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Research paper

Smoking is a significant determinant of low serum vitamin D in young and middle-aged healthy males

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ABSTRACT

OBJECTIVE: We aimed to determine the prevalence of 25(OH)D (D_2 and D_3 independently) inadequacy in healthy young/middle-aged men and to investigate its relationship with BMD, bone markers, demographic and lifestyle parameters such as age, BMI, smoking, alcohol consumption and dietary calcium intake. DESIGN: We determined 25(OH)D levels using LC-MS/MS, a robust method for measurement of both 25(OH)D_3 and 25(OH)D_2, iPTH, osteocalcin, beta C terminal cross-linked telopeptides of type I collagen (b-CTXs), procollagen type 1 amino-terminal propeptide (PINP), BMD at L_2-L_4 and proximal femur, smoking habits, daily dietary calcium intake and alcohol consumption in 181 randomly selected healthy men aged 20-50y. RESULTS: The prevalence of vitamin D deficiency (25(OH)D <20ng/ml) was 50.3%. Only 8.8% of the participants had vitamin D sufficiency (25(OH)D ≥30ng/ml). We found a strong correlation between 25(OH)D and smoking in the totality of participants (p<0.001). 25(OH)D level was lower by approximately 4.3 ng/dl (p<0.001) in a smoker compared to a non-smoker among the totality of participants, while this value increased to 9.2ng/ml in the 40-50y subgroup (p=0.003). A multinomial logistic regression model demonstrated that a young smoker (20-29y) had 58% increased likelihood of having vitamin D deficiency compared to a non-smoker of the same age group (p=0.041). CONCLUSIONS: A high prevalence of vitamin D deficiency was identified in a young and middle-aged male population. Smoking is a significant determinant of serum 25(OH)D, while it increases significantly the likelihood of having vitamin D deficiency. In our hands, vitamin D levels are not a determinant of bone turnover and BMD in this population.

Key words: BMD, BMI, LC-MS/MS, Men, Smoking, Vitamin D

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INTRODUCTION

The important role of vitamin D in bone health has long been recognized.[1] Several recent studies have suggested that inadequate serum 25-hydroxyvitamin D (25(OH)D) concentration is associated with bone loss through, among others, secondary hyperparathyroidism and resulting high bone remodeling.

However, most of the studies indicating these relationships have been performed in elderly people—mainly women—while data on healthy young/middle aged men are limited.[2-7]

Vitamin D exists in two forms: vitamin D_2 or ergocalciferol which is found naturally in foods of plant origin, and vitamin D_3 or cholecalciferol which is mainly synthesized in the skin by exposure to ultraviolet-B light and is also abundant in foods of animal origin. The main source of vitamin D is via exposure of the human skin to sunlight between 10am and 3pm in the spring, summer and fall.[8] It is important to know that circulating 25(OH)D concentration is the best indicator of whole body vitamin D status and is used for the classification of vitamin D status into deficient (25(OH)D <20ng/ml), insufficient (25(OH)D <30ng/ml) or sufficient (25(OH)D ≥30mg/ml) vitamin D status.[9] Although the structural differences between D_2 and D_3 alter their metabolism, in general, the biologic activity of their active metabolites is comparable.[8] However, recent studies found different associations between these two forms with respect to such conditions as Alzheimer's disease and cardiovascular risk factors in childhood.[10,11]

Hypovitaminosis is a worldwide health problem with the estimated percentages of people suffering from vitamin D deficiency ranging from 31% in Australia to 98% in Mongolia.[12] It is assumed that people living in countries with high amounts of sunlight may have a lower risk of vitamin D deficiency. However, recent studies have indicated that the prevalence of vitamin D deficiency even in tropical countries is as high as that observed in Western populations.[13]

Therefore, the objectives of our study were: 1) to determine the prevalence of 25(OH) D (D_2 and D_3 independently) inadequacy in young/middle-aged men living in urban and suburban areas of Athens, Greece, and 2) to investigate its relationship with BMD, parathyroid function and bone turnover markers as well as demographic and lifestyle parameters such as age, BMI, smoking, alcohol consumption and dietary calcium intake.

