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Free 25-hydroxyvitamin D is low in obesity, but there are no adverse consequences for bone health.

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Abbreviations

25OHD	25-hydroxyvitamin D
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
BMD	Bone mineral density
Bone ALP	Bone alkaline phosphatase
CTX	C-terminal telopeptide of type I collagen
DBP	Vitamin D binding protein
DXA	Dual-energy X-ray absorptiometry
HR-pQCT	High resolution peripheral quantitative tomography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
PINP	procollagen type I N propeptide
PTH	Parathyroid hormone
SPPB	Short physical performance battery

Abstract

Background: The mechanism and clinical significance of low circulating 25-hydroxyvitamin D (25OHD) in obese people are unknown. Low total 25OHD may be due to low vitamin D binding proteins (DBP) or faster metabolic clearance. Obese people have higher bone mineral density (BMD), suggesting that the low total 25OHD may not have the expected adverse consequences for bone.

Objective: The aims of this study were to determine whether 1) vitamin D metabolism and 2) its association with bone health differ by body weight.

Design: We conducted a cross-sectional observational study of 223 normal weight, overweight and obese men and women ages 25 to 75 in South Yorkshire, UK in fall/spring. A subgroup of 106 were also assessed in winter. We used novel techniques including an immunoassay for free 25OHD, stable isotope for $25OHD_3$ half-life, and high resolution quantitative tomography (HR-pQCT) to make a detailed assessment of vitamin D physiology and bone health.

Results: Total serum 25OHD was lower in obese and overweight than normal weight people in fall/spring (geometric means 45.0 and 40.8 vs 58.6 nmol/l, $p<0.001$), but not in winter. Serum 25OHD was inversely correlated with BMI in fall/spring and winter.

Free 25OHD measured by immunoassay or calculated from DBP and albumin was lower in obesity. DBP, DBP genotype, and $25OHD_3$ half-life did not differ between BMI groups. Bone turnover was lower and bone density was higher in obese people.

Conclusions: Total and free 25OHD and $1,25(OH)_2D$ are lower at higher body weight, and this can't be explained by lower DBP or shorter half-life of $25OHD_3$. However, obese people had lower bone turnover and higher bone density than normal weight.

We speculate that low 25OHD in obesity is due to greater pool of distribution. Lower 25OHD in obesity may not reflect at-risk skeletal health.

Keywords: vitamin D, obesity, vitamin D binding protein, half-life, bone density, bone turnover

Introduction

Vitamin D is essential for intestinal absorption of dietary calcium and skeletal mineralisation. Vitamin D deficiency causes undermineralisation, increased bone resorption, osteomalacia and rickets. Vitamin D insufficiency is associated with increased risk of osteoporosis (1) and possibly poorer muscle function and other adverse health outcomes (2).

Serum total 25-hydroxyvitamin D (25OHD) is the most commonly used biomarker for vitamin D status; it has a long plasma half-life and reflects both skin synthesis and oral intake. Recommended sufficiency levels are 50 to 75 nmol/l (20 to 30 ng/ml) (3), (4).

Serum total 25OHD is lower in obese people, and inversely correlated with BMI. This has been reported in adults and children of different ethnic groups all over the world. (5-13). However, the causes and clinical significance of the low 25OHD, and hence the value of total 25OHD as a biomarker of vitamin D status in different body weights is not clear.

Possible causes of low serum 25OHD in obesity are lower vitamin D supply (less sunlight exposure (14) or lower dietary intake (15)), greater volume of distribution, reduced biological availability or more rapid clearance.

More than 99% of circulating 25OHD and 1,25$(OH)_2$D are bound to vitamin D binding protein (DBP) and albumin, and the remaining free fraction is the most biologically available. Also, genetic polymorphism results in three DBP phenotypes, with differing circulating DBP levels and affinity for 25OHD (16, 17). Lower concentrations of binding proteins would reduce total 25OHD measurements, but free 25OHD might be unchanged. It is not clear whether DBP levels differ by body weight (18, 19).

Parathyroid hormone (PTH) may be increased in obesity (15, 20), and higher PTH could increase the metabolic clearance rate of 25OHD.

