Scandinavian Journal of Clinical & Laboratory Investigation, 2014; 74: 177–183

informa
healthcare

ORIGINAL ARTICLE

Serum free and bio-available 25-hydroxyvitamin D correlate better with bone density than serum total 25-hydroxyvitamin D

MARTIN S. JOHNSEN[1], GURI GRIMNES[1,2], YNGVE FIGENSCHAU[3], PETER A. TORJESEN[4], BJØRG ALMÅS[5] & ROLF JORDE[1,2]

[1]*Tromsø Endocrine Research Group, Department of Clinical Medicine, University of Tromsø, Tromsø,* [2]*Division of Internal Medicine, University Hospital of North Norway, Tromsø,* [3]*Institute of Medical Biochemistry, University of Tromsø and Department of Laboratory Medicine, University Hospital of North Norway, Tromsø,* [4]*Hormone Laboratory, Department of Endocrinology, Oslo University Hospital, Oslo, and* [5]*Hormone Laboratory, Haukeland University Hospital, Bergen, Norway*

Abstract

In the circulation 25-hydroxyvitamin D (25(OH)D) is bound to vitamin D-binding protein (DBP) and albumin. Only a small fraction is in the unbound, free form. According to the 'free-hormone-hypothesis' only the free form is biologically active. Genetic differences in DBP may affect the binding to 25(OH)D and thereby the amount of free 25(OH)D. In the present study sera were obtained from 265 postmenopausal women with low bone mass density (BMD). Serum 25(OH)D, DBP and albumin were measured and the free and bio-available (free + albumin-bound) 25(OH)D calculated. Based on genotyping of the polymorphisms rs7041 and rs4588, the six common DBP phenotypes were identified and the free and bio-available 25(OH)D calculated according to the corresponding binding coefficients. Relations between measures of 25(OH)D and PTH and BMD were evaluated with linear regression adjusted for age and BMI. The calculated amount of free and bio-available 25(OH)D was 0.03% and 13.1%, respectively, of the measured total serum 25(OH)D. Adjusting for DBP phenotype affected the calculated free and bio-available 25(OH)D levels up to 37.5%. All measures of 25(OH)D correlated significantly with PTH, whereas a significant association with BMD was only seen for the free and bio-available 25(OH)D measures. Adjusting for the DBP phenotypes improved the associations. These relations were almost exclusively seen in subjects not using vitamin D and/or calcium supplements. In conclusion, the free and bio-available forms of 25(OH)D may be a more informative measure of vitamin D status than total 25(OH)D. Adjustment for DBP phenotype may improve this further.

Key Words: *Endocrinology, polymorphism, genetic, vitamin D, vitamin D-binding protein, 25-hydroxyvitamin D*

Introduction

Vitamin D is both a hormone and a vitamin. It can be obtained from the diet or produced endogenously from 7-dehydrocholesterol in the skin during UVB exposure. Vitamin D is hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D), the metabolite used to evaluate the vitamin D status. Further hydroxylation to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the active metabolite, takes place in the kidneys [1]. The vitamin D metabolites are transported in the circulation by vitamin D-binding protein (DBP) which is a water-soluble carrier-protein. About 85–90% of 25(OH)D and $1,25(OH)_2D$ are bound to DBP. A considerable amount is also bound to albumin and less than 1% of 25(OH)D and $1,25(OH)_2D$ circulate in the bloodstream freely [2].

According to the free-hormone hypothesis it is the free fraction of a hormone that is the biological active component. Because albumin binds 25(OH)D weakly one may assume that 25(OH)D dissociates from albumin during tissue perfusion [3]. Therefore, bio-available 25(OH)D refers to the sum of the free and the albumin-bound fraction of 25(OH)D. If the free hormone hypothesis holds true for vitamin D metabolites, one should expect that the free and/or bio-available fractions would correlate more strongly

Correspondence: Rolf Jorde, Medical Department, University Hospital of North Norway, 9038 Tromsø, Norway. Fax: + 47 776 69730. E-mail: rolf.jorde@unn.no

(Received 23 September 2013; accepted 23 November 2013)

ISSN 0036-5513 print/ISSN 1502-7686 online © 2014 Informa Healthcare
DOI: 10.3109/00365513.2013.869701

with biological actions of vitamin D than the total serum 25(OH)D.