SUBJECTS AND METHODS

Population studied

Study participants were selected from the civilian personnel of the Hellenic Air Force living in urban and suburban areas of Athens and undergoing annual routine blood and urine tests. Recruitment took place over three months (September 2012 to November 2012). All the participants were healthy men aged 20-50 years, having normal blood counts and normal results for liver and kidney function tests. Exclusion criteria were any treatment or medical complications known to affect vitamin D and bone metabolism, such as primary hyperparathyroidism, cancer, malabsorption syndrome, hyperthyroidism, diabetes mellitus, pituitary, adrenal, gonadal and rheumatic diseases, as well as a history of immobility for more than one month. In addition, participants had not taken vitamin D and/or calcium supplements for the last 12 months. After screening of 216 individuals, a total of 192 individuals fulfilled the inclusion criteria. Out of these 181 attended the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Ethics Committee of the University of Athens. Written informed consent was obtained from all the participants of the survey. All men underwent a general physical examination. Measurements of body weight were obtained to the nearest 0.1 kg using a standard balance beam, and measurements of height were obtained to the nearest 0.1 cm using the wall-mounted stadiometer. Body mass index (BMI) was calculated as weight (kilograms) divided by height squared (square meters).

Biochemical determinations

Venous blood samples were collected in the morning between 0800 and 0900 hours under standardized conditions after an overnight fast. Serum samples were prepared immediately after phlebotomy and stored at -85°C for the measurement of the serum levels of calcium, phosphate, albumin, alkaline phosphatase

(ALP), intact parathyroid hormone (iPTH), beta C terminal cross-linked telopeptides of type I collagen (b-CTX), amino-terminal propeptide of type I collagen (P1NP), osteocalcium (OC) and 25-hydroxyvitamin D2 ($25(OH)D_2$) and 25-hydroxyvitamin D_3 ($25(OH)D_3$).

Serum intact PTH levels were measured using sandwich immunoassay (PTH STAT; Roche Diagnostics). Serum levels of b-CTX, intact-OC and P1NP were determined using an ECLIA Cobas e601 analyzer (Roche Diagnostics). The intra-assay and inter-assay CVs were 2% and 2.5%, respectively, for P1NP, 1.5% and 1.8%, respectively, for b-CTX, 1% and 2%, respectively, for OC.

The levels of $25(OH)D_3$ and $25(OH)D_2$ were determined in serum of participants using Liquid-Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) technology. The assay was carried out according to Chromsystems Instruction Manual for LC-MS/MS Analysis (Mass Chrom $25(OH)D_3/D_2$ in serum/plasma) and use of LC-MS/MS APT5000 (Applied Biosystems). Because the lower limit of quantification (LLOQ) is MS/MS system dependent, we calculated the LLOQ for Mass Chrom $25(OH)D_3/D_2$ in serum/plasma (Chromsystems) in LC-MS/MS API5000. To this end, we diluted the calibrator 1 (Lot no.1409 Chromsystems 3PLUS 1 Multilevel Plasma Calibrator set $25(OH)D_3/D_2$) with distilled H_20 (1:20 and 1:30 dilution). The signal to noise (S/N) ratio was calculated and it was found to be >10 over 5 consecutive batches for both $25(OH)D_3$ and $25(OH)D_2$. Based on the above, we calculated that the LLOQ is / was 0.5ng/ml for the $25(OH)D_3/D_2$ assay. The inter- and intra-assay CVs were <7% and <5%, respectively. 25(OH)D concentrations were calculated as the sum of $25(OH)D_2$ and $25(OH)D_3$.

Bone Mineral Densitometry

Bone mineral density was measured at the lumbar spine (L2-L4) and proximal femur, using dual-X-ray absorptiometry (DXA), on a Lunar DPX densitometer (Lunar, Madison, WI, USA). Values for results of DXA measurements were expressed as BMD (g/cm2) and T score and Z scores of a healthy reference population, as supplied by the manufacturer. Short-term precision for spine and proximal femur measurements had a coefficient of variation (CV) of 1% to 2%. Age, body weight, height were recorded by the same physician.

Other measurements

All subjects were medically examined and interviewed using a standardized questionnaire to collect information on smoking habits, dietary calcium intake and alcohol consumption. Smoking was categorized as a dichotomous variable: non-smokers (never smokers and ex-smokers, i.e. responders who had stopped smoking at least one year before the study) and current smokers.

