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Active Vitamin D (1,25-Dihydroxyvitamin D) and Bone Health in Middle-Aged and Elderly Men: The European Male Aging Study (EMAS)

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Context: There is little information on the potential impact of serum 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] on bone health including turnover.

Objective: The objective of the study was to determine the influence of $1,25(OH)_2D$ and 25-hydroxyvitamin D [25(OH)D] on bone health in middle-aged and older European men.

Design, Setting, and Participants: Men aged 40–79 years were recruited from population registers in 8 European centers. Subjects completed questionnaires that included questions concerning lifestyle and were invited to attend for quantitative ultrasound (QUS) of the heel, assessment of height and weight, and a fasting blood sample from which $1,25(OH)_2D$, 25(OH)D, and PTH were measured. $1,25(OH)_2D$ was measured using liquid chromatography tandem mass spectrometry. Bone markers serum N-terminal propeptide of type 1 procollagen (P1NP) and crosslinks (β-cTX) were also measured. Dual-energy x-ray absorptiometry (DXA) of the hip and lumbar spine was performed in 2 centers.

Main Outcome Measure(s): QUS of the heel, bone markers P1NP and β-cTX, and DXA of the hip and lumbar spine were measured.

Results: A total of 2783 men, mean age 60.0 years (SD 11.0) were included in the analysis. After adjustment for age and center, $1,25(OH)_2D$ was positively associated with 25(OH)D but not with PTH. 25(OH)D was negatively associated with PTH. After adjustment for age, center, height, weight, lifestyle factors, and season, $1,25(OH)_2D$ was associated negatively with QUS and DXA parameters and associated positively with β-cTX. $1,25(OH)_2D$ was not correlated with P1NP. 25(OH)D was positively associated with the QUS and DXA parameters but not related to either bone turnover marker. Subjects with both high $1,25(OH)_2D$ (upper tertile) and low 25(OH)D (lower tertile) had the lowest QUS and DXA parameters and the highest β-cTX levels.

Conclusions: Serum $1,25(OH)_2D$ is associated with higher bone turnover and poorer bone health despite being positively related to 25(OH)D. A combination of high $1,25(OH)_2D$ and low 25(OH)D is associated with the poorest bone health. **(*J Clin Endocrinol Metab* 98: 995–1005, 2013)**

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Abbreviations: BMD_a, areal bone mineral density; BMI, body mass index; BUA, broadband ultrasound attenuation; CI, confidence interval; β-cTX, β-C-terminal cross-linked telopeptide; CV, coefficient of variation; DXA, dual-energy x-ray absorptiometry; E_2, estradiol; EMAS, European Male Aging Study; LC-MS/MS, liquid chromatography-tandem MS; MS, mass spectrometry; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; PASE, Physical Activity Scale for the Elderly; P1NP, N-terminal propeptide of type 1 procollagen; QUS, quantitative ultrasound; SOS, speed of sound; T, testosterone.

Vitamin D deficiency is common, particularly among the elderly (1). Vitamin D status is most commonly characterized by measuring serum 25-hydroxyvitamin D [25(OH)D], the most abundant circulating metabolite. The influence of 25(OH)D on bone health has been extensively examined (1), particularly in postmenopausal women, with fewer studies in men (2–11).

1,25-Dihydroxyvitamin D [1,25$(OH)_2$D] is the metabolically active molecule responsible for most of the actions of vitamin D and is derived from 25(OH)D by 1α-hydroxylation primarily in the kidney (12). The main effect of 1,25$(OH)_2$D is to increase calcium absorption from the gut (13). 1,25$(OH)_2$D binds to the vitamin D receptor in the epithelial cells of the duodenum causing the synthesis of calcium binding proteins that regulate active intestinal calcium absorption (13, 14). It also stimulates calcium reabsorption in the kidney. The production of 1,25$(OH)_2$D is stimulated by PTH and its concentrations directly influenced by serum calcium and phosphate (15). In addition to regulating serum calcium uptake in the intestine and kidney, evidence from in vitro and animal studies suggest that 1,25$(OH)_2$D may also regulate calcium resorption from bone by having direct effects on bone cells (13, 14, 16). There is, however, little information on the potential impact of serum 1,25$(OH)_2$D on bone health including turnover.

Compared with 25(OH)D, serum concentrations of 1,25$(OH)_2$D are 1000-fold lower and its half-life much shorter at approximately 7 hours (12). Measurement of 1,25$(OH)_2$D has typically been by RIA, often preceded by HPLC, thus making it time consuming. Such measurement also typically required large volumes of serum. Recent advances in mass spectrometry (MS) have provided more accurate measurements of many metabolic hormones, but to date very few MS-based assays for 1,25$(OH)_2$D have been developed. Consequently, epidemiological data are scarce, but there is some evidence that 1,25$(OH)_2$D declines with age in some (17–19) but not all studies (20, 21). Among the few studies that have measured both vitamin D metabolites and also PTH, some provide evidence of a positive association between 1,25$(OH)_2$D and 25(OH)D (17–19) and between 1,25$(OH)_2$D and PTH (17, 18). There are very few studies examining the influence of 1,25$(OH)_2$D on bone health, and the data are conflicting. A small study of healthy men aged 30–92 years found no association between 1,25$(OH)_2$D and radial or vertebral bone mineral content (21). One study showed a doubling of the risk of hip fracture in postmenopausal women with low serum 1,25$(OH)_2$D (22), and another study found lower 1,25$(OH)_2$D levels in hip fracture patients compared with controls (23).

The European Male Aging Study (EMAS) is a large population-based study of aging in middle-aged and older European men, which incorporates an extensive range of clinical, biochemical, health, and lifestyle information, including a new state-of-the-art MS-based measurement of serum 1,25$(OH)_2$D. We used data from EMAS to examine the interrelationships between 1,25$(OH)_2$D, 25(OH)D, and PTH. We compared the influence of 1,25$(OH)_2$D, 25(OH)D, and PTH on bone health measured using quantitative ultrasound (QUS) of the heel, dual-energy x-ray absorptiometry (DXA) of the hip and lumbar spine, and serum markers of bone turnover.