$1,25(OH)_2D$ increases calcium absorption in the intestines and reabsorption in the kidneys. It also increases calcium reabsorption in bone, and together with increased calcium levels, this inhibits parathyroid hormone (PTH) secretion from the parathyroid gland [4]. PTH increases osteoclast activity [5]. Thus, adequate vitamin D levels are important for bone health by ensuring sufficient inhibition of PTH secretion. There is also evidence that vitamin D metabolites have multiple other biological effects like maintaining the immune system [6], preventing cardiovascular disease [7] and diabetes [8], and even decreased mortality [9]. In most [10–12] but not all studies [13,14] bone mass density (BMD) shows a positive correlation with vitamin D status. In the present study we wanted to test whether this association becomes stronger using the free and bio-available fraction of serum 25(OH)D as compared to the total serum levels of 25(OH)D. In addition, the impact of the free and bio-available fraction of serum 25(OH)D on PTH levels were investigated.

DBP is a highly polymorphic protein with six major phenotypes, all with different binding affinities for 25(OH)D [15]. These phenotypes are the results of two single nucleotide polymorphisms (SNPs) in the *DBP* gene (globulin-complex gene, *GC* gene), rs7041 and rs4588 [16]. We have therefore genotyped the subjects for these SNPs in order to apply the correct DBP binding coefficients for each individual in the calculations of free and bio-available 25(OH)D.

Materials and methods

Subjects

The blood samples were collected at baseline from post-menopausal women aged 50–80 years old who participated in an osteoporosis intervention study [17]. Among the 297 subjects included in the osteoporosis study, 32 were missing data needed to calculate free 25(OH)D, leaving 265 subjects for the present analysis. The subjects were included from January 2007 until February 2009. All subjects had a T-score in total hip or lumbar spine ≤ -2. Exclusion criteria relevant to this study were suspected hyperparathyroidism (serum calcium >2.55 mmol/L, serum calcium >2.50 mmol/L combined with plasma PTH >5.0 pmol/L, or serum calcium >2.45 mmol/L combined with plasma PTH >7.0 pmol/L). Further exclusion criteria were use of steroids, renal stone disease, systolic blood pressure >175 mmHg or diastolic blood pressure >105 mmHg, serum creatinine >110 μmol/L, chronic diseases like ischemic heart disease, diabetes, granulomatous disease, and cancer. No ethnic data were collected, but the large majority of the subjects were Caucasian and a few were Sami women.

Measurements

The subjects filled in a questionnaire on use of vitamin D and calcium supplements. Height and weight were measured with light clothing and no shoes. Total body, total hip, and lumbar spine (L1-L4) BMD were measured using dual X-ray absorptiometry (DEXA) (GE Lunar Prodigy, Lunar Corporation, Madison, WI, USA).

Serum was stored in aliquots at -70°C for later analyses of total serum 25(OH)D and DBP. Serum 25(OH)D was measured at the Hormone Laboratory, Haukeland University Hospital, using an in-house developed liquid chromatography double mass spectrometry method [18]. The reference range is 50–113 nmol/L. The laboratory takes part in the external quality program DEQAS [19]. DBP was analyzed at the Hormone Laboratory, Aker University Hospital by an in house competitive radioimmunoassay according to Kauppinen-Mäkelin et al. [20], reference range 3.0–5.3 μmol/L.

The other analyses were performed consecutively at the Department of Medical Biochemistry at the University Hospital of North Norway. Plasma PTH was measured using an automated clinical chemistry analyzer (Immulite 2000, Siemens Healthcare Diagnostics, Los Angeles, CA, USA), reference range 1.1–7.5 pmol/L, and serum calcium was analyzed using an automated analyzer (Hitachi 917) with reagents from Boehringer Mannheim, reference range 2.15–2.55 mmol/L. Albumin was measured by a colorimetric method (bromcresol green) using an automated analyzer, Cobas 800 (c702, Roche Diagnostics, Mannheim, Germany). The method was standardized to reference material BCR470/CRM470 from Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). The reference interval was that elaborated jointly with The Nordic Reference Interval Project (NORIP), 18–39 years: 36.0–48.0 g/L, 40–69 years: 36.0–45.0 g/L and ≥ 70 years: 34.0–45.0 g/L.