There is a paradox in body weight, vitamin D and bone; low 25OHD would be expected to be associated with higher bone and lower BMD, but BMI and fat mass are positively correlated with BMD (21), and higher body weight is generally protective against fracture (22).

The aims of this study were to apply newly available techniques (including an immunoassay for free 25OHD and a stable isotope method for $25OHD_3$ half-life) to determine how vitamin D metabolism is affected by body weight, and a detailed assessment of bone (with multiple biochemical markers of bone turnover, dual energy X-ray absorptiometry (DXA) and high resolution peripheral quantitative CT (HR-pQCT)) to determine whether lower 25OHD affects bone health in obesity.

Methods

We conducted a cross-sectional study of healthy Caucasian men and women (ages 25 to 40 and 55 to 75) from South Yorkshire, UK (latitude 53° N).

Participants were approached through poster adverts, emails to hospital staff, mailing from general practice surgeries and a database of volunteers. Participants were recruited in three BMI categories: normal weight (BMI 18.5 to 24.9 kg/m^2), overweight (BMI 25 to 29.9 kg/m^2), and obese (BMI >30 kg/m^2). Exclusion criteria were: pregnancy or breast feeding within the last year, conditions (including diabetes) or medication (including hormonal contraception) known to affect vitamin D or bone metabolism, immobilisation, high alcohol intake, and competitive athletes. Older women were at least five years postmenopausal. There were no restrictions on supplement intake, and supplement use was included in the dietary calcium and vitamin D assessment. (For recruitment detail see **Supplemental Table 1**).

The study was approved by South Yorkshire Research Ethics Committee, conducted according to the Declaration of Helsinki, and all subjects gave written informed consent.

All participants were assessed in fall or spring (19 September to 31 October 2012, and 2 April to 16 May 2013) when UV-B is available. Fasting morning blood samples were taken for measurement of serum total and free 25OHD, $1,25(OH)_2D$, DBP, albumin, PTH, biochemical markers of bone turnover and DBP genotype. Statistical analyses were adjusted for date of visit. Sunlight exposure, dietary vitamin D intake and muscle function were also assessed.

A subgroup of 106 participants were also assessed in winter (11 December 2012 to 1 April 2013), to assess vitamin D status when there is negligible UV-B and avoid perturbation of the isotope tracer study by sunlight exposure. Fasting morning blood samples were taken for measurement of 25OHD, and $25OHD_3$ half-life was assessed with an isotope tracer.

Measurements

Short physical performance battery (SPPB) score (maximum score 12) was calculated from narrow walk and chair stand tests (23). Grip strength was measured using a digital dynamometer (Seahan Corp., Masan).

The sunlight questionnaire was supplied by Prof Lanham-New, University of Surrey, UK (5). It assesses habitual sunlight exposure by season and during holidays. Questionnaire assessment of sunlight exposure has been shown to correlate with vitamin D status (24).

Dietary vitamin D intake was assessed with DIETQ (Tinuviel Software, UK). This is a semi-quantitative habitual food frequency intake questionnaire with computerised analysis based on the UK nutrient database (25).

25OHD was measured in the Manchester Institute of Human Development, UK by liquid chromatography tandem mass spectrometry (LC-MS/MS). This laboratory participates in DEQAS and the assay is calibrated against the NIST standard. $25OHD_2$ was undetectable in most subjects.

Free (unbound) 25OHD was determined by immunoassay (26) (Future Diagnostics, Netherlands, inter-assay CV at 13.2pg/ml 5.3%). Free 25OHD can also be estimated by calculation from total 25OHD, DBP, albumin and their binding affinities, but this approach has limitations due to genetic variation in DBP, and the direct measurement by immunoassay is more closely correlated with serum PTH and calcium (27).

$1,25(OH)_2D$ was measured by manual immunoassay after immunoextraction (ImmunoDiagnostic Systems, UK, inter-assay CV 6.0%, intra-assay CV 2.6%).

DBP was measured by Quantikine manual immunoassay (R&D Systems, UK, inter-assay CV 3.3%, intra-assay CV 3.9%).