In order to assess calcium intake, the consumption of foods representing the major sources of daily calcium intake, such as typical Greek cheeses (feta cheese and kasseri cheese), yogurt and milk was recorded in a weekly food-frequency questionnaire. This was determined through an individual face to-face interview. A fixed range of food containers, i.e. a glass for milk and a cup for yogurt, was used to standardize portion sizes, each containing ≈300 mg of calcium. A similar amount of calcium was contained in the reference servings of feta and kasseri cheese (≈70 and 40 g, respectively). Color photographs were shown to the participants to demonstrate the standard sizes of the cheese servings. The number of servings eaten weekly was recorded and calcium intake per week was estimated and expressed as mg of calcium per week.

Questions about the consumption of beer, wine and spirits were included in each questionnaire, which permitted us to evaluate the weekly consumption of ethanol expressed as gr of alcohol per week.

Statistical analysis

Data were expressed as mean±standard deviation (SD) for continuous variables and as percentages for categorical data. The Kolmogorov-Smirnov test was utilized for normality analysis of the parameters.

Bivariate analyses were made by using the Student t-test and Pearson correlation coefficients to analyze the relation between the dependent variable [25(OH)D] and the quantitative and qualitative demographic and clinical variables respectively. Multiple linear regression analysis (enter method) was performed to determine a multivariate summary model of determinants of dependent variable. All assumptions of linear regression analysis were also examined.

Moreover, multinomial logistic regression, using as dependent variable the three categories of 25(OH)D status (deficiency, insufficiency, sufficiency: <20, 20-29, ≥30 ng/ml, respectively), was used to analyze the relationship between the dependent variable and the quantitative, qualitative demographic and clinical variables. All tests are two-sided; statistical significance was set at $p<0.05$. All analyses were carried out using the statistical package SPSS vr 16.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill, USA).

RESULTS

Descriptive characteristics of participants are shown in Table 1. The average age of the 181 men in this study was 34.69±7.38 (range 20–50) years (Table 1). Approximately 9.3% of the men were obese (BMI >30Kg/m^2) and 52.48% were classified as overweight (25≤BMI ≤30 kg/m^2). Approximately 93.4% had a dietary calcium intake of <600mg/day and 35.3% were cigarette smokers.

Of the 181 subjects in whom 25OHD$_2$ and 25(OH)D$_3$ were measured, 158 had 25OHD$_2$ concentrations less than the lower limit of quantification (<0.5ng/ml).

Regarding the remaining 23 subjects, the mean concentration of D$_2$ was 0.72±0.2ng/ml.

The mean levels of 25(OH)D and PTH were 19.81±6.96 ng/ml (range 5.26-38.3) and 23.63±9.01 pg/ml (range 5.53-58.92), respectively. Individuals aged 20-29 years had a mean concentration of 25(OH)D 18.7 ng/ml, participants aged 30-39 years had 19.76ng/ml, whereas participants aged 40-50 years had the highest mean concentration of 25(OH)D at 21.18ng/ml. There was a positive gradient with age, although not significant (p=0.271).

The overall prevalence of vitamin D deficiency, defined as 25(OH)D levels less than 20ng/ml, was 50.3%, while the prevalence of vitamin D insufficiency (25(OH)D = 20-29ng/ml) was 40.9%. Only 8.8% of the studied population had sufficient levels of vitamin D (25(OH)D ≥30ng/ml), while 4.4% had severe vitamin D deficiency (25(OH)D <10ng/ml).

Looking at the three age subgroups (Table 2), participants aged 20-29 years had the highest prevalence of vitamin D deficiency (56.8%) and the lowest prevalence of vitamin D sufficiency (6.8%) in contrast to individuals aged 40-50 years (43.6% and 12.8%, respectively).

Table 2. Age groups and levels of 25(OH)D

25(OH)D (ng/ml)	Total participants	years		
		20-29	30-39	40-50
<20	91 (50.3%)	25 (56.8%)	49 50.0%)	17 (43.6%)
20-29	74 (40.9%)	16 (36.4%)	41 (41.8%)	17 (43.6%)
≥30	16 (8.8%)	3 (6.8%)	8 (8.2%)	5 (12.8%)

All values are presented as n (%).