DNA was prepared from whole blood. Genotyping was performed by KBioscience (http://www.kbioscience.co.uk) using KBioscience Competitive Allele-Specific PCR genotyping system [21].

Calculations of free and bio-available serum 25(OH)D

Calculations of free and albumin-bound 25(OH)D were done using a general formula developed by Vermeulen et al. [22] and tailored for calculating free 25(OH)D by Powe et al. [23]:

Free 25(OH)D = total 25(OH)D/((binding coefficient albumin $\times$ [albumin]) + (binding coefficient DBP $\times$ [DBP]))

Albumin-bound 25(OH)D = [free 25(OH)D] × binding coefficient albumin × [albumin].

Bio-available 25(OH)D was calculated as the sum of free 25(OH)D and albumin-bound 25(OH)D. The binding coefficient used for albumin was 6×10^5 and for DBP (without regard to DBP phenotype) 7×10^8 [2]. In order to assign binding coefficients for the various DBP (or Gc) phenotypes, the subjects were genotyped for the two SNPs rs7041 and rs4588. For these two SNPs the following haplotypes are possible outcomes:

	rs 7041	
rs4588	T	G
A	TA (2)	GA
C	TC (1f)	GC (1s)

The GA- variant does not occur in humans on a regular basis, and therefore only the following diplotypes are relevant:

	TA	TC	GC
TA	TA-TA	TA-TC	TA-GC
TC	TA-TC	TC-TC	GC-TC
GC	TA-GC	GC-TC	GC-GC

Based on the binding coefficients for Gc-1S, Gc-1F and Gc-2 [15], and the binding coefficient of combined haplotypes to be the mean of the two, the following binding coefficients for the specific DBP phenotypes were used in our calculations of 'SNP-adjusted' free and bio-available 25(OH)D:

Diplotype	Phenotype	Binding coefficient
GC/GC	Gc-1S/Gc-1S	6×10^8
GC/TC	Gc-1S/Gc-1F	4.8×10^8
GC/TA	Gc-1S/Gc-2	8.6×10^8
TC/TC	Gc-1F/Gc-1F	3.6×10^8
TC/TA	Gc-1F/Gc-2	7.4×10^8
TA/TA	Gc-2/Gc-2	11.2×10^8

Statistics

Normal distribution was evaluated by assessing histograms visually, and all variables were considered normally distributed.

Comparisons between groups were performed with Student's paired and unpaired *t*-tests. Linear regression was used to evaluate relations with adjustments for body mass index (BMI) and age. In this model the interaction between use of supplements (calcium and/or vitamin D) and measures of serum 25(OH)D and BMD appeared ($p = 0.08$). In this study, intake of cod liver oil was not included as a supplement as in Norway this most often is a life-long habit. Data are, where relevant, presented for subjects with and without supplements.

The statistical analyses were performed with SPSS. All tests were done two-tailed and a *p*-value < 0.05 was considered statistically significant. The data are shown as mean (SD) unless otherwise specified.

Ethics

The study was approved by the Regional Committee for Medical and Health Research Ethics (REK Nord).

Results

Twenty-five subjects were taking calcium supplements alone, 18 subjects were taking vitamin D supplements alone, and 54 subjects were taking both calcium and vitamin D. In those taking calcium the mean supplement intake was 804 (268) mg/day, and in those taking vitamin D the mean supplement intake was 393 (247) IU/day. Although there appeared to be an interaction between use of supplements, measures of serum 25(OH)D and BMD, the distribution curves for 25(OH)D, DBP and albumin appeared similar in users and non-users of supplements (Supplementary Figures 1–15, available online at http://informahealthcare.com/doi/abs/10.3109/00365513.2013.869701). One hundred and sixty-eight subjects had no supplements. The characteristics of the subjects are shown in Table I. As expected, serum calcium was significantly higher, plasma PTH lower and measures of serum 25(OH)D higher in those on supplements. In the subjects not taking supplements the free and bio-available 25(OH)D were 0.03% and 13.1%, respectively, of the total serum 25(OH)D.

In those not using vitamin D supplements the mean serum 25(OH)D levels were non-significantly higher in those included during the summer months (May–September) ($n = 70$) compared to those included during the winter months (October–April) ($n = 123$) (70.1 [17.7] vs. 65.0 [23.4] nmol/L, respectively), and similarly in those using vitamin D supplements 84.2 (15.7) ($n = 31$) and 80.5 (26.5) nmol/L ($n = 41$), respectively.