Materials and Methods

Subjects

The subjects included in this analysis were recruited for participation in EMAS. Details concerning the study design and recruitment have been described previously (24). Briefly, men were recruited from population-based sampling frames in 8 centers: Florence (Italy), Leuven (Belgium), Łódź (Poland), Malmö (Sweden), Manchester (United Kingdom), Santiago de Compostela (Spain), Szeged (Hungary), and Tartu (Estonia). Stratified random sampling was used with the aim of recruiting equal numbers of men in each of 4 10-year age bands: 40–49, 50–59, 60–69, and 70–79 years. Subjects were invited by letter to complete a postal questionnaire and attend for an interviewer-assisted questionnaire, clinical assessments and a fasting blood sample. The overall response rate was 45%. Ethical approval for the study was obtained in accordance with local institutional requirements in each center. All subjects provided written informed consent.

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Study questionnaires and clinical data

The postal questionnaire included questions concerning current smoking, alcohol consumption in the previous year (response set = every day/5–6 days per week/3–4 days per week/1–2 days per week/less than once a week/not at all) and also whether they were currently being treated for a range of medical conditions, which included diabetes and prostate disease. The interviewer assisted questionnaire included the Physical Activity Scale for the Elderly (PASE) and also asked about current medications (25). Subjects also completed the Reubens physical performance test (26). Height was measured to the nearest 1 mm using a stadiometer (Leicester height measure, SECA UK Ltd, Birmingham, United Kingdom) and body weight to the nearest 0.1 kg using an electronic scale (SECA model number 8801321009; SECA UK Ltd). A single fasting morning (before 1000 hours) venous blood sample was obtained from all subjects.

Assessment of 25(OH)D and 1,25(OH)$_2$D$_3$

Serum 25(OH)D levels were determined using a RIA (RIA kit; DiaSorin, Stillwater, Minnesota). Intra- and interassay coefficients of variation (CVs) for 25(OH)D were 11% and 8%, respectively. The detection limit of the RIA kit was 2.0 ng/mL. 1,25-(OH)$_2$D$_3$ was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as a lithium adduct according to the method described by Casetta et al (27). In contrast to this earlier method, methanol instead of acetonitrile was used for protein precipitation of 200 μL serum samples. The injected volume of supernatant was increased from 90 μL to 180 μL and injected on a Shimadzu Prominence HPLC Shimadzu, Kyoto, Japan) (coupled to an AB Sciex API 5500 QTRAP tandem mass spectrometer (Sciex, Warrington, United Kingdom). The use of ultrapure methanol (Fisher; Optima liquid chromatography-mass spectrometry) further helped to increase sensitivity due to reduced ion suppression in the LC-MS/MS interface (28). The 1,25-(OH)$_2$D$_3$ standard dissolved in ethanol was calibrated by measuring the UV absorbance at 264 nm, using a molar absorbance of 18 300. Calibrators (6.25–250 pg/mL) were dissolved in a surrogate matrix containing bovine serum albumin (60 g/L) dissolved in physiological water with the addition of 0.2% serum with a 1,25-(OH)$_2$D$_3$ concentration lower than 10 pg/mL. The internal standard peak area of calibrators or serum samples did not fluctuate more than 20% relative to a water blank. Calibration curves were linear through zero over the entire measuring range from 6.25 to 250 pg/mL. The signal to noise ratio of a 6.25 pg/mL calibrator was greater than 10, allowing the definition of a limit of quantification of less than 6.25 pg/mL. Carryover as measured in a blank after the injection of the highest calibrator level was lower than the limit of detection, the latter defined as 3 times the background noise level. Potential interferences from 24(R),25(OH)$_2$D$_3$ and 25(S),26(OH)$_2$D$_3$ but not 1,25(OH)$_2$-3-epi-D$_3$ were chromatographically resolved from the 1,25(OH)$_2$D$_3$ peak. The interday imprecision of pooled serum at high and low serum concentrations were, respectively, 10.1% CV (n = 9) for serum, with a mean concentration of 7.16 pg/mL, and 5.9% CV (n = 20) for serum, with a mean concentration of 55.8 pg/mL. Seventy-six samples were measured with both this new LC-MS/MS method and the traditional liquid chromatography-RIA method as described by Bouillon et al (29), which resulted in a linear fit of 1.84 + 1.006x as well as an excellent coefficient of correlation of r = 0.91.

Hormone measurements

Measurements of testosterone (T) and estradiol (E_2) were carried out by gas chromatography mass spectrometry. SHBG was measured by the Modular E170 platform electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). The free and bioavailable (non-SHBG bound) T and E_2 levels were derived from total hormone, SHBG, and albumin concentrations using mass action equations and association constants. Further details are described elsewhere (30). In addition, samples were transported in frozen state to a single laboratory for measurement of PTH and IGF-I (University of Santiago de Compostela). Serum was assayed for PTH using a chemiluminescence immunoassay (Nichols Advantage Bio-Intact PTH assay; Quest Diagnostics, Madison, New Jersey). Interassay CV for PTH was 2.8%. The detection limit of the chemiluminescence immunoassay was 1.6 pg/mL. Serum was assayed for IGF-I using chemiluminescence as previously described (31).

QUS of the heel

QUS of the left heel was performed with the Sahara clinical sonometer (Hologic, Inc, Waltham, Massachusetts) using a standardized protocol in all centers. Outputs included broadband ultrasound attenuation (BUA; measured in decibels per megahertz) and speed of sound (SOS; measured in meters per second). The in vivo CVs were 2.8% and 0.3% for BUA and SOS, respectively. Repeat measurements were performed on a roving phantom at each of the 8 centers (32). Standardized CVs for within-machine variability ranged by center: for SOS, from 1.0% to 5.6%, and BUA from 0.7% to 2.7%. Standardized CVs for between-machine variability were 4.8% for BUA and 9.7% for SOS (32).

Dual-energy x-ray absorptiometry

Areal bone mineral density (BMD_a) scans were carried out in the Manchester and Leuven subsets of EMAS (n = 676). Both sites used DXA QDR 4500A devices from the same manufacturer (Hologic, Inc). BMD_a was measured at the lumbar spine (L1 to L4) and proximal femur (total region). The precision errors in Leuven were 0.57% and 0.56% at the lumbar spine and total femur region, respectively. In Manchester, these precision errors were 0.97% and 0.97%, respectively. Both devices were cross-calibrated with the European spine phantom (33).

