclosure Statement

The authors declare that no competing financial interests exist.

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Address correspondence to:
La-or Chailurkit, Ph.D.
Division of Endocrinology and Metabolism
Department of Medicine
Faculty of Medicine
Ramathibodi Hospital
Mahidol University
Rama 6th Road
Bangkok 10400
Thailand

E-mail: laor.cha@mahidol.ac.th

Received Date : 4-May-2012
Returned for Revision: 1-Jun-2012
Finally Revised Date : 21-Sep-2012
Accepted Date : 21-Sep-2012
Article category : B

Causal inference of the effect of adiposity on bone mineral density in adults

Daruneewan Warodomwichit[*], Chanika Sritara[†], Ammarin Thakkinstian[‡], La-or Chailurkit[*], Sukit Yamwong[*], Wipa Ratanachaiwong[¶], Boonsong Ongphiphadhanakul[*] and Piyamitr Sritara[*]

[*] *Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand,* [†] *Department of Radiology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand,* [‡] *Section for Clinical Epidemiology and Biostatistics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand and* [¶] *Medical and Health Office, Electricity Generating Authority of Thailand, Nonthaburi, Thailand*

Running title: Causal effect of adiposity on bone mineral density

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cen.12061

Correspondence: Boonsong Ongphiphadhanakul, M.D., Department of Medicine, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand.

Tel.: +66 2 201 2206; Fax +66 2 201 1715;

E-mail: boonsong.ong@mahidol.ac.th

Keywords: obesity-associated gene, bone mineral density, causal inference, Mendelian randomization

Summary

Objective The causal effect of adipose tissue on bone mass and the direction of its net influence have not been directly assessed in adult humans. Using the Mendelian randomization analysis, we assessed the causality of adiposity in measurements of bone mass in adult males and females.

Design and Methods Subjects consisted of 2,154 adults aged 25–54 years from a cross-sectional cohort of the employees of the Electricity Generating Authority of Thailand. Body composition was determined after at least 3 h of fasting using multi-frequency bioelectrical impedance analysis. Bone mineral density was assessed by dual energy X-ray absorptiometry. A polymorphism in the fat mass and obesity-associated gene (*FTO* rs9939609) was used as an instrument in the Mendelian randomization analysis.

Results The genotype distribution of the *FTO* rs9939609 polymorphism was 61.1% TT, 33.9% AT and 5.0% AA. The average body mass index (BMI) , body fat mass and percent body fat were 23.9 kg/m^2 (SD = 3.6), 17.9 kg (SD = 6.6) and 26.8 % (SD = 7.2) , respectively. The *FTO* rs9939609 polymorphism was significantly correlated with BMI (coefficient = 0.673 kg/m^2, $P < 0.001$), body fat mass (coefficient = 0.948 kg, $P < 0.001$) and percent body fat (coefficient = 0.759 %, $P < 0.01$) . An instrumental variable (IV) regression model, using BMI as the intermediate phenotype, suggested that *FTO* was a strong IV. Also, the *FTO*-BMI polymorphism was significantly associated with total hip and femoral neck bone mineral density (BMDs) but was not correlated with total spine BMD, with estimated correlation coefficients of 0.0189 (95% CI: 0.0046, 0.0332), 0.0149 (95% CI: 0.0030, 0.0268) and 0.0025 (95% CI: -0.0131, 0.0136) g/cm^2, respectively. The variances of BMDs explained by the *FTO*-BMI were 19.0%, 21.3%, and 1.1%, respectively. Similar trends were also observed for the *FTO*-body fat mass and *FTO*-percent body fat correlations.

Conclusions Mendelian randomization analysis suggests that adiposity might be causally related to bone mineral density at the femur but not at the spine.

Introduction

Osteoporosis is a health problem worldwide. A number of risk factors are related to susceptibility to osteoporosis, including estrogen deficiency, sedentary lifestyle and reduced adiposity. Adipose tissue is now considered an endocrine organ. In addition to its conventional role in energy storage, adipose tissue secretes a number of bioactive proteins which influence a variety of biological processes, including energy homeostasis and inflammation. As far as bone is concerned, leptin, an adipokine secreted from adipose tissue, has been shown to diminish bone formation through a central nervous system delay in animal models. In humans, a number of adipokines, including leptin,[1,2] adiponectin[3–5] and omentin-1,[6] have been shown in observational studies to be variably related to bone mineral density (BMD).