Using binding coefficients specific for the six DBP phenotypes ('SNP adjusting') had a considerable effect on the estimated serum free and bio-available 25(OH)D levels. Thus, phenotype Gc-1S/Gc-2 'SNP adjustment' led to a calculated increase in free 25(OH)D of 37.5% and an increase in bio-available 25(OH)D of 26.8%. For the phenotype Gc-1F/Gc-1F a decrease of 34.7% and 34.6% for calculated free 25(OH)D and bio-available 25(OH)D, respectively, was found (Table II). The serum DBP differed according to phenotype as expected [24], with the Gc-2/Gc-2 phenotype having the lowest and the GC-1 phenotypes the highest levels (Table II).

Table I. Characteristics of the subjects taking vitamin D and/or calcium supplements and the subjects not taking supplements.

	Subjects taking supplements ($n = 97$)	Subjects not taking supplements ($n = 168$)
Age (years)	61.8 (9.6)	63.3 (6.9)
BMI (kg/m^2)	24.8 (3.4)	24.7 (3.3)
Serum calcium (mmol/L)	2.37 (0.09)	2.35 (0.07)*
Plasma PTH (pmol/L)	4.64 (1.43)	5.27 (1.76)**
Serum albumin (g/L)	44.5 (2.2)	44.4 (2.1)
Serum DBP (μmol/L)	3.79 (0.54)	3.90 (0.62)
BMD total body (g/cm^{-2})	1.007 (0.053)	0.997 (0.056)
BMD L1–L4 (g/cm^{-2})	0.890 (0.071)	0.877 (0.066)
BMD total hip (g/cm^{-2})	0.791 (0.070)	0.789 (0.081)
Serum total 25(OH)D (nmol/L)	79.54 (22.20)	66.02 (21.70)***
Serum albumin-bound 25(OH)D (nmol/L)	10.55 (2.90)	8.60 (2.98)***
Serum bio-available 25(OH)D (nmol/L)	10.57 (2.91)	8.62 (2.99)***
Serum free 25(OH)D (pmol/L)	26.3 (7.4)	21.5 (7.4)***
DBP-bound 25(OH)D (nmol/L)	68.96 (19.68)	57.39 (19.04)***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ vs. subjects taking supplements, Student's *t*-test.

In the linear regression model with BMI and age as covariates, almost all the 25(OH)D measures correlated significantly and similarly with plasma PTH, in both users and non-users of supplements. There were no significant relations with serum calcium and measures of serum 25(OH)D (data not shown). For BMD there were no significant associations with total 25(OH)D. In subjects not using supplements, all measures of free and bio-available 25(OH)D had a significant positive association with BMD total body. In this group, the 'SNP-adjusted' free and bio-available 25(OH)D were also significantly and positively associated with BMD total hip (Table III). Serum DBP was significantly and negatively associated with BMD total body and BMD total hip (Table III). No significant relations were seen between serum albumin and BMD or PTH (Table III).

Discussion

The main objective of this study was to examine whether free or bio-available 25(OH)D would correlate better with plasma PTH and BMD than total 25(OH)D; and secondly, to examine if using specific binding coefficients for the different DBP phenotypes in calculating the free and bio-available fraction of 25(OH)D would improve the results.

All measures of 25(OH)D had a strong and negative association with PTH. For BMD only the free and bio-available measures of 25(OH)D had a significant positive association, whereas no significant associations were seen for total 25(OH)D. Although comparisons of standardized beta coefficients should be done with caution, it is noteworthy that at the hip the standardized beta-coefficients were five times higher for free and bio-available 25(OH)D and BMD than what was observed for total 25(OH)D. It should be noted that these significant associations were primarily seen in the subjects not using supplements. This is reasonable, as supplements will have a rapid effect on the serum 25(OH)D levels, whereas an effect on bone will come more slowly. In line with this, the high and almost equal relation between all measures of serum 25(OH)D and PTH reflects the rapid response in PTH secretion to changes in vitamin D status, as that seen following vitamin D supplementation.

Table II. Measures of serum 25(OH)D according to DBP phenotype.