Bone marker measurements

To assess bone resorption, serum β-C-terminal cross-linked telopeptide (β-cTX) was measured on the Elecsys 2010 automated analyzer (Roche Diagnostics GmbH) as previously described (34). The intraassay CV evaluated by repeated measurements of several serum samples was less than 5.0%. The detection limit was 10 pg/mL. To evaluate bone formation, measurements were performed on the Elecsys 2010 with a 2-site assay using monoclonal antibodies raised against intact human N-terminal propeptide of type 1 procollagen (P1NP) purified from human amniotic fluid. The interassay CV was less than 3.0% and the lower detection limit less than 5 ng/mL.

Analysis

The association between 1,25(OH)$_2$D and 25(OH)D as well as 1,25(OH)$_2$D, 25(OH)D, and PTH was initially assessed visually using scatter plots and superimposing linear lines and lo-

cally weighted scatter plot smooth curves. The strength of the associations was then determined using linear regression after adjusting for age and center. For ease of interpretation and comparison, $1,25(OH)_2D$, 25(OH)D, and PTH were standardized into Z scores (per SD). These variables were also categorized into quintiles to assess the potential threshold effects.

The association between $1,25(OH)_2D$, 25(OH)D, PTH, and factors that could potentially confound associations with bone parameters were assessed using linear regression adjusting for age and center. These factors included height (centimeters), weight (kilograms), body mass index (BMI) (kilograms per square meter), PASE score (per 100), time to walk 50 feet (seconds), smoking (percentage), alcohol consumption (categorized by number of days consumed alcohol), and, after standardizing to Z scores, serum calcium, creatinine, total and free T, total and free E_2, SHBG, and IGF-I. Multivariable linear regression was then used to determine the association between $1,25(OH)_2D$, 25(OH)D, PTH, and QUS parameters (BUA and SOS), DXA (total hip and lumbar spine), and bone turnover parameters (PINP and β-cTX) with the bone measures as dependent variables, adjusting for age, center, season of measurement, and factors found to be associated with the bone outcomes in the previous analysis. All continuous variables were standardized into Z scores (per SD).

To assess the influence of the combination of $1,25(OH)_2D$ and 25(OH)D on bone health, subjects were categorized into 4 groups: 1, normal 25(OH)D and $1,25(OH)_2D$; 2, normal 25(OH)D and high $1,25(OH)_2D$; 3, low 25(OH)D and normal $1,25(OH)_2D$; and 4, low 25(OH)D and high $1,25(OH)_2D$. Low 25(OH)D was determined as those in the lowest tertile of 25(OH)D (<17.7 ng/mL) and high $1,25(OH)_2D$ was determined as those in the highest tertile of $1,25(OH)_2D$ (>64.6 pg/mL). Tertiles were chosen because it provided greater statistical power than quintiles, although broadly similar results were obtained when subjects were categorized using quintiles. A similar categorization was used to assess the combination of low 25(OH)D and high PTH. These models included age, center, season of measurement, and factors found to be associated with the bone outcomes. Results of all linear regression analyses are expressed as standardized β-coefficients and 95% confidence intervals (CIs). Statistical analysis was performed using STATA version 9.2 (http://www.stata.com).

Table 1. Subject Characteristics

Variable	Subjects (n = 2783) Mean (SD)	Range
Age at interview, y	60.0 (11.0)	40.1–82.7
Height, cm	173.5 (7.4)	147.0–199.5
Weight, kg	83.3 (13.8)	43.0–175.0
BMI, kg/m^2	27.6 (4.0)	17.7–51.9
PASE score (0–1100)	193.2 (90.2)	0.0–592.5
$1,25(OH)_2D$, pg/mL	59.3 (16.5)	13.6–164.6
25(OH)D, ng/mL	24.4 (12.4)	2.0–84.4
PTH, pg/mL	28.4 (12.1)	1.1–96.8
Creatinine, μmol/L	90.9 (16.5)	25.0–176.0
Calcium, mmol/L	2.4 (0.1)	1.2–3.3
T, nmol/L	16.4 (6.0)	0.2–46.8
Free T, pmol/L	288.9 (88.4)	1.5–695.1
E_2, pmol/L	73.5 (24.7)	9.9–229.0
Free E_2, pmol/L	1.3 (0.4)	0.1–4.3
SHBG, nmol/L	42.9 (19.8)	8.8–200.0
IGF-I, ng/mL	132.2 (43.1)	7.6–363.2
QUS		
BUA, dB/MHz	80.4 (18.9)	6.3–201.7
SOS, m/s	1550.1 (34.2)	1458.7–1784.4
DXA		
Total hip, g/cm^2	1.018 (0.145)	0.4–1.4
Lumbar spine, g/cm^2	1.066 (0.182)	0.5–1.6
Bone markers		
P1NP, ng/mL	42.4 (20.8)	6.2–473.9
β-cTX, pg/mL	360.6 (182.4)	10.0–1330.0
Current smokers	21.1%	
Alcohol consumption[a]	55.5%	
Taking corticosteroids	3.6%	
Taking vitamin D/calcium	0.6%	

[a] More than 1 d/wk.

Results

Subjects

A total of 2783 men with a mean age of 60.0 years (SD 11.0) had complete $1,25(OH)_2D$, 25(OH)D, QUS, and bone marker data. Characteristics of the subjects are shown in Table 1. Mean BMI was 27.6 kg/m^2. A little more than one fifth of the subjects reported that they currently smoke, whereas 56% of the men reported consuming alcohol on at least 1 day per week, 4% reported currently taking corticosteroids, and 0.6% was on calcium and/or vitamin D supplementation. Mean $1,25(OH)_2D$ was 59.3 pg/mL (SD 16.5), 25(OH)D 24.4 ng/mL (SD 12.4), and PTH 28.4 pg/mL (SD 12.1). As expected, there was some variation in $1,25(OH)_2D$ and 25(OH)D levels according to the season in which they were measured (Fig. 1). The highest 25(OH)D was observed in the summer and autumn (mean 29.6 and 29.9 ng/mL, respectively) and the lowest in the winter and spring months (mean 20.9 and 20.4 ng/mL, respectively). Levels of $1,25(OH)_2D$ followed a similar pattern. In addition, there was significant variation in $1,25(OH)_2D$, 25(OH)D, and PTH by center (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org); however, there did not appear to be any trend toward decreasing levels 25(OH)D with increasing latitude.