Table 1. Demographic characteristics of the total participants

	Mean	SD	Median	Min	Max
Age (years)	34.69	7.38	34.00	20.00	50.00
Weight (kg)	83.24	10.85	83.00	57.00	120.00
Height (m)	1.79	0.06	1.80	1.62	1.97
BMI (Kg/m^2)	25.94	2.82	25.62	19.05	37.13
BMD L_2L_4	1.22	0.13	1.22	0.89	1.58
BMD neck	1.02	011	1.03	0.73	1.50
25(OH) Vit D (ng/ml)	19.81	6.96	19.90	5.26	38.30
b-CTXs (ng/ml)	0.36	0.19	0.32	0.03	1.56
Osteocalcin (ng/ml)	18.32	7.07	16.98	6.35	53.74
P1NP (ng/ml)	52.11	25.51	48.33	13.25	238.40
Alcohol (g/week) ethanol intake	56.42	54.42	45.598	0.00	385.79
Calcium (mg/week)	2300.11	1228.85	2240	0.00	5600.00
PTH (pg/ml)	23.63	9.01	22.56	5.53	58.92

Table 3 shows the correlation of 25(OH)D with demographic factors such as age, weight, height and BMI as well as lifestyle/dietary factors such as alcohol consumption and dietary calcium intake. We found no significant correlation between 25(OH)D levels and the aforementioned variables in the totality of participants as well as in the three age subgroups, with the exception of the younger subgroup (20-29 years) who had a marginally significant negative correlation between 25(OH)D and age.

Moreover, there was no correlation between 25(OH) D and BMD in the femoral neck and the lumbar spine either between 25(OH)D and serum PTH levels or dietary calcium intake in the total population as well as in the age subgroups. Calcium intake was not associated with BMD, PTH and bone turnover markers.

Finally, no correlation was demonstrated between 25(OH)D and bone turnover markers: serum osteocalcin, P1NP, b-CTXs levels, in the total population. However, there was a significant positive correlation between 25(OH)D and osteocalcin (r=0.431 p=0.003), P1NP (r=0.504, p=0.0005), b-CTXs (r=0.347 p=0.021) in participants aged 20-29 years.

By contrast, we found a strong correlation between 25(OH)D and smoking in the totality of participants (p<0.001) as well as in the three age subgroups 20-29y, 30-39y and 40-50y (p=0.004, p=0.044, p=0.017, respectively) (Table 4).

Multiple linear regression analysis revealed that age, BMI, smoking, alcohol consumption and calcium intake accounted for 10%, 27%, 9% and 28% of the serum 25(OH)D level variability in the total population and three age subgroups (20-29y, 30-39y and 40-50y), respectively, while only smoking was a significant determinant of serum 25(OH)D for all age subgroups, except the 30-39 year-olds. Interestingly, 25(OH)D level was lower by approximately 4.3 ng/dl in a smoker compared to a non-smoker for the total population and the 20-29y subgroup (p<0.001, and p=0.040, respectively), while this value increased to 9.2 ng/ml in the 40-50y subgroup (p=0.003) (Table 5).

The multinomial logistic regression model for the association between demographic characteristics and 25(OH)D levels demonstrated that a smoker had a 58% increased likelihood of having vitamin D deficiency compared to a non-smoker for the 20-29y age subgroup (p=0.041) (Table 6) and 63% for the 40-50y subgroup, although the latter did not reach statistical significance (data not shown).

Table 3. Pearson's Correlation coefficient between 25(OH)D levels and quantitative variables

	Total	years		
	Participants (N=181)	**20-29 (N=44)**	**30-39 (N=98)**	**40-50 (N=39)**
Age (years)	0.122	**-0.310***	-0.174	-0.050
Weight (Kg)	-0.036	0.147	-0.146	-0.098
Height (m)	-0.083	0.081	-0.097	-0.197
BMI(Kg/m^2)	0.011	0.108	-0.112	-0.008
Alcohol (g/week)	0.003	-0.231	-0.074	0.219
BMD $_{l2l4}$	0.087	-0.050	0.170	-0.006
BMD $_{neck}$	0.120	0.160	0.137	0.207
b-CTXs (ng/ml)	0.123	**0.347***	0.166	0.049
Osteocalcin (ng/ml)	0,099	**0.431****	0.107	-0.039
Calcium (mg/week)	0,042	0.089	0.051	0.034
PTH (pg/ml)	0.030	0.039	-0.007	0.029
P1NP (ng/ml)	0.133	**0.504****	0.087	0.044