C-terminal telopeptide of type I collagen (CTX, bone resorption marker), procollagen type I N propeptide (PINP) and osteocalcin (bone formation markers) were measured by automated immunoassay (Cobas e411, Roche Diagnostics, Germany). Inter-assay CVs were: CTX 4.0%, PINP 4.1%, osteocalcin 2.2%. Bone alkaline phosphatase (bone ALP, bone formation marker) was measured by automated immunoassay (iSYS, ImmunoDiagnostic Systems, inter-assay CV 4.5%).

Albumin, creatinine, calcium and PTH were measured by autoanalyser (Cobas c701, Roche Diagnostics, inter-assay precision <2.0% all tests).

DBP genotyping was done by Sheffield Children's Hospital, UK. The pyrosequencing assay was developed using PSQ software version 1.0.6 (Qiagen) to detect rs4588 and rs7041 polymorphisms.

25OHD half-life was measured with a 24 mcg orally administered tracer stable isotope of $25OHD_3$ (3-^2H-25-hydroxyvitamin D_3 (6, 19, 19-d3)). The tracer was given dissolved in olive oil with a standard breakfast. Venous blood was taken at 6±1, 9±2, 27±2 and 30±2 days after

123 administration. $25OHD_3$ half-life was calculated from the terminal slope of the disappearance
124 of d3-$25OHD_3$, as $t1/2=ln(2)/kB$, where kB is the natural logarithm of the slope of the line of
125 best fit from day 5 to day 30 (28). Tracer preparation and LC-MS/MS measurements (29)
126 were performed at MRC Human Nutrition Research, Cambridge, UK.

127 Bone mineral density and fat mass were assessed by dual energy X-ray absorptiometry
128 (DXA) and high resolution peripheral quantitative tomography (HR-pQCT).

129 Whole body, lumbar spine and hip DXA were performed with a Discovery densitometer
130 (Hologic Inc, Waltham MA, USA). The short-term precision for the spine and hip are 1.0%
131 and 1.1%.

132 HR-pQCT images of the distal radius and tibia (4% site, non-dominant, non-fractured) were
133 obtained using XtremeCT (Scanco Medical AG, Switzerland). Images were analysed with
134 Scanco software (version 6). The short term precision of the BMD measurements is 0.2 to
135 5.5% (30).

136 *Statistics*

137 Normality was assessed using histograms. Skewed variables were log10 transformed for
138 analysis.

139 Variables that differed between the three BMI groups were identified with analysis of
140 variance (ANOVA). Effects of age group and gender were tested with analysis of covariance
141 (ANCOVA). Post-hoc testing for differences between pairs of BMI groups was adjusted for
142 multiple comparisons using the Tukey method.

143 Relationships between variables and BMI (as a continuous variable) were examined with
144 univariate linear models. Multiple linear regression models were used to adjust for age (as a
145 continuous variable) and gender.

Correlations between variables were calculated with Spearman's Rank test, and 95% confidence intervals were calculated by bootstrapping.

Statistical analyses were performed with SPSS Version 21 and R Version 3.2.1.

The fall/spring study (n=223) had 90% power at 5% two-sided significance to detect a 0.22 correlation coefficient between BMI and 25OHD. For ANOVA, 65 participants per BMI group had 90% power to detect a standardised effect size of 0.26 at 5% two-sided significance.

The winter study (n=106) had 90% power at 5% two-sided significance to detect a 0.30 correlation coefficient between BMI and 25OHD. For ANOVA, 32 participants per BMI group had 90% power to detect a standardised effect size of 0.37 at 5% two-sided significance.

For missing data report see **Supplemental Table 2.**

Results

Characteristics of study participants are given in **Table 1**. Dietary calcium intake did not differ between BMI groups (mg/day mean and 95% CI: normal weight 1072 (1002 to 1145), overweight 1074 (998 to 1158), obese 1055 (1001 to 1112)). The subset also assessed in winter were representative of the whole group (n=106: normal BMI = 34, overweight = 32, obese = 40; younger = 46, older = 60; male = 50, female = 56).

Total $25OHD_3$ was lower in obese and overweight people than normal weight people in fall/spring, but not in winter (**Figure 1**). In fall/spring, 56% of overweight and obese people had $25OHD_3$ below 50nmol/l, compared with 37% of normal weight. In winter, 75% of

overweight and obese people had $25OHD_3$ below 50nmol/l, compared with 62% of normal weight.