Although results from animal models and observational studies in humans suggest that adiposity influences bone mass, the effects of adipokines on bone mass are different. Results from observational studies can be confounded by factors which influence both adiposity and bone mass, such as body size and weight. Moreover, the causality of adipose tissue on bone mass and the direction of net influence have not been directly assessed in adult humans.

The fat mass and obesity-associated (*FTO*) locus on chromosome 16 (16q12.2) has been identified from genome–wide association studies as a major candidate gene for obesity in children and adults.[7–9] The *FTO* rs9939609 polymorphism is of particular interest, since it was found to be associated with obesity in different ethnic groups.[8,10–13] The finding of common genetic variants of *FTO*, which have been consistently associated with adiposity, provided an opportunity to conduct a Mendelian randomization study of obesity and bone outcomes. Therefore, utilizing the Mendelian randomization analysis, we assessed the causality of adiposity in the attainment of BMD in adults.

Materials and methods

The study design was a cross-sectional cohort of the employees of the Electricity Generating Authority of Thailand (EGAT). Prior to commencement, the study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University. All subjects gave written informed consent. Survey data were collected using a self-administered questionnaire, physical examination, electrocardiography, chest radiography and blood analysis.

BMD measurements

After changing into lightweight clothing without dense objects, each participant underwent dual-emission X-ray absorptiometry (DXA) (Hologic QDR 4500W; Bedford, MA) to obtain BMD values of the lumbar spine (L1–L4) and left proximal femur (femoral neck and total hip). The DXA procedure complied with the ISCD Position Statement.[14] For lumbar spine DXA, each participant lay flat on the midline of the imaging table, with legs elevated by a supporting pillow so that the spine was straight. The DXA scan included T12 and L5 vertebrae and both iliac crests. For the proximal femur DXA, the participant lay supine with the left foot fixed to a positioning device to keep the hip internally rotated and adducted so that the femoral shaft was straight, with minimal visualization of the lesser trochanter while the ischium and the greater trochanter were included on the scan. The scans were then analyzed according to the manufacturer's recommendations. Each morning prior to the scheduled DXA scans, a quality–control procedure was performed as recommended by the manufacturer to ensure machine precision of more than 98.5%. The BMD coefficients of variation were 0.82%, 2.52% and 1.51% for the lumbar spine, femoral neck and total hip, respectively.

Adiposity measurements

Anthropometric variables including weight, height and waist circumferences were measured using standard techniques in all studies. Body mass index (BMI) was derived by weight (kg)/height $(m)^2$. Body composition was determined after at least 3 h of fasting using multi-frequency bioelectrical impedance analysis with eight-point tactile electrodes (InBody 720; Biospace, Seoul, Korea).

FTO genetic analysis

DNA was extracted by phenol-chloroform method.[15] Individual genotyping of all subjects was performed using real-time PCR (TaqMan® MGB probes): 10 ng of DNA was added into the PCR reaction, consisting of TaqMan Universal Master Mix (1x) and TaqMan MGB probes for *FTO* rs9939609 SNP (1x) in a total volume of 10 μL. The real-time PCR reaction protocol was 10 min at 95 °C, 40 cycles of 15 s at 92 °C, and 1 min at 60 °C using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were expressed as mean (or median, where appropriate) and frequency for continuous and categorical data, respectively. Hardy–Weinberg equilibrium was assessed using an exact test. [16] Relationships between the *FTO* polymorphism and variables were assessed for both males and females using linear regression and a chi-square test (or exact test, where appropriate) for continuous and categorical data, respectively.