Bold indicates statistical significant correlation * p<0.05 ** p<0.005

DISCUSSION

Our results revealed a high incidence (50.3%) of vitamin D deficiency (<20ng/ml), while the mean levels of 25(OH)D were 19.81ng/ml. Participants aged 20-29 years had the highest incidence of vitamin D deficiency (57%). The high incidence of vitamin D deficiency in our study is in line with results of stud-

Table 4. Smoking and 25(OH)D

	v	**Smoking**	**25(OH)D (ng/ml) Mean±SD**	**p-value**
Total participants	117	no	21.36±6.56	**<0.001**
Participants	64	yes	17.00±6.83	
20-29 years	26	no	20.93±6.51	**0.004**
	18	yes	15.50±4.40	
30-39 years	61	no	20.87±6.54	**0.044**
	37	yes	17.93±7.52	
40-50 years	30	no	22.70±6.69	**0.017**
	9	yes	16.10±7.82	

Table 5. Multiple linear regression model for the association between demographic and clinical characteristics and levels of Vitamin D per age group

	Reference category	Coefficient B	SE B	p-value
Total population				
Constant	----	20.82	5.05	<0.001
Age (years)	----	0.10	0.08	0.195
BMI (Kg/m^2)	----	-0.13	0.19	0.500
Smoking	no	-4.24	1.08	**<0.001**
Alcohol (g/week)	----	0.04	0.14	0.769
Calcium (mg/week)	----	0.001	0.001	0.752
Age group 20-29				
Constant	----	32.75	14.20	0.027
Age (years)	----	-0.70	0.45	0.126
BMI (Kg/m^2)	----	0.23	0.33	0.486
Smoking	no	-4.33	2.03	**0.040**
Alcohol (g/week)	----	-0.21	0.29	0.478
Calcium (mg/week)	----	0.001	0.001	0.443
Age group 30-39				
Constant	----	11.75	12.26	0.341
Age (years)	----	0.52	0.27	0.072
BMI (Kg/m^2)	----	-0.30	0.32	0.344
Smoking	no	-2.46	1.49	0.103
Alcohol (g/week)	----	-0.12	0.24	0.622
Calcium (mg/week)	----	0.00	0.00	0.880
Age group 40-49				
Constant	----	41.48	19.73	0.043
Age (years)	----	-0.25	0.36	0.495
BMI (Kg/m^2)	----	-0.36	0.35	0.323
Smoking	no	-9.16	2.89	**0.003**
Alcohol (g/week)	----	0.48	0.23	**0.050**
Calcium (mg/week)	----	0.00	0.00	0.817

Table 6. Multinomial logistic regression model for the association between demographic and clinical characteristics and levels of Vitamin D (20-29 age group)

25(OH)D (ng/ml)	ORs	(95% CI)		p-value
<20				
Age (years)	2.17	0.45	10.42	0.333
BMI (Kg/m^2)	0.57	0.15	2.17	0.414
Alcohol (g/week)	1.02	0.37	2.82	0.975
Calcium (mg/week)	1.00	0.99	1.00	0.744
Smoking (no, reference category)	1.58	1.05	2.02	**0.041**
20-29				
Age (years)	1.79	0.38	8.47	0.462
BMI (Kg/m^2)	0.60	0.16	2.24	0.446
Alcohol (g/week)	1.08	0.39	2.98	0.875
Calcium (mg/week)	1.00	0.99	1.00	0.775
Smoking (no, reference category)	1.44	1.01	1.90	**0.050**

X^2=18,74, df=10, p=0.044; Cox and Snell R^2=0.353

BMI: body mass index; CI: confidence interval; ORs: odds ratios In MLR analysis, Vitamin D value above 30 was set as the reference category.

ies from countries at a similar latitude to Greece.[14,15]

Our study showed undetectable levels of 25(OH)D_2 in the majority of the male population. Studies on the levels of 25(OH)D_2 in adults are limited and the results are mixed; however, the majority have demonstrated higher concentrations than that found in our study.[16-18] It should be mentioned that the low proportion of individuals in the cohort with 25(OH)D_2 levels >0.5 ng/mL (12%) did not enable evaluation of the correlation between vitamin D_2 and the studied variables.