Total $25OHD_3$ in fall/spring was inversely correlated with BMI (adjusted for date of visit, age and gender; model adjusted $R^2 = 0.339$, $p<0.001$). For every five unit increase in BMI, total $25OHD_3$ decreased by 10.0% (95% CI: 5.7 to 14.0%, $p<0.001$). After the same adjustments, total $25OHD_3$ was also negatively correlated with whole body fat mass (model adjusted $R^2 =0.334$, $p<0.001$). For every 10kg increase in fat mass, total $25OHD_3$ decreased by 11% (95% CI: 6 to 15%, $p<0.001$).

Although total $25OHD_3$ did not differ by BMI group in winter, $25OHD_3$ was negatively correlated with BMI (adjusted for age and gender; model adjusted R^2 0.172, $p<0.001$). For every five unit increase in BMI, $25OHD_3$ decreased by 8.2% (95% CI: 0.5 to 15.3%, $p=0.038$).

Dietary vitamin D and sunlight exposure did not differ by BMI group (**Table 2**). The average hours of sunlight (irradiance measurement above 120 w/m2) in Sheffield during the period of the study measurements were 4.6 in fall/spring and 1.9 in winter (Data kindly provided by Weston Park Weather Station, Sheffield).

DBP and albumin did not differ by BMI group, and adjustment for age and gender did not change this result (**Table 2**). DBP genotype distribution (Gc1-1 47%, Gc2-1 42%, Gc2-2 11%) was similar to other reported white European populations (16). Genotype distribution did not differ by BMI group and BMI did not differ by genotype. Total $25OHD_3$ concentration did differ by genotype (mean nmol/l and 95% CI: Gc1-1 52.2 (47.2 to 57.6), Gc2-1 45.3 (40.9 to 50.3), Gc2-2 39.4 (32.1 to 48.3) $p=0.024$).

$25OHD_3$ half-life did not differ by BMI group (**Table 2**).

Free 25OHD was lower in the obese and overweight groups than normal weight in fall/spring. BMI was negatively correlated with free 25OHD (adjusted for date of visit, age and gender; model adjusted $R^2 = 0.296$, $p<0.001$). For every five unit increase in BMI, free 25OHD decreased by 12.3% (95% CI: 7.7 to 16.6%, $p<0.001$). When total 25OHD was added to the model the relationship between free 25OHD and BMI was no longer significant ($R^2 = 0.619$, $p=0.16$).

Total $1,25(OH)_2D$ was also lower in the obese and overweight groups than normal weight in fall/spring (**Table 3**).

PTH did not differ by BMI group (**Table 3**) and was not correlated with BMI. Adjusting for age and gender did not change this result. CTX and osteocalcin were lower in the obese group than normal weight and overweight. Bone ALP and PINP did not differ between BMI groups (**Table 3**).

BMD by DXA at the whole body, lumbar spine and hip, and by HR-pQCT at the distal radius and tibia was higher in the overweight and obese groups than normal weight (**Table 3**).

Grip strength did not differ by BMI group. Adjustment for age and gender did not change this result. SPPB score was lower in the overweight and obese groups than normal weight. However, SPPB score was not correlated with 25OHD (Spearman's rho -0.122, 95% CI: -0.261 to 0.014, p=0.073).

Discussion

This is the first study to use the free 25OHD assay and stable isotope half-life method to investigate the effect of body weight on vitamin D metabolism.

As expected, total serum 25OHD is was lower at higher body weight (lower in obese than normal weight people in fall/spring, and negatively correlated with BMI in fall/spring and in winter). We also identified that the biologically available free serum 25OHD and active hormone $1,25(OH)_2D$ were lower in obesity. However, PTH was similar across BMI groups, (other studies have described higher PTH in obesity (15, 20, 31)), bone turnover was not higher (bone resorption was lower than normal weight and formation was similar), and BMD by DXA and HR-pQCT was higher at all measured sites. We have previously shown that bone microarchitecture is more favourable for bone strength in obese people, with greater cortical thickness and trabecular number (31).

We investigated several possible mechanisms for the effects of body weight on vitamin D status. Dietary vitamin D intake and sunlight exposure were similar across BMI groups. A previous UK study also found that sunlight exposure did not vary with BMI (32).