Mendelian randomization analysis[17] was applied to assess causal relationships between *FTO*, adiposity (i.e. BMI and body fat mass) and BMD. Instrumental variable (IV) regression with two-stage least squares method was applied to explore these causal relationships, using the *FTO* polymorphism with additive effect as the IV and BMI/body fat mass as the endogenous variables.[18,19] These models were also adjusted for confounding variables (i.e. alcohol, age and gender), since univariate analysis suggested that they were associated with intermediate phenotype and/or BMD. In the first-stage regression, the F–statistic (hereafter called F-First) was used to assess whether the *FTO* polymorphism was sufficiently strong to be an IV. A value of F-First greater than 10 indicated that the *FTO* was a strong IV, and thus the estimated causal relationships should be valid. In addition, linear regression with ordinary least squares (OLS) method was also applied to directly assess the association between *FTO*, adiposity and BMD. The Durbin–Wu–Hausman statistic was applied to compare the results between the IV and OLS regression approaches. All analyses were performed using STATA version 12.0. A *P*-value of less than 0.05 was considered statistically significant.

Results

Table 1 describes the clinical characteristics, adiposity and BMD of the study subjects (n = 2,154). The mean age of the subjects was 40.2 years (SD = 6.9). The average body mass index (BMI) , body fat mass and percent body fat were 23.9 kg/m^2 (SD = 3.6), and 17.9 kg (SD = 6.6) and 26.8 % (SD = 7.2)), respectively. The correlation matrix between parameters of adiposity and BMD is shown in Table 2. Measures of adiposity, including BMI, body fat mass and percent body fat, were all significantly related to BMD at all skeletal sites.

The association of measures of adiposity and BMD can be confounded by variables such as age, gender, alcohol consumption and cigarette smoking. For being an IV, the *FTO* polymorphism should not be associated with these potential confounders. Therefore, associations between the subject characteristics and the *FTO* genotypes were assessed. As described in Table 3, none of these potential confounders were associated with the *FTO* polymorphism. The *FTO* genotype frequencies complied with Hardy–Weinberg equilibrium rules ($P = 0.510$).

Relationships between adiposity (i.e. BMI, body fat mass and percent body fat) and the *FTO* polymorphism were explored (Table 4). The mean BMI was significantly higher in minor homozygous and heterozygous genotypes compared to the major homozygous genotype ($P < 0.001$). Applying linear regression analysis by fitting *FTO* as an additive effect suggests that the *FTO* polymorphism was significantly correlated with BMI (coefficient = 0.637 kg/m^2, $p < 0.001$); indicating that carrying an A allele would increase BMI of 0.637 kg/m^2. This trend was also observed with body fat mass and percent body fat, i.e. mean body fat mass or percent body fat in minor homozygous and heterozygous genotypes was significantly higher than in the major homozygous genotype ($P < 0.001$).

Table 5 describes the results from OLS and IV regression analyses by measures of adiposity and skeletal sites. In the IV regression model using BMI as the intermediate phenotype, *FTO* was a strong IV for total hip, femoral neck, and total spine BMD, with F-statistics of 25.7, 21.9, and 21.8, respectively. The *FTO*-BMI was also significantly associated with total hip and femoral neck BMDs but not with total spine BMD, with estimated correlation coefficients of 0.0189 (95% CI: 0.0046, 0.0332), 0.0149 (95% CI:

0.0030, 0.0268) and 0.0025 (95% CI: −0.0131, 0.0136) g/cm^2, respectively. The variances of BMDs explained by the *FTO*-BMI were 19.0%, 21.3%, and 1.1%, respectively. Similar trends were also observed for *FTO*-body fat mass, i.e. the *FTO*-body fat mass was significantly associated with total hip and femoral neck BMDs but not with total spine BMD, with correlation coefficients of 0.0122 (95% CI: 0.0023, 0.0221), 0.0086 (95% CI: 0.0005, 0.0167) and 0.0012 kg (95% CI: −0.0074, 0.0098), respectively. Similar results for percent body fat was obtained. The F-First statistics showed that *FTO* was a strong IV for all BMDs.

The results of OLS regression suggest that both BMI and body fat mass correlate significantly with all BMD sites after adjusting for *FTO* and other covariables. Although the magnitude of adiposity effects from IV estimates were higher than the OLS estimates for all BMDs except total spine BMD, Durbin–Wu–Hausman tests did not reach statistical significance (Table 5).