Association between $1,25(OH)_2D$, 25(OH)D, and PTH

$1,25(OH)_2D$ was positively correlated with 25(OH)D (β-coefficient = 0.457 pg/mL; $P < .001$) (Fig. 2A). The association persisted after adjustment for age and center, and there was no evidence of threshold effects when 25(OH)D was categorized into quintiles or an interaction with PTH when PTH was categorized into quintiles (data not shown). There was a modest correlation between $1,25(OH)_2D$ and PTH ($\beta = -.060$ pg/mL; $P = .021$) (Fig. 2B), which was attenuated after adjustment for age and center. 25(OH)D was negatively associated with PTH ($\beta = -.194$ ng/mL; $P < .001$) (Fig. 2C). This relationship

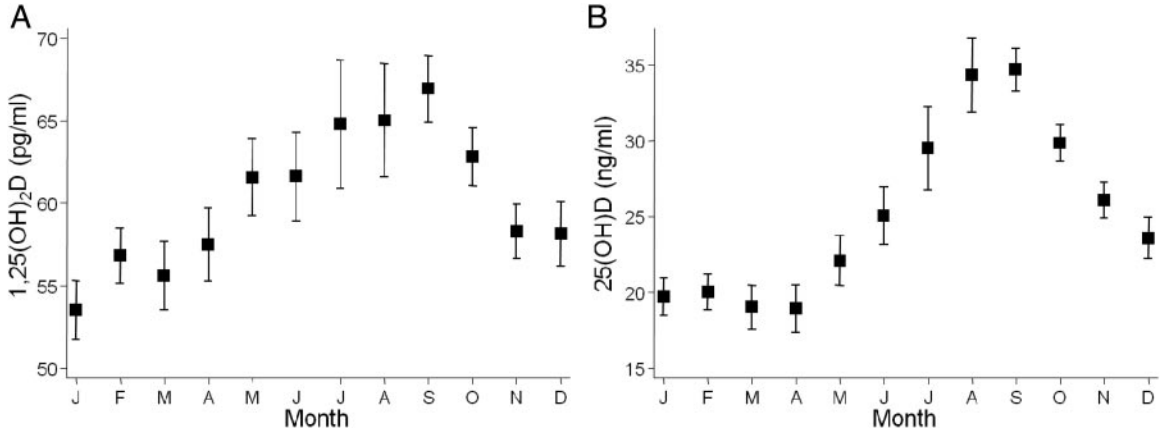

Figure 1. 1,25$(OH)_2$D (A) and 25(OH)D (B) levels by month of measurement. Values are mean and 95% CI.

persisted after adjustment for age and center, with no evidence of threshold effects. When an interaction with 1,25$(OH)_2$D was explored, the negative association between 25(OH)D and PTH was more marked in subjects in the highest quintile of 1,25$(OH)_2$D compared with those in the lowest quintile (β for difference in slope = −.140; P = .007). Further adjustment for serum calcium levels had no influence on the results.

Association between 1,25$(OH)_2$D, 25(OH)D, and PTH and age, anthropometric, lifestyle, and hormonal factors

The association between 1,25$(OH)_2$D, 25(OH)D, and PTH and age, anthropometric, lifestyle, hormonal, and biochemical factors are shown in Table 2. 1,25$(OH)_2$D decreased and PTH increased with age, but there was no association between 25(OH)D and age. 1,25$(OH)_2$D was