	DBP phenotypes					
	Gc-1S/Gc-1S ($n = 73$	Gc-1S/ Gc-1F ($n = 59$)	Gc-1S/ Gc-2 ($n = 83$)	Gc-2/Gc-2 ($n = 12$)	Gc-1F/Gc-2 ($n = 22$)	Gc-1F/ Gc-1F ($n = 16$)
Serum total 25(OH)D (nmol(L)	75.9 (23.7)	73.0 (24.4)	69.3 (21.7)	62.2 (17.0)	61.0 (19.5)	70.4 (21.8)
Serum free 25(OH)D (pmol/L)	25.0 (8.3)	22.6 (7.8)	23.2 (7.6)	25.0 (7.2)	20.2 (5.8)	20.2 (6.6)
Serum free 25(OH)D SNP adjusted (pmol/L)	28.6 (9.5)*	18.8 (6.5)*	31.9 (10.4)*	42.1 (11.9)*	19.2 (5.5)*	13.2 (4.3)*
Serum bio-available 25(OH)D (nmol/L)	10.0 (3.4)	9.0 (3.0)	9.3 (3.0)	10.3 (3.1)	8.3 (2.3)	8.1 (2.7)
Serum bio-available 25(OH)D SNP adjusted (nmol/L)	11.5 (3.8)*	7.5 (2.5)*	12.8 (4.2)*	17.3 (5.1)*	7.9 (2.2)*	5.3 (1.8)*
Serum DBP (μmol/L)	3.83 (0.53)	4.10 (0.57)	3.76 (0.54)	3.00 (0.31)	3.73 (0.54)	4.46 (0.59)

*$p < 0.001$ versus unadjusted measure. Student's paired *t*-test.

Table III. Standardized beta-coefficients and r^2 values from the linear regression model with age and BMI as covariates.

	Dependent variables			
	Total BMD	BMD L1-L4	BMD Total hip	Plasma PTH
Subjects not taking vitamin D or calcium supplements ($n = 168$)				
Serum total 25(OH)D	0.078	0.016	0.031	−0.295***
Serum free 25(OH)D	0.167*	0.033	0.130	−0.298***
Serum free 25(OH)D SNP adjusted	0.204**	0.013	0.144*	−0.224**
Serum bio-available 25(OH)D	0.168*	0.025	0.136	−0.297***
Serum bio-available 25(OH)D SNP adjusted	0.205**	0.007	0.152*	−0.224**
Serum DBP	−0.249***	−0.074	−0.242**	0.077
Serum albumin	0.011	−0.098	0.065	−0.043
r^2	0.195–0.254	0.025–0.034	0.192–0.229	0.029–0.115
Subjects taking vitamin D or calcium supplements ($n = 97$)				
Serum total 25(OH)D	−0.096	−0.067	0.097	−0.186
Serum free 25(OH)D	−0.103	−0.061	0.169	−0.164
Serum free 25(OH)D SNP adjusted	0.023	0.067	0.101	−0.294***
Serum bio-available 25(OH)D	−0.085	−0.058	0.198*	−0.162
Serum bio-available 25(OH)D SNP adjusted	0.033	0.065	0.121	−0.294**
Serum DBP	0.071	−0.020	−0.152	−0.019
Serum albumin	0.110	0.015	0.134	0.042
r^2	0.095–0.105	0.028–0.032	0.089–0.108	0.040–0.126

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

specific assays, which at least for free 25(OH)D, hopefully will be available in the near future. And finally, the results were not completely consistent at all BMD measurement sites and need confirmation in other studies.

On the other hand, we included a fairly large group of subjects and made adjustments and stratified for relevant confounders. Also, adjusting the free and bio-available 25(OH)D levels for the different DBP binding coefficients is a novel and biologically logical approach.

The results of the study may have important clinical consequences. The free and/or bio-available fractions of 25(OH)D may be more strongly linked to important biological effects than the total fraction and may be beneficial to assess for certain patient groups, such as postmenopausal females or others at risk of osteoporosis or low BMD. However, DBP is an expensive analysis. The increasing volume of 25(OH)D analyses being performed does not allow measurement and calculation of free and/or bio-available 25(OH)D instead of total 25(OH)D for the general patient groups without a considerable cost. Genotyping of specific DBP phenotypes in order to more correctly calculate the free and/or bio-available 25(OH)D levels, would increase the costs of analysis even further.