Discussion

In the present study, using a Mendelian randomization analysis, we have demonstrated that adiposity is likely to play a causal role in the determination of bone mass. The magnitude of the effect, however, is relatively small. Although causality is suggested by these analyses, the underlying mechanism(s) still require further investigation. In addition to being the source of adipokines, adipose tissue is able to convert androgens to estrogens through the *CYP19* aromatase enzyme,[20] which may account for the observation that obese postmenopausal women have lower bone turnover, higher bone mass and fewer fractures.[21] Given the myriad of possible mechanisms affecting the influence of adiposity on bone mass, it remains unclear which one plays a predominant role; and it is likely that additional underlying possibilities still remain to be discovered. On the other hand, it is well accepted that body size is also associated with higher areal BMD. It is therefore unclear whether adiposity directly leads to high bone mass or is simply related to BMD because of the confounding effect of body size and weight. Using the instrumental variable analysis, our results suggest that there is a net positive effect of adiposity on BMD which is not likely to be confounded by body size and weight, a finding that is in agreement with a previous study using a different approach.[22]

It is noteworthy that in the present study the causal role of adiposity in the determination of bone mass is only apparent in the femur. Of the skeletal sites used for BMD measurement, the vertebra is comprised of more trabecular bone compared to the femur, which suggests that adiposity may predominantly influence cortical bone. The underlying reason for this phenomenon is not entirely clear. Metabolic differences exist between skeletal sites rich in trabecular bone and cortical bone. Trabecular BMD changes more (in either direction) in response to bone stimuli, including exercise[23] and antiresorptive[24] or bone–forming agents such as intermittent teriparatide.[24] However, unlike other bone active stimuli, parathyroid hormone (PTH), particularly when continuously elevated, affects cortical bone to a greater extent than trabecular bone.[25] Since a number of studies[27–29] have found that adiposity and serum PTH are correlated, it is conceivable that the influence of adiposity predominantly on cortical bone may be partly mediated through the effect of PTH. Such a hypothesis cannot be readily tested in the present study since serum PTH data are lacking. Further investigations involving the potential causal relationships among adiposity, PTH and bone mass are warranted.

The Mendelian randomization analysis has been increasingly utilized in health research to assess causality based on genetic observational studies. A proper Mendelian randomization study must comply with a number of assumptions.[24,30] In the present study the *FTO* genotype was strongly associated with adiposity, which satisfies one of the assumptions for a proper instrumental variable. The associations between the *FTO* gene and osteoporosis phenotypes have recently been described.[31] The effect is likely to be mediated through the influence of adiposity. However, since the function of the *FTO* gene is not entirely known, the possibility remains that there might be other *FTO* gene–determined confounders of adiposity and bone mass; this would render the use of the *FTO* gene as an instrumental variable in this case less valid.

In conclusion, the Mendelian randomization analysis suggested that adiposity might be causally related to bone mineral density in the femur but not in the spine.

Acknowledgements

This study was supported by the Thailand Research Fund and Mahidol University.

Conflicting interests and financial disclosure

Nothing to declare.

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Table 1. Clinical characteristics of cohort

Characteristic	Mean (SD)
Age (years)	40.0 (7.4)
Body weight (kg)	66.1 (12.5)
Height (cm)	166.2 (7.8)
BMI (kg/m^2)	23.9 (3.6)
Body fat mass (kg)	17.9 (6.6)
Percent body fat (%)	26.8 (7.2)
Cigarettes smoking/day, median (range)	0 (0–50)
Alcohol consumption, number (%)	982/2,325 (42.2)
Lumbar spine BMD (g/cm^2)	0.975 (0.118)
Femoral neck BMD (g/cm^2)	0.801 (0.121)
Total femur BMD (g/cm^2)	0.925 (0.129)