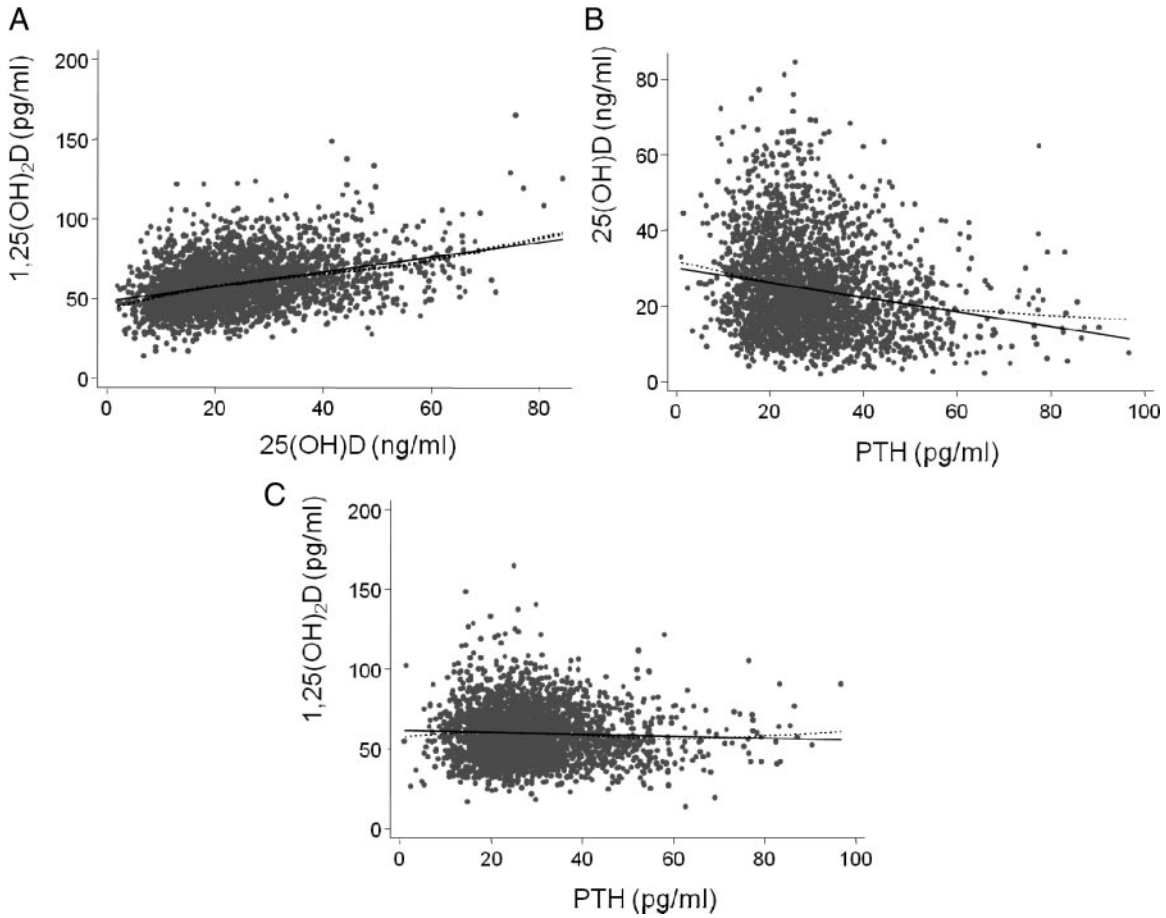

Figure 2. Association between 1,25$(OH)_2$D and 25(OH)D (A), 25(OH)D and PTH (B), and 1,25$(OH)_2$D and PTH (C). The solid lines represent the linear relationship, and the dashed lines represent locally weighted scatterplot smoothing (LOWESS).

Table 2. Association Between 1,25(OH)$_2$D, 25(OH)D, PTH and Age, Anthropometry, Lifestyle, and Hormonal Factors

	β-Coefficient (95% CI)[a]		
	1,25(OH)$_2$D (per SD)	**25(OH)D (per SD)**	**PTH (per SD)**
Age (y)[b]	−.007 (−.010, −.004)[c]	.000 (−.003, .003)	.014 (.011, .017)[c]
Height (cm)	−.012 (−.017, −.006)[c]	.005 (−.000, .011)	.005 (−.001, .010)
Weight (kg)	−.008 (−.011, −.006)[c]	−.004 (−.007, −.002)[d]	.007 (.004, .010)[c]
BMI (kg/m^2)	−.022 (−.031, −.013)[c]	−.020 (−.029, −.012)[c]	.022 (.013, .031)[c]
PASE score (per 100)	.110 (.062, .158)[c]	.133 (.086, .180)[c]	−.040 (−.089, .010)
Time to walk 50 feet (sec)	−.014 (−.026, −.002)[e]	−.027 (−.039, −.016)[c]	.010 (−.003, .022)
Current smoker (yes vs no)	−.172 (−.262, −.083)[c]	−.267 (−.355, −.179)[c]	−.081 (−.172, .011)
Alcohol consumption/wk			
None	−.189 (−.308, −.069)[d]	−.134 (−.253, −.016)[e]	.010 (−.113, .132)
<1 day	−.047 (−.153, .058)	−.063 (−.168, .041)	.060 (−.048, .168)
1–2 days	Referent	Referent	Referent
3–4 days	.027 (−.100, .155)	.009 (−.118, .136)	.079 (−.051, .210)
5–6 days	−.040 (−.199, .119)	−.029 (−.187, .128)	.109 (−.054, 0.271)
Every day	.096 (−.028, .219)	−.009 (−.131, .114)	−.025 (−.151, .102)
Creatinine (per SD)	−.120 (−.159, −.082)[c]	.082 (.044, .121)[c]	.086 (.046, .125)[c]
Calcium (per SD)	.051 (.008, .094)[e]	.086 (.043, .130)[c]	−.136 (−.178, −.093)[c]
Total T (per SD)	.043 (.006, .079)[e]	.054 (.018, .090)[d]	−.051 (−.088, −.013)[d]
Free T (per SD)	.025 (−.015, .065)	.062 (.022, .101)[d]	−.015 (−.056, .026)
Total E_2 (per SD)	.013 (−.024, .049)	−.024 (−.060, .012)	.038 (.001, .075)[e]
Free E_2 (per SD)	−.009 (−.046, .028)	−.039 (−.075, −.002)[e]	.073 (.036, .111)[c]
SHBG (per SD)	.028 (−.010, .067)	.009 (−.029, .047)	−.058 (−.097, −.019)[d]
IGF-I (per SD)	−.010 (−.047, .028)	.071 (.034, .108)[c]	−.054 (−.092, −.015)[d]

[a] Adjusted for age and center except where adjusted for center only.

[b] Adjusted for center.

[c] $P < .001$.

[d] $P < .01$.

[e] $P < .05$.

associated with height. Body weight and BMI were positively associated with PTH and negatively associated with both 1,25(OH)$_2$D and 25(OH)D. Higher levels of physical activity as measured by PASE score were positively associated with 1,25(OH)$_2$D and 25(OH)D, whereas poor physical performance as measured by the time to walk 50 feet and also smoking and no alcohol intake were negatively associated.

25(OH)D was positively associated with serum creatinine, calcium, total T, free T, and IGF-I. 1,25(OH)$_2$D was positively associated with serum calcium and total T and negatively associated with creatinine, whereas PTH was positively associated with serum creatinine, total E_2, and free E_2 and negatively associated with calcium, total T, SHBG, and IGF-I (Table 2).

Association between 1,25(OH)$_2$D,25(OH)D, PTH, and bone health parameters

After adjustment for age, center, height, weight, PASE score, current smoking, alcohol consumption, and season of measurement, higher levels of 1,25(OH)$_2$D were associated with higher levels of the bone resorption marker β-cTX (Table 3). 1,25(OH)$_2$D did not appear to be related to the bone formation marker P1NP. In contrast, 25(OH)D was not associated with markers of bone turnover. Higher levels of PTH were associated with higher levels of both markers of bone turnover (Table 3).