In conclusion, our results indicate that free and bio-available 25(OH)D may be a more informative measure of the vitamin D status in relation to BMD, and also that adjusting for DBP phenotype may be a further improvement. However, the findings need confirmation in larger studies.

Acknowledgements

The superb assistance by the staff at the Clinical Research Unit, from Marian Remijn and Line Wilsgaard at the DEXA laboratory, Inger Myrnes and Astrid Lindvall at the Department of Medical Biochemistry at the University Hospital of North Norway, Otto Bårholm at the Hormone Laboratory, Haukeland University Hospital, and Bente Holme at the Hormone Laboratory, Oslo University Hospital, is greatly appreciated.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

The study was funded by a grant from The Norwegians Women Public Health Association in Troms, The North Norway Regional Health Authority, the University of Tromsø, and The Research Council of Norway.

References

[1] Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 1999;96:507–15.

[2] Bikle DD, Gee E, Halloran B, Kowalski MA, Ryzen E, Haddad JG. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. J Clin Endocrinol Metab 1986;63:954–9.

[3] Ferrini RL, Barrett-Connor E. Sex hormones and age: a cross-sectional study of testosterone and estradiol and their bioavailable fractions in community-dwelling men. Am J Epidemiol 1998;147:750–4.

[4] DeLuca HF. Overview of general physiologic features and functions of vitamin D. Am J Clin Nutr 2004; 80(6 Suppl.):1689S–96S.

[5] Bellido T, Saini V, Pajevic PD. Effects of PTH on osteocyte function. Bone 2013;54:250–7.

[6] Cantorna MT, Zhu Y, Froicu M, Wittke A. Vitamin D status, 1,25-dihydroxyvitamin D3, and the immune system. Am J Clin Nutr 2004;80(6 Suppl.):1717S–20S.

[7] Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidiroglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, Macdonald HM, Forouhi NG, Loos RJ, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasan RS, Soranzo N, Bojunga J, Psaty BM, Lorentzon M, Foroud T, Harris TB, Hofman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Järvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hyppönen E, Spector TD. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet 2010;376:180–8.

[8] Blanton D, Han Z, Bierschenk L, Linga-Reddy MV, Wang H, Clare-Salzler M, Haller M, Schatz D, Myhr C, She JX, Wasserfall C, Atkinson M. Reduced serum vitamin D-binding protein levels are associated with type 1 diabetes. Diabetes 2011;60:2566–70.

[9] Autier P, Gandini S. Vitamin D supplementation and total mortality: a meta-analysis of randomized controlled trials. Arch Intern Med 2007;167:1730–7.

[10] Nakamura K, Tsugawa N, Saito T, Ishikawa M, Tsuchiya Y, Hyodo K, Maruyama K, Oshiki R, Kobayashi R, Nashimoto M, Yoshihara A, Ozaki R, Okano T, Yamamoto M. Vitamin D status, bone mass, and bone metabolism in home-dwelling postmenopausal Japanese women: Yokogoshi Study. Bone 2008;42:271–7.

[11] von Mühlen DG, Greendale GA, Garland CF, Wan L, Barrett-Connor E. Vitamin D, parathyroid hormone levels and bone mineral density in community-dwelling older women: the Rancho Bernardo Study. Osteoporos Int 2005; 16:1721–6.

[12] Hannan MT, Litman HJ, Araujo AB, McLennan CE, McLean RR, McKinlay JB, Chen TC, Holick MF. Serum 25-hydroxyvitamin D and bone mineral density in a racially and ethnically diverse group of men. J Clin Endocrinol Metab 2008;93:40–6.

[13] Garnero P, Munoz F, Sornay-Rendu E, Delmas PD. Associations of vitamin D status with bone mineral density, bone turnover, bone loss and fracture risk in healthy postmenopausal women. The OFELY study. Bone 2007; 40:716–22.

[14] Hosseinpanah F, Rambod M, Hossein-nejad A, Larijani B, Azizi F. Association between vitamin D and bone mineral density in Iranian postmenopausal women. J Bone Miner Metab 2008;26:86–92.