Table 2. Correlation matrix between measures of adiposity and BMD

	BMI	Body fat mass(kg)	Body fat mass(%)	Lumbar BMD	Femoral neck BMD	Total femur BMD
BMI	1.0000					
Body fat mass(kg)	0.85 (< 0.001)	1.0000				
Body fat mass(%)	0.51 (<0.001)	0.82 (<0.001)				
Lumbar BMD	0.19 (< 0.001)	0.14 (< 0.001)	0.03 (0.13)	1.0000		
Femoral neck BMD	0.39 (< 0.001)	0.23 (< 0.001)	-0.02 (0.39)	0.62 (< 0.001)	1.0000	
Total femur BMD	0.39 (< 0.001)	0.22 (< 0.001)	-0.03 (0.162)	0.57 (< 0.001)	0.81 (< 0.001)	1.0000

Table 3. Description and comparison of characteristics of subjects, according to *FTO* genotypes

Characteristic	*FTO* genotype			*P*-value
	TT (n = 1315)	AT (n = 731)	AA (n = 108)	
Age, mean (SD)	40.1 (6.9)	40.0 (6.9)	40.3 (6.8)	0.500
Gender				
Male	949 (72.2%)	532 (72.8%)	79 (73.2%)	0.955
Female	366 (27.8%)	199 (27.2%)	29 (26.8%)	
Alcohol consumption				
Yes	565 (43.0%)	305 (41.7%)	46 (42.6%)	0.941
No	749 (57.0%)	426 (58.3%)	62 (57.4%)	
Cigarette smoking, median (range)	0 (0,50)	0 (0,30)	0 (0,15)	0.526

Table 4. Relationship between adiposity and *FTO* genotypes

Adiposity	*FTO* rs9939609			B (linear regression**)	P value
	TT	AT	AA		
BMI	23.7(3.7)*	24.3(3.6)	25.4(4.2)	0.637	< 0.001
Body fat mass (kg)	17.6(6.6)	18.3(6.9)	20.2(7.9)	0.948	< 0.001
Percent body fat (%)	26.6(7.2)	27.1(7.1)	28.6(8.2)	0.759	0.006

*mean (SD), **additive effect of *FTO* locus

Table 5. Linear and IV regression analysis of the relationships between BMD and BMI (A), body fat mass (B) and percent body fat
(C)

A

BMI (kg/m^2)	Linear regression			IV regression				
	β	95% CI	*P*-value	β	95% CI	*P*-value	F-First*	WH *P*-value**
Total hip BMD (g/cm^2)	0.0138	0.0124, 0.0153	< 0.001	0.0189	0.0046, 0.0332	0.010	25.734	0.486
Femoral neck BMD (g/cm^2)	0.0119	0.0107, 0.0132	< 0.001	0.0149	0.0030, 0.0268	0.014	21.864	0.629
Total spine BMD (g/cm^2)	0.0069	0.0056, 0.0083	< 0.001	0.0025	−0.0131, 0.0136	NS	21.826	0.313

B

Body fat mass (kg)	Linear regression			IV regression				
	β	95% CI	*P*-value	β	95% CI	*P*-value	F-First*	WH *P*-value**
Total hip BMD (g/cm^2)	0.0052	0.0043, 0.0061	<0.001	0.0122	0.0023, 0.0221	0.016	15.378	0.142
Femoral neck BMD (g/cm^2)	0.0045	0.0041, 0.0055	<0.001	0.0086	0.0005, 0.0167	0.037	15.377	0.348
Total spine BMD (g/cm^2)	0.0026	0.0019, 0.0034	<0.001	0.0012	-0.0074, 0.0098	0.790	15.303	0.725

C

Percent body fat (%)	Linear regression			IV regression				
	β*	95% CI	*P*-value	β	95% CI	*P*-value	F-First**	WH *P*-value***
Total hip BMD (g/cm^2)	0.0035	0.0026,0.0044	<0.001	0.0134	0.0019, 0.0250	0.023	17.188	0.067
Femoral neck BMD (g/cm^2)	0.0032	0.0024,0.0040	<0.001	0.0094	0.0002, 0.0187	0.046	17.188	0.168
Total spine BMD (g/cm^2)	0.0013	0.0004, 0.0021	0.002	0.0013	-0.0079, 0.0104	0.784	17.090	0.997

* β =Regression coefficient, **First-stage regression F-statistic, *** Durbin–Wu–Hausman test comparing ordinary least square vs. instrumental variable regression