Higher 1,25(OH)$_2$D was associated with lower QUS parameters at the heel and DXA BMD$_a$ at the lumbar spine (Table 4). Similar results were observed for QUS BUA and SOS, so only the results for SOS are presented here. When categorized into quintiles, those in the highest (vs lowest) quintile of 1,25(OH)$_2$D had significantly lower SOS and total hip and lumbar spine BMD$_a$. However, higher 25(OH)D was associated with higher SOS at the heel and DXA BMD$_a$ at the total hip and lumbar spine. There was some inconsistency across the categories, although there was no evidence of any threshold effects when 25(OH)D was categorized into quintiles (Table 4). PTH was unrelated to the SOS, but higher PTH levels were associated with lower BMD$_a$ at the total hip although not the lumbar spine. Compared with those in the lowest quintile of PTH, those in the fourth quintile had lower BMD$_a$ at both the total hip and lumbar spine (Table 4). Further adjustment for creatinine, serum calcium, and total T made no difference to the 1,25(OH)$_2$D or 25(OH)D results (data not shown), but further adjustment for serum creatinine, calcium, and total T attenuated the associations between PTH and hip and lumbar spine BMD$_a$ (data not shown).

When the subjects were categorized by both vitamin D metabolite levels, those in the lowest tertile of 25(OH)D

Table 3. Association of $1,25(OH)_2D$, 25(OH)D, and PTH with Bone Turnover

	β-Coefficient (95% CI)[a]	
	P1NP (per SD)	β-cTX (per SD)
$1,25(OH)_2D$ (per SD)	.017 (−.025, .058)	.162 (.124, .201)[b]
$1,25(OH)_2D$ quintiles		
1: <45.6	Referent	Referent
2: 45.6–54.0	−.006 (−.129, .116)	.049 (−.064, .163)
3: 54.1–61.7	.013 (−.111, .138)	.167 (.052, .282)[c]
4: 61.8–72.2	.089 (−.037, .216)	.288 (.170, .405)[b]
5: >72.2	.079 (−.050, .209)	.496 (.376, .615)[b]
25(OH)D (per SD)	−.039 (−.084, .007)	−.029 (−.071, .014)
25(OH)D quintiles		
1: <14.1	Referent	Referent
2: 14.1–19.4	−.075 (−.199, .048)	−.083 (−.199, .033)
3: 19.5–25.3	.011 (−.115, .138)	−.012 (−.131, .107)
4: 25.4–33.7	−.080 (−.211, .051)	−.112 (−.235, .011)
5: >33.7	−.097 (−.238, .044)	−.090 (−.222, .043)
Vitamin D categories		
Mid- or highest tertile 25(OH)D/mid- or lowest tertile $1,25(OH)_2D$	Referent	Referent
Mid- or highest tertile 25(OH)D/highest tertile $1,25(OH)_2D$	.047 (−.054, .148)	.280 (.186, .374)[b]
Lowest tertile 25(OH)D/mid- or lowest tertile $1,25(OH)_2D$	−.005 (−.108, .099)	.055 (−.041, .151)
Lowest tertile 25(OH)D/highest tertile $1,25(OH)_2D$	.114 (−.057, .286)	.453 (.294, .612)[b]
PTH (per SD)	.121 (.081, .160)[b]	.196 (.159, .233)[b]
PTH quintiles		
1: <18.84	Referent	Referent
2: 18.84–23.89	.094 (−.029, .216)	.097 (−.018, .211)
3: 23.90–29.11	.191 (.068, .314)[c]	.222 (.107, .336)[b]
4: 29.12–36.31	.266 (.141, .392)[b]	.316 (.199, .434)[b]
5: >36.31	.359 (.234, .485)[b]	.527 (.410, .644)[b]
25(OH)D/PTH categories		
Mid- or highest tertile 25(OH)D/mid- or lowest tertile PTH	Referent	Referent
Mid- or highest tertile 25(OH)D/highest tertile PTH	.203 (.098, .308)[b]	.276 (.178, .374)[b]
Lowest tertile 25(OH)D/mid- or lowest tertile PTH	−.053 (−.163, .057)	−.076 (−.179, .026)
Lowest tertile 25(OH)D/highest tertile PTH	.181 (.057, .305)[c]	.306 (.191, .422)[b]

Highest tertile of $1,25(OH)_2D$ is greater than 64.6 pg/mL, lowest tertile of 25(OH)D is less than 17.7 ng/mL, and highest tertile of PTH is greater than 31.20 pg/mL.

[a] Adjusted for age, center, height, weight, PASE score, current smoking, alcohol consumption, and season of measurement.

[b] $P < .001$.

[c] $P < .01$.

and the highest tertile of $1,25(OH)_2D$ had higher β-cTX as well as lower SOS at the heel and lower BMD_a at the total hip and lumbar spine compared with the subjects in the middle to high tertiles of 25(OH)D and middle to low tertiles of $1,25(OH)_2D$ (Tables 3 and 4).

When the subjects were categorized by 25(OH)D and PTH levels, those in the lowest tertile of 25(OH)D and the highest tertile of PTH levels had higher bone turnover markers and lower heel SOS, hip, and lumbar spine BMD_a compared with those in the middle to high tertiles of 25(OH)D and middle to low tertiles of PTH.

Excluding those on antiosteoporotic medication or those receiving calcium/vitamin D supplementation made no difference to any of the results.

Discussion

In this population-based sample of middle-aged and older European men, $1,25(OH)_2D$ was positively associated with 25(OH)D but not with PTH. 25(OH)D was negatively related to PTH. Both metabolites of vitamin D showed similar seasonal variation. Higher $1,25(OH)_2D$ was associated with higher β-cTX levels, lower QUS parameters at the heel, and lower DXA BMD_a at the lumbar spine. In contrast, higher 25(OH)D was not associated with bone turnover but correlated significantly with higher QUS parameters and DXA BMD_a at the hip and lumbar spine. Subjects in the lowest tertile of 25(OH)D and the highest tertile of $1,25(OH)_2D$ had the highest β-cTX and the lowest QUS and DXA BMD_a values. PTH was positively related to markers of bone turnover and weakly negatively associated with BMD_a at the hip. As expected, subjects in the lowest tertile of 25(OH)D and the highest tertile of PTH had higher bone turnover and lower QUS and DXA BMD_a.