Lower total 25OHD in obesity was not due to differences in protein binding; free 25OHD was also lower and serum albumin, DBP and DBP genotype did not differ by BMI group.

$25OHD_3$ half-life did not differ by BMI group, so lower 25OHD in obesity is not due to more rapid metabolic clearance.

After cutaneous synthesis and absorption, vitamin D is distributed into fat, muscle and other tissues (33), and when volume of distribution is greater, less vitamin D may be available for 25-hydroxylation. 25OHD is also distributed into fat and muscle, and into serum (34) and all of these compartments are increased in obesity. Consistent with this, other investigators have reported that the summer rise in circulating 25OHD is blunted in obesity (32, 35). When exposed to UV-B, normal weight and obese people have similar cutaneous synthesis of vitamin D (49), but the serum 25OHD rise is smaller in obese people (18), consistent with our observation that the 25OHD difference between normal weight and obese is greater in

fall/spring than in winter. This theory is supported by evidence that serum 25OHD response to oral vitamin D dosing is BMI-dependent (31, 36).

Due to the greater volume of distribution, if whole body vitamin D and 25OHD were similar in obese and normal weight people, measured serum concentrations would be lower in obese people (and conversely, people with low BMI may have relatively high serum 25OHD but lower whole body stores). Therefore, BMI may need to be considered when using serum 25OHD as a marker of vitamin D status.

It is possible that the lower serum 25OHD in obesity does reflect true vitamin D deficiency, but that adverse skeletal effects are countered by positive skeletal effects of obesity, such as increased loading, oestrogen synthesis from adipocyte aromatase, or adipocyte hormones such as leptin.

Physical function score was poorer in obese people, but not correlated with 25OHD. Vitamin D and calcium supplementation may improve physical functioning in older people, but there is less evidence for benefit in young adults (34-36). Other factors such as less physical activity and fat infiltration of muscle might contribute to poorer function. It is possible that vitamin D maintains muscle integrity in older adults by preventing intramuscular fat accumulation (37), which might be relevant to muscle function in obesity.

There are some limitations to this study. Dietary and sunlight exposure habits differ by geography and culture, and it is very possible that lower dietary vitamin D and sunlight exposure contribute to low 25OHD in obese people elsewhere. We did not measure volume of distribution directly; this would require an intravenous isotope and there are none available for human use. We did not measure intestinal calcium absorption. We used the R+D DBP assay; other DBP assays may give different results because the influence of DBP genotype varies by assay (38), but all participants were Caucasian which will have minimised genotype

variation (the genotype distribution varies by ethnic group) and DBP genotype distribution did not differ between the BMI groups. We also excluded effects of protein binding by direct measurement of free 25OHD.

We have not assessed effects of low 25OHD beyond the musculoskeletal system. Vitamin D deficiency has been associated with diseases such as cancer and metabolic syndrome, where obesity is also a risk factor. However, there is not yet evidence for a causative role of vitamin D deficiency (39).

In conclusion, it is well recognised that total serum 25OHD is low in obesity, but we have shown that biologically available free serum 25OHD and the active hormone $1,25(OH)_2D$ are also lower at higher body weight. The likely cause of lower 25OHD in obesity is greater volume of distribution. The lower 25OHD in obesity was not associated with higher PTH or bone turnover, lower bone density or poorer physical function. BMI affects the relationship between serum 25OHD and bone health and lower serum 25OHD at higher body weight may not indicate at-risk skeletal health.

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Table 1: Participant characteristics by BMI group.

BMI group	Female/Male (number)	Age (years)	Height (m)	BMI (kg/m^2)	Fat mass (kg)
Normal (18.5 to 24.9 kg/m^2)	43/34	55.9 (16.0)	1.68 (0.09)	22.8 (1.4)	19.2 (3.5)
Overweight (25.0 to 29.9 kg/m^2)	28/35	50.6 (15.2)	1.72 (0.09)	27.6 (1.3)	27.6 (5.7)
Obese (>30.0 kg/m^2)	42/41	56.6 (15.4)	1.69 (0.10)	35.4 (4.3)	40.7 (9.2)

Results given as mean (SD)

Table 2: Possible contributors to low vitamin D in obesity.

