Title
24,25-Dihydroxyvitamin D3 and Vitamin D Status of Community-Dwelling Black and White Americans

Authors
Berg, Anders H
Powe, Camille E
Evans, Michele K
et al.

24,25-dihydroxyvitamin D₃ and Vitamin D Status of Community Dwelling Black and White Americans

Anders H. Berg, MD, PhD[1,*], **Camille E. Powe, MD, MPH**[2], **Michele K. Evans, MD**[3], **Julia Wenger, MPH**[4], **Guillermo Ortiz, MD**[4], **Alan B. Zonderman, PhD**[3], **Pirianthini Suntharalingam**[1], **Kathryn Lucchesi, PhD, RPh**[4], **Neil R. Powe, MD, MPH, MBA**[5], **S. Ananth Karumanchi, MD**[6,7], and **Ravi I. Thadhani, MD, MPH**[4,*]

[1]Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School [2]Division of Endocrinology, Massachusetts General Hospital, Boston, Massachusetts [3]Laboratory of Epidemiology and Population Sciences, National Institute on Aging, National Institutes of Health, Baltimore Maryland [4]Division of Nephrology, Massachusetts General Hospital, Boston, Massachusetts [5]Department of Medicine, San Francisco General Hospital and University of California, San Francisco, California [6]Division of Nephrology, Beth Israel Deaconess Medical Center, Boston, Massachusetts [7]Howard Hughes Medical Institute

Abstract

BACKGROUND—24,25-dihydroxyvitamin D (24,25(OH)$_2$D) is a metabolite of 25-hydroxyvitamin D (25D). Blacks frequently have low total 25D without manifestations of vitamin D deficiency, suggesting that total serum 25D may incorrectly reflect vitamin D status in different racial groups. The ratio of serum 24,25(OH)$_2$D to 25D (Vitamin D Metabolite Ratio [VMR]) represents a new candidate biomarker for vitamin D status.

METHODS—We measured 24,25(OH)$_2$D$_3$ and 25D$_3$ by mass spectrometry in a random community cohort of black (n=212) and white (n=164) Americans to evaluate VMR as a marker for vitamin D status. We measured parathyroid hormone concentrations by immunoassay to compare VMR and 25D$_3$ against a physiological indicator of vitamin D deficiency.

RESULTS—Serum 24,25(OH)$_2$D$_3$ strongly correlated with 25D$_3$ in both black and white subjects (r = 0.90, p<0.001 and r = 0.86, p<0.001 respectively). Blacks had lower mean 25D$_3$ than whites (17.0±7.8 vs. 27.5±11.3 ng/mL (42.4±19.5 vs. 68.6±28.2 nmol/L), p<0.001) and lower mean 24,25(OH)$_2$D$_3$ (2.1±1.3 vs. 3.6±2.0 ng/mL (5.1±3.1 vs. 8.7±4.8 nmol/L)), p<0.001). In contrast to total 25D$_3$ concentrations, mean VMR values were similar in blacks and whites (11.9±4.0 vs. 12.5±3.4, p=0.16, respectively) and were negatively correlated with parathyroid hormone concentrations in both races (r$_s$= −0.26, p<0.001 and r$_s$= −0.25, p<0.001, respectively).

CONCLUSIONS—Our results provide further evidence that measurement of total 25D for assessment of vitamin D status in patients of African descent deserves reevaluation, and suggests that alternative measures such as VMR should be considered.

*Co-corresponding authors: Anders H. Berg, MD, PhD, Beth Israel Deaconess Medical Center, 330 Brookline Avenue YA309, Boston, MA 02115, 617-285-6989, ahberg@bidmc.harvard.edu. Ravi I. Thadhani, MD, MPH, Massachusetts General Hospital, 55 Fruit Street GRB-1008G, Boston, MA 02215, rthadhani@mgh.harvard.edu.