[15] Arnaud J, Constans J. Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). Hum Genet 1993; 92:183–8.
[16] Li F, Jiang L, Willis-Owen SA, Zhang Y, Gao J. Vitamin D binding protein variants associate with asthma susceptibility in the Chinese Han population. BMC Med Genet 2011; 12:103.
[17] Grimnes G, Joakimsen R, Figenschau Y, Torjesen PA, Almås B, Jorde R. The effect of high-dose vitamin D on bone mineral density and bone turnover markers in postmenopausal women with low bone mass – a randomized controlled 1-year trial. Osteoporos Int 2012;23:201–11.
[18] Grimnes G, Almaas B, Eggen AE, Emaus N, Figenschau Y, Hopstock LA, Hutchinson MS, Methlie P, Mihailova A, Sneve M, Torjesen P, Wilsgaard T, Jorde R. Effect of smoking on the serum levels of 25-hydroxyvitamin D depends on the assay employed. Eur J Endocrinol 2010;163:339–48.
[19] Fraser WD, Milan AM. Vitamin D assays: past and present debates, difficulties, and developments. Calcif Tissue Int 2013;92:118–27.
[20] Kauppinen-Mäkelin R, Tähtelä R, Löyttyniemi E, Kärkkäinen J, Välimäki MJ. A high prevalence of hypovitaminosis D in Finnish medical in- and outpatients. J Intern Med 2001;249:559–63.
[21] Jorde R, Schirmer H, Wilsgaard T, Joakimsen RM, Mathiesen EB, Njølstad I, Løchen ML, Figenschau Y, Berg JP, Svartberg J, Grimnes G. Polymorphisms related to the serum 25-hydroxyvitamin D level and risk of myocardial infarction, diabetes, cancer and mortality. The Tromsø Study. PLoS One 2012;7:e37295.
[22] Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. J Clin Endocrinol Metab 1999;84:3666–72.
[23] Powe CE, Ricciardi C, Berg AH, Erdenesanaa D, Collerone G, Ankers E, Wenger J, Karumanchi SA, Thadhani R, Bhan I. Vitamin D-binding protein modifies the vitamin D-bone mineral density relationship. J Bone Miner Res 2011;26:1609–16.
[24] Lauridsen AL, Vestergaard P, Nexo E. Mean serum concentration of vitamin D-binding protein (Gc globulin) is related to the Gc phenotype in women. Clin Chem 2001; 47:753–6.
[25] Bhan I, Powe CE, Berg AH, Ankers E, Wenger JB, Karumanchi SA, Thadhani RI. Bioavailable vitamin D is more tightly linked to mineral metabolism than total vitamin D in incident hemodialysis patients. Kidney Int 2012;82:84–9.
[26] Dastani Z, Berger C, Langsetmo L, Fu L, Wong BY, Malik S, Goltzman D, Cole DE, Richards JB. In healthy adults, biological activity of vitamin D, as assessed by serum PTH, is largely independent of DBP concentrations. J Bone Miner Res 2014;29:494–9.
[27] Chun RF, Lauridsen AL, Suon L, Zella LA, Pike JW, Modlin RL, Martineau AR, Wilkinson RJ, Adams J, Hewison M. Vitamin D-binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D. J Clin Endocrinol Metab 2010;95:3368–76.
[28] Chun RF, Peercy BE, Adams JS, Hewison M. Vitamin D binding protein and monocyte response to
[29] 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D: analysis by mathematical modeling. PLoS One 2012;7:e30773.
[30] Bikle DD, Gee E. Free, and not total, 1,25-dihydroxyvitamin D regulates 25-hydroxyvitamin D metabolism by keratinocytes. Endocrinology 1989;124:649–54.
[31] Jeffery LE, Wood AM, Qureshi OS, Hou TZ, Gardner D, Briggs Z, Kaur S, Raza K, Sansom DM. Availability of 25-hydroxyvitamin D(3) to APCs controls the balance between regulatory and inflammatory T cell responses. J Immunol 2012;189:5155–64.
[32] Kuchuk NO, van Schoor NM, Pluijm SM, Chines A, Lips P. Vitamin D status, parathyroid function, bone turnover, and BMD in postmenopausal women with osteoporosis: global perspective. J Bone Miner Res 2009;24:693–701.
[33] Gutiérrez OM, Farwell WR, Kermah D, Taylor EN. Racial differences in the relationship between vitamin D, bone mineral density, and parathyroid hormone in the National Health and Nutrition Examination Survey. Osteoporos Int 2011;22:1745–53.

Supplementary material available online

Supplementary Figures 1–15