BMI group	**Normal** n = 77	**Overweight** n=63	**Obese** n=83
Dietary vitamin D intake (µg)	3.61 (3.01, 4.34)	3.05 (2.50, 3.72)	2.72 (2.24, 3.31)
Annual sunlight exposure score	90.48 (82.44, 98.53)	96.34 (86.69, 105.98)	92.33 (84.54, 100.13)
Summer sunlight exposure score	48.45 (43.96, 53.74)	51.15 (45.37, 56.94)	47.55 (42.65, 52.46)
Vitamin D binding protein (µg/ml)	136.0 (124.9,147.0)	124.9 (112.3, 137.6)	130.5 (120.7, 140.4)
Albumin (g/l)	46.0 (45.3, 46.8)	45.7 (45.0, 46.4)	45.1 (44.2, 45.9)
$25OHD_3$ half-life (days)	17.8 (16.6, 19.1)	17.0 (15.8, 18.2)	18.2 (17.0, 19.1)

Dietary vitamin D and sunlight scores given as geometric mean (95% CI). ANOVA all $p>0.05$.

Table 3: Possible consequences of low vitamin D in obesity

BMI group	Normal n=77	Overweight n=63	Obese n=83
Free 25OHD[2] (pmol/l)	10.6[a] (9.4, 12.0)	7.5[b] (6.5, 8.6)	7.8[b] (6.9, 8.8)
Total 1,25(OH)$_2$D[1] (pmol/l)	95.0[a] (87.1, 103.7)	79.4[b] (72.3, 87.1)	78.5[b] (72.3, 85.3)
PTH (ng/l)	41.4[a] (38.4, 44.7)	41.4[a] (37.6, 45.5)	43.5[a] (40.5, 46.7)
CTX[1] (ng/l)	0.45[a] (0.40, 0.50)	0.47[a] (0.43, 0.51)	0.38[b] (0.35, 0.42)
Osteocalcin[1] (ng/ml)	23.0[a] (21.3, 24.8)	22.0[a] (20.5, 23.6)	19.1[b] (18.0, 20.4)
PINP (ng/ml)	40.8[a] (36.9, 45.2)	41.4[a] (38.3, 44.8)	37.8[a] (34.8, 41.0)
Bone ALP (ng/ml)	12.8[a] (11.7, 13.9)	12.9[a] (11.8, 14.0)	12.7[a] (11.8, 13.7)
Whole body DXA BMD[2] (g/cm^2)	1.07[a] (1.05, 1.09)	1.14[b] (1.11, 1.16)	1.16[b] (1.13, 1.18)
Lumbar spine DXA BMD[2] (g/cm^2)	0.95[a] (0.91, 0.98)	1.04[b] (1.01, 1.08)	1.09[c] (1.06, 1.13)
Total hip DXA BMD[2] (g/cm^2)	0.88[a] (0.85, 0.91)	1.00[b] (0.97, 1.03)	1.06[c] (1.03, 1.09)
Distal radius HR-pQCT BMD[2] (mgHA/cm^3)	272.0[a] (258.6, 286.0)	303.0[b] (290.6, 315.9)	315.0[c] (303.9, 326.5)
Distal tibia HR-pQCT BMD[2] (mgHA/cm^3)	280.0[a] (269.5, 290.8)	312.2[b] (298.3, 326.7)	327.6[b] (316.8, 338.8)
Grip strength (kg)	22.1[a] (20.3, 23.9)	24.1[a] (21.6, 26.6)	23.1[a] (21.0, 25.1)
Short physical performance battery score[2]	9.5[a] (9.1, 9.9)	9.1[b] (8.7, 9.4)	8.3[c] (8.0, 8.7)

Measurements taken in fall/spring. Results given as geometric mean (95% CI). ANCOVA adjusted for age, gender (and date of visit for biochemistry) [1]$p<0.01$, [2]$p<0.001$.

Means not sharing a common superscript letter are significantly different at $p<0.05$ based on post-hoc testing adjusted for multiple comparisons using the Tukey method.

Figure legends

Figure 1: Total $25OHD_3$ (LC-MS/MS) by BMI group in fall/spring (n =223) (A) and in winter (n=106) (B).

Results shown as geometric mean and 95% confidence interval. ANCOVA adjusted for date of visit (April/May vs September/October), age group and gender.