Keywords

Vitamin D; 24, 25-dihydroxyvitamin D; parathyroid hormone; racial disparities; ethnic background; mass spectrometry

INTRODUCTION

Vitamin D insufficiency has been widely associated with negative health outcomes including higher mortality (1–5), although cause and effect have yet to be firmly established (6). Among the possible consequences of vitamin D insufficiency, the strongest evidence is for a negative effect on skeletal health (7–9). Clinical investigations of vitamin D supplementation to decrease fracture risk, however, have been inconclusive (2, 10–12). The implications of having low serum concentrations of total 25-hydroxyvitamin D (25D) in black Americans are particularly uncertain. Blacks consistently have lower total 25D than whites and often meet standard criteria for diagnosis of vitamin D insufficiency (i.e., 25D <20 ng/mL (<48.4 nmol/L)) (3, 13, 14), however blacks also have paradoxically higher bone mineral density and a lower risk of osteoporosis and fragility fractures compared to whites (15–18).

This paradox was partially reconciled by recent findings from the Healthy Aging in Neighborhoods of Diversity Across the Lifespan (HANDLS) study (19). Although black Americans have significantly lower mean total 25D concentrations compared to whites, their concentrations of bioavailable 25D may be equivalent (19). These findings have raised important questions as to whether measurement of serum total 25D provides a reliable indicator of vitamin D sufficiency for people of all races and genetic backgrounds (20).

Recent evidence suggests that adequacy of vitamin D may be reflected by concentrations of serum $24,25(OH)_2D$ (21, 22). $24,25(OH)_2D$ is the major product of catabolism of 25D, and because enzymatic synthesis of $24,25(OH)_2D$ is directly proportional to concentrations of 25D substrate, concentrations of both metabolites in circulation are strongly correlated (23). Furthermore, expression of the 24-hydroxylase enzyme (CYP24A1) that converts 25D to $24,25(OH)_2D$ is regulated in part by vitamin D receptor activity (24, 25). Because production of $24,25(OH)_2D$ depends upon both concentrations of 25D and on vitamin D-regulated expression of CYP24A1, concentrations of $24,25(OH)_2D$ may be an even better indicator of vitamin D sufficiency than 25D itself.

Recent findings also suggest that adequacy of vitamin D may be reflected by the ratio of $24,25(OH)_2D$ and 25D serum concentrations (hereinafter referred to as the Vitamin D Metabolite Ratio, or VMR) (21, 22). This ratio should depend primarily upon CYP24A1 expression, which is downregulated in vitamin D deficiency, and thus the VMR would be predicted to decrease in vitamin deficient states. Multiple studies have shown that VMR tends to be disproportionately decreased in patients with low 25D concentrations and in patients who have functional vitamin D deficiency because of chronic kidney disease (CKD) (21–23, 26, 27). Low VMR also may be predictive of responsiveness to vitamin D supplementation (21, 27), and it has been demonstrated that patients with CKD do not increase VMR concentrations in response to vitamin D supplementation as much as control

subjects, consistent with the model that defective kidney production of $1,25(OH_2)D$ results in a persistent decrease in $24,25(OH)_2D$ catabolism (22).

Measurement of VMR may also be an indicator of vitamin D sufficiency in African Americans who have low 25D concentrations but are not functionally deficient. African Americans expressing the Gc1F variant of vitamin D binding protein (DBP) have significantly lower concentrations of 25D compared to whites but show no signs of vitamin D deficiency (19, 28–31). We hypothesized that lower serum total 25D concentrations may be related to reduced binding by serum DBP, but that these patients may have sufficient vitamin concentrations due to increased 25D bioavailability. DBP is also the major protein carrier for circulating $24,25(OH)_2D$ (32, 33), and we would predict that effects of DBP binding on vitamin metabolites would affect 25D and $24,25(OH)_2D$ equally, and thus both 25D and $24,25(OH)_2D$ may be lower in African Americans compared to whites, but their VMR values may be equivalent.

The significance of the differences in 25D concentrations between black and white Americans is still a matter of investigation. In the present study, we tested whether there are also differences in concentrations of $24,25(OH)_2D$ and VMR values between racial groups.