We observed a significant but modest decline in $1,25(OH)_2D$, but not 25(OH)D, with age in keeping with some (17–19) but not all studies (20, 21). This implies that

Table 4. Association of $1,25(OH)_2D$, 25(OH)D and PTH With QUS SOS and DXA-Assessed BMD_a

	β-Coefficient (95% CI)[a]		
	QUS SOS (per SD)	DXA Total Hip BMD_a (per SD)	DXA Lumbar Spine BMD_a (per SD)
$1,25(OH)_2D$ (per SD)	−.077 (−.116, −.038)[b]	−.051 (−.142, .041)	−.111 (−.209, −0.013)[d]
$1,25(OH)_2D$ quintiles			
1: <45.6	Referent	Referent	Referent
2: 45.6–54.0	−.022 (−.137, .094)	−.285 (−.538, −.032)[d]	−.213 (−.483, .057)
3: 54.1–61.7	−.047 (−.163, .070)	−.154 (−.407, .099)	−.175 (−.444, .095)
4: 61.8–72.2	−.080 (−.199, .039)	−.123 (−.394, .149)	−.138 (−.429, .152)
5: >72.2	−.212 (−.333, −.090)[c]	−.281 (−.551, −.011)[d]	−.403 (−.692, −.115)[c]
25(OH)D (per SD)	.073 (.031, .116)[c]	.164 (.078, .249)[b]	.102 (.009, .194)[d]
25(OH)D quintiles			
1: <14.1	Referent	Referent	Referent
2: 14.1–19.4	.046 (−.071, .162)	−.071 (−.374, .232)	−.064 (−.391, .262)
3: 19.5–25.3	.112 (−.007, .232)	.211 (−.087, .509)	.303 (−.019, .624)
4: 25.4–33.7	.099 (−.024, .222)	.244 (−.061, .549)	.180 (−.147, .507)
5: >33.7	.159 (.026, .291)[d]	.336 (.042, .630)[d]	.258 (−.058, .574)
Vitamin D categories			
Mid- or highest tertile 25(OH)D/mid- or lowest tertile $1,25(OH)_2D$	Referent	Referent	Referent
Mid- or highest tertile 25(OH)D/highest tertile $1,25(OH)_2D$	−.156 (−.251, −.061)[c]	−.128 (−.321, .065)	−.227 (−.433, −.021)[d]
Lowest tertile 25(OH)D/mid- or lowest tertile $1,25(OH)_2D$	−.126 (−.223, −.029)[d]	−.319 (−.545, −.094)[c]	−.255 (−.496, −.014)[d]
Lowest tertile 25(OH)D/highest tertile $1,25(OH)_2D$	−.375 (−.536, −.214)[b]	−.625 (−1.050, −.201)[c]	−.887 (−1.340, −.435)[b]
PTH (per SD)	−.023 (−.061, .015)	−.098 (−.184, −.011)[d]	−.032 (−.125, .061)
PTH quintiles			
1: <18.84	Referent	Referent	Referent
2: 18.84–23.89	−.046 (−.164, .072)	−.091 (−.358, .176)	−.071 (−.358, .216)
3: 23.90–29.11	.006 (−.112, .124)	−.262 (−.526, .002)	−.026 (−.310, .258)
4: 29.12–36.31	−.044 (−.164, .077)	−.366 (−.634, −.098)[c]	−.294 (−.582, −.007)[d]
5: >36.31	−.066 (−.186, .054)	−.230 (−.504, .045)	−.085 (−.380, .210)
25(OH)D/PTH categories			
Mid- or highest tertile 25(OH)D/mid- or lowest tertile PTH	Referent	Referent	Referent
Mid- or highest tertile 25(OH)D/highest tertile PTH	−.024 (−.125, .076)	−.054 (−.255, .146)	−.085 (−.300, .130)
Lowest tertile 25(OH)D/mid or lowest tertile PTH	−.096 (−.201, .009)	−.252 (−.515, .011)	−.251 (−.534, .032)
Lowest tertile 25(OH)D/highest tertile PTH	−.148 (−.266, −.030)[d]	−.480 (−.763, −.198)[c]	−.404 (−.708, −.100)[c]

Highest tertile of $1,25(OH)_2D$ is greater than 64.6 pg/mL, lowest tertile of 25(OH)D is less than 17.7 ng/mL, and highest tertile of PTH is greater than 31.20 pg/mL.

[a] Adjusted for age, center, height, weight, PASE score, current smoking, alcohol consumption, and season of measurement.

[b] $P < .001$.

[c] $P < .01$.

[d] $P < .05$.

renal capacity to synthesize $1,25(OH)_2D$, in addition to 25(OH)D production in the skin in response to sunlight, may be relatively well conserved, even in elderly community-dwelling men. Sunlight exposure, however, also appeared to have an influence on serum $1,25(OH)_2D$ as reflected by our observation of seasonal variation in $1,25(OH)_2D$ levels very similar to that of 25(OH)D. Although the seasonal variation of serum 25(OH)D levels is well established (35, 36), the influence of season on $1,25(OH)_2D$ has been a matter of debate in the literature (18–20, 37), with some studies reporting seasonal differences in 25(OH)D-deficient subjects only (1, 37), which is consistent with the endocrinological principles of negative feedback. We observed seasonal variation in $1,25(OH)_2D$ at all levels of 25(OH)D, including in men who were 25(OH)D replete (data not shown).

In our study, as in others (17–19), $1,25(OH)_2D$ was positively associated with 25(OH)D, also in agreement

with the substrate-dependent nature of $1,25(OH)_2D$ synthesis. However, only approximately 12% of the variation of $1,25(OH)_2D$ was explained by 25(OH)D, implying that other factors such as diet (calcium and phosphate intake), serum calcium and phosphate concentrations, immobility, and renal function as well as genetic background may also determine $1,25(OH)_2D$ levels (12). We observed differences in the levels of both $1,25(OH)_2D$ and, as previously reported (38), 25(OH)D between European centers. These differences were, however, not associated with latitude, and no other specific patterns emerged, with some centers having low 25(OH)D but high $1,25(OH)_2D$, whereas others had both high 25(OH)D and $1,25(OH)_2D$, providing further evidence that many factors determine vitamin D status at a population level.