According to our data, smokers had lower serum 25(OH)D concentrations than non-smokers. Interestingly, in the totality of participants, smoking was the only significant determinant of serum 25(OH)D among the tested variables (BMI, age, smoking, alcohol consumption and calcium intake). Furthermore, 25(OH)D level was expected to be lower by 4.2 ng/dl in a smoker by comparison with a non-smoker for all age-groups but this value increased to 9.2 ng/dl for the 40-50y subgroup. This suggests the need for young and especially middle-aged smokers to be screened for vitamin D deficiency.

The negative correlation between 25(OH)D levels and smoking could possibly be explained by the fact that smoking is usually accompanied by a less healthy lifestyle (less physical activity, alcohol consumption and bad dietary habits) leading to reduced sun exposure and thus synthesis of vitamin D. However, a causative role of smoking in vitamin D deficiency could not be excluded; recent studies have in fact shown that metabolic derivatives of nathphalane (a metabolite in cigarette smoke) such as tetralones can inhibit CYP27A1 activity.[19]

In line with our results, Jaaskelainen et al. studying 5714 subjects (47% men) aged 30-79 years found that smokers had lower serum 25(OH)D concentrations than non-smokers.[20] Moreover, Thuesen et al in a recent large population study showed that odds ratios of vitamin D severe deficiency (25(OH)D <10ng/ml)/ vitamin D deficiency (25(OH)D <20ng/ml) associated with daily smoking was 1.47 and 1.36, respectively.[21] In contrast, Scragg et al. in a sample of 295 men aged 35-64 years found that smoking was not correlated with 25(OH)D levels,[22] while data from recent studies also agreed with the absence of correlation between smoking and 25(OH)D serum concentrations.[23,24] The inconsistency among the various studies could be explained by the different way that smoking is defined, heterogeneity in smoking intensity as well as by the different methodology used to measure serum 25(OH)D. Notably, the Tromso study revealed that determination of serum 25(OH)D using ECLIA (electrochemiluminiscence) resulted in falsely elevated levels of 25(OH)D in smokers, something which does not occur using LC-MS/MS.[25] An overestimation of 25(OH)D concentration—due to the methodology used—could possibly overlook detection of a negative correlation between smoking and 25(OH)D levels.

We found no significant correlation between serum 25(OH)D concentration and age, although there was a positive 25(OH)D gradient with age. This observation is inconsistent with earlier studies, which have indicated that serum 25(OH)D concentrations decrease with increasing age.[25,26] However, the KNHANES study including 2504 males aged >20 years found that vitamin D deficiency was most prevalent in the age group of 20–29, with a rate of 65%, and least prevalent in the older age subgroups.[27] Our findings could be explained by increased prevalence of health-promoting physical activity in older subgroups, thus it is possible that they spend more time outdoors. Moreover, age is positively linked to the daily dietary intakes of vitamin D.[23]

Our data demonstrated no correlation between BMI and 25(OH)D concentration. Findings from previous studies on the association between serum levels of vitamin D and obesity are conflicting.[25,28-30] The Tromso study, although confirming the inverse relationship between BMI and 25(OH)D, noted that this correlation became significant in men with higher BMI levels and more pronounced in subjects with BMI levels greater than 35.[25] In our study, although the range of BMI values was wide, the number of obese subjects with BMI >30 was small (n=17), this probably not allowing us to draw statistically significant results.

Similarly to other studies,[31,32] we did not find any significant correlation between serum 25(OH)D and calcium, phosphate, PTH as well as bone turnover markers either of bone formation (serum OC, PINP, ALP) or resorption (serum CTXs) in the totality of participants. Looking at the younger subgroup (20-29y), who had the lower 25(OH)D levels, we did not find any differences in the indices of bone remodeling between smokers and non-smokers, although smoking has been associated with reduced OPG production and increased bone remodeling, as shown by Lappin et al.[33]

In terms of the routine measurements of calcium, phosphate, ALP and PTH, studies have demonstrated that these parameters are not adequate to identify patients with hypovitaminosis D[7,32] and are thus not reliable predictors of hypovitaminosis D.[31] Studies of the relationship between vitamin D status and bone turnover have yielded conflicting results,[7,34-36] which may be attributed, at least in part, to differences in dietary calcium intake. Notably, several studies supported an inverse relationship between 25(OH)D and serum PTH (and consequently bone turnover markers as a result of secondary hyperparathyroidism) when dietary Ca intake is adequate;[37,38] it should be noted that in our study, the daily dietary calcium consumption was very low (on average 328mg/day).