MATERIALS AND METHODS

Study Population

HANDLS is a population-based cohort study supported by the Intramural Research Program of the National Institute on Aging (N=3720) (34). Study participants were 30 to 64 years of age, living in Baltimore, Maryland, recruited from 13 contiguous U.S. Census tracts. Participants from the original HANDLS cohort were randomly sampled from within age, race, gender, and socioeconomic status strata, excluding those who did not self-identify as black or white. Participants selected for this ancillary study included all subjects for which there was sufficient serum available for analysis. The Medstar Research Institute's Institutional Review Board approved the protocol. The Partners Committee on Human Research exempted the present study from review.

Data Collection

We used cross-sectional data from HANDLS collected between 2004 and 2008. After a home-based interview, participants underwent an examination on a mobile research vehicle where blood was sampled, height and weight measured, and bone densitometry performed. Blood samples were typically drawn between 9:15AM – 10:30AM. Only subjects who completed the examination were included in the study.

Laboratory Analysis

Blood samples were drawn at the examination into serum separator tubes without anticoagulant, centrifuged at $1430 \times g$ for 15 minutes, and 1.8 milliliters of serum transferred to Nunc Cryotubes and stored at −80C for future analysis. 100 μL of serum was mixed with $25D_3–[^2H_6]$ and $24R,25-(OH)_2D_3–[^2H_6]$ isotopic internal standards dissolved in 5% bovine serum albumin (IsoSciences, Inc., King of Prussia, PA). Total $25D_3$ and $24,25D_3$

were extracted away from DBP and other serum binding factors by protein precipitation with 250 μL methanol and cleared by centrifugation. Vitamin D metabolites were isolated from extracted supernatants by solid phase extraction chromatography (Strata C-18E 96-well SPE plates, Phenomenex, Inc., Torrences, CA), and eluted with 1 mL ethyl acetate containing 0.1 mg/mL 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD). PTAD-derivatized samples were dried under vacuum and redissolved with 100 μL of 50% ethanol. Samples were then analyzed for vitamin D metabolites using reverse phase chromatography coupled to tandem mass spectrometry in multiple reaction monitoring mode (intra-assay CV 1.1% and 3.5% for $25D_3$ and $24,25(OH)_2D_3$, respectively). Assays were calibrated using $25D_3$ and $24R,25-(OH)_2D_3$ commercial standards (Cerilliant, Inc., Round Rock, TX). Intact PTH levels were measured using the Cobas electrochemiluminescense immunoassay on the Modular Analytics E170 automated analyzer (Roche Diagnostics, Indianapolis, IN, USA; inter-assay CV 2.5%). Additional method details are described in the online Supplementary Materials file.

Statistical Analysis

The characteristics of the study participants are presented as means ± standard deviations or numbers and percentages and were compared according to race with the use of t-tests or chi-square tests. As PTH was non-normally distributed, it is presented as a median (quartile 1, quartile 3) and compared with a Mann-Whitney U test between races. The relationship between $25D_3$ and $24,25D_3$ was summarized with the use of Pearson product-moment correlation while Spearman's rank correlation coefficient was used to summarize the relationship between $24,25D_3$ and PTH by race. All participants were divided into tertiles of PTH and general linear models (GLM) were used to examine interactions between PTH and race in predicting $25D_3$, $24,25(OH)_2D_3$, or the ratio of $24,25(OH)_2D_3$ to $25D_3$. Additionally, the association between the ratio of $24,25(OH)_2D_3 : 25D_3$ and $25D_3$ was examined with the use of GLM, including an interaction term between race and $25D_3$. Due to a possible inflection point, associations between $25D_3$ and $24,25(OH)_2D_3$ were examined after stratifying by $25D_3$ concentrations less then and greater than 12 ng/mL within each race. Results of GLM models are presented as means ± 95% confidence intervals. Statistical analyses were conducted with the use of SAS software, version 9.4 (SAS Institute). Two-tailed P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

Assay Validation

To sensitively measure both $25D_3$ and $24,25(OH)_2D_3$ we developed an isotope-dilutional tandem mass spectrometric assay adapted from recently described methods (23). The assay uses derivatization with 4-Phenyl-1,2,4-triazole-3,5-dione (PTAD) to increase ionization and sensitivity, and methylamine to improve chromatographic separation of vitamin D metabolites (23). The