We found only a modest correlation between $1,25(OH)_2D$ and PTH that was attenuated by adjustment for other factors. Previous studies are also discordant, with some finding an association (17, 18) and others not (19). The mechanism for a lack of association is unclear, however, because PTH is considered the major driver of bone resorption in men with low 25(OH)D. The absence of a strong $1,25(OH)_2D$-PTH relationship is interesting because the rise of PTH in response to 25(OH)D is often used to define a threshold of serum 25(OH)D. We observed a relationship between 25(OH)D and PTH in keeping with several studies (11, 39, 40).

This is the first study to examine the association between $1,25(OH)_2D$ and bone turnover. Serum $1,25(OH)_2D$ was positively associated with the bone resorption marker β-cTX. This higher rate of bone turnover did not, however, translate into lower QUS/DXA parameters across the physiological range of $1,25(OH)_2D$: only the highest concentrations of $1,25(OH)_2D$ (above 72 pg/mL) were associated with lower QUS/DXA parameters. These findings are consistent with the notion that $1,25(OH)_2D$, in addition to its well-established stimulatory effect on intestinal calcium absorption in response to calcium intake, may also increase bone resorption. Indeed, recent data from in vitro and animal studies suggest that $1,25(OH)_2D$ may have a direct effect on osteoblasts and hence bone resorption because of its well-established interaction with the receptor activator of nuclear factor-κB/receptor activator of nuclear factor-κB ligand signaling pathway (13, 14). The observation of an association between $1,25(OH)_2D$ and bone resorption in this cohort may indeed reflect a physiological adaptive mechanism to changes in calcium status, which appears independent of PTH and leads to bone loss only in men with the highest $1,25(OH)_2D$ levels. We did not observe an association between 25(OH)D and bone turnover, in contrast to a Dutch study of older men and women (11). Although this was slightly surprising, given the relationship between 25(OH)D and PTH, it is possible that the previously observed threshold effect between 25(OH)D and bone turnover markers may not apply to healthy middle-aged and older men.

Data on $1,25(OH)_2D$ and bone health are scarce and conflicting. A study of 62 healthy men aged 30–92 years found no association between $1,25(OH)_2D$ and radial or vertebral bone mineral content (21). The Study of Osteoporotic Fractures Research Group provided evidence to suggest that the risk of hip fracture increased by a factor of 2.1 (95% CI 1.2–3.5) in postmenopausal women with low serum $1,25(OH)_2D$ [$\leq$23 pg/mL (55 pmol/L)] (22); however, these conclusions were based on a relatively small nested case-control analysis using a Study of Osteoporotic Fractures subset of 133 women who subsequently had hip fractures. Another study, which included men, found lower $1,25(OH)_2D$ levels in hip fracture patients compared to controls (23). In contrast, the association we observed between 25(OH)D and QUS/DXA parameters has been well documented (2–11), although whether this reflects a causal relationship or merely the fact that 25(OH)D is an excellent marker of general health is still a matter for debate. Our observation of a lack of association between 25(OH)D and markers of bone turnover is also in keeping with some (6) but not all studies (11).

The influence of PTH on bone is well established (1, 41, 42). There are, however, few studies examining the relationship between PTH and bone turnover in men, but our observation, which we have previously reported (34), of a positive association is in accord with data from a study of community-dwelling French men aged 55–85 years (6). Our observation of a weak association with BMD_a at the hip is also concordant with some other community-based studies of men (3, 5, 6, 42).

What are the implications of these data? These results contribute to the understanding of the influence of $1,25(OH)_2D$ on bone health in middle-aged and elderly men. As far as we are aware, this is the first population-based study to show that high levels of $1,25(OH)_2D$ are associated with poorer bone health. Men in the highest tertile of $1,25(OH)_2D$ and lowest tertile of 25(OH)D had a lumbar spine BMD_a almost 1 SD lower, which could equate to a 2-fold increase in risk of fracture (43). A possible explanation for this is that low 25(OH)D (and consequently poorer bone health) is being compensated for by higher PTH, which in turn increases $1,25(OH)_2D$ levels.

Our study has a number of advantages. It is large and population based and used standardized methods in assessment of QUS, DXA, vitamin D metabolites, PTH, and lifestyle and other characteristics. In addition, we have previously described a new, highly accurate LC-MS/MS method for measuring $1,25(OH)_2D$ (27) and in this study

examine its performance in a community-based sample for the first time. The addition of lithium salt conjugated favorably with $1,25(OH)_2D$, thus increasing its ability to be ionized and measured by the mass spectrometer. This enabled accurate measurement of low concentrations of $1,25(OH)_2D$ in a large number of samples using only 200 μL of serum without the need for time-consuming derivatization.

There are, however, a number of limitations to be considered when interpreting the results. The overall response rate for participation was 45%. It is possible that those invited but who did not take part may have differed with respect to levels of the bone health measurements and vitamin D/PTH than those who took part, and therefore, the data concerning the absolute levels of these parameters need to be interpreted with caution. Any factors influencing participation, however, are unlikely to have influenced the results of the analysis, which was based on an internal comparison of those who participated. This study, like most epidemiological studies, was based on a single assay of $1,25(OH)_2D$/25(OH)D and PTH levels. The epiforms of $1,25(OH)_2D$ could not be differentiated using our LC-MS/MS method. Some measurement error for serum PTH may have occurred despite the use of morning fasting samples that might not have fully corrected for the diurnal variation of PTH (44). This would have tended to reduce the chances of finding associations between $1,25(OH)_2D$/25(OH)D, PTH, and BMD_a rather than produce spurious associations. We did not have accurate data on dietary calcium, dietary/serum phosphate, or any other markers of 1α-hydroxylase activity, which could influence serum $1,25(OH)_2D$ levels. Given the cross-sectional design of the study, it is not possible to determine the temporal or causal nature of the observed relationships. Finally, the study was based on assessment of middle-aged and older European men and extrapolation beyond this group should be undertaken with caution.

In summary, in this population sample of middle-aged and older European men, higher $1,25(OH)_2D$ levels were associated with higher bone turnover and poorer bone health despite also being modestly associated with higher 25(OH)D. A combination of high $1,25(OH)_2D$ and low 25(OH)D was associated with the poorest bone health.

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