We observed a positive correlation between bone turnover markers and 25(OH)D concentration in the younger age group (20-29y) which cannot be explained. In contrast to our results, Solarz et al. studying football players aged 19-34 years found no correlation between 25(OH)D levels and bone turnover markers (OC, PINP, β-CTX), although both parameters were higher in this group compared with physically inactive men.[39]

We have found no correlation between BMD in either the lumbar spine or proximal femur and 25(OH) D, indicating that other factors (e.g. testosterone levels) may play a more important role in BMD regulation in this age group. Moreover, a recent study by Gal-

lagher et al conducted in young women suggested that active transport of calcium is saturated at very low serum 25(OH)D levels <5 ng/mL.[40] This very efficient calcium absorption at very low levels of serum 25(OH)D could explain why normal subjects do not develop osteomalacia. Of note, studies on the relation of BMD with 25(OH)D levels in men aged 20-50y are limited and yield conflicting results, although it is a high risk group for vitamin D inadequacy.[7,41] Further studies are needed to examine the correlation of BMD changes over time with serum 25(OH)D levels in the course of the study.

A strength of our study is the method for the determination of 25(OH)D. Due to its hydrophobic character and strong protein binding, measurement of 25(OH)D is technically demanding. Serum 25(OH) D concentration can be measured by competitive binding assay, radioimmunoassay (RIA), automated immunoassay (chemiluminiscence: CLIA, electrochemiluminiscence: ECLIA, enzymeimmunoassay: EIA) which have recently been launched, as well as high performance liquid chromatography (HPLC) and more recently LC-MS/MS. The specificity and accuracy of these methods are variable.[42]

Regarding the immunoassays, the accuracy of the method will depend on the specificity of the antibody (Ab) used (how well the Ab recognizes D_2 and D_3). On the other hand, HPLC and LC-MS/MS can report D_2 and D_3 independently. Notably, recent studies observed that immunoassays, particularly those on automated platforms, are prone to matrix effects and can lead to false results.[42,43] In a recent study performed by Snellman et al, they analyzed specimen from 204 subjects (102 twin pairs) using three different methodologies: HPLC-APCI-MS (Agilent/Hewlet-Packard), RIA (IDS) and CLIA (DiaSorin-Liaison). Interestingly, using a cut-off of 20 ng/mL, 8% of the subjects were insufficient using HPLC, 22% with RIA and 43% by CLIA, indicating that depending on the methodology used, a subject can be classified as deficient or not.[44] In the present study, we used LC-MS/MS which is reliable and robust for the measurement of both $25(OH)D_3$ and $25(OH)D_2$, while it is considered to be the gold standard for measurement of total 25(OH)D concentration.[45] Another strength of this study is that it offers data on an array of biological, behavioral and environmental correlates.

When interpreting our data it is appropriate to consider certain limitations of our study. One limitation is that we rely on just one measurement of 25(OH)D concentration taken during the fall season. However, according to a recent study by Major et al, a single blood sample obtained in spring or fall provides a reasonable average for 25(OH)D over a 1-yr period.[46] Another limitation of our study is its cross-sectional design. Hence, the associations presented between independent factors and outcome variables do not necessarily represent causal relationships. We presented data from a sample of healthy, young/middle aged Greek men living in the urban and suburban areas of Attica. Therefore, data presented in this study might not be applicable to general populations or other ethnicities. A study that involves a broader range of age and BMI is important for further validation of these findings.

In conclusion, a high prevalence of vitamin D deficiency was identified in the Greek young/middle aged male population. Our data suggest that vitamin D status is not a determinant of bone metabolism and BMD in young/middle aged men. Smoking is a significant determinant of serum 25(OH)D, while it increases the likelihood of having vitamin D deficiency by approximately 60% in the young male population.

DECLARATION OF INTEREST

There is no conflict of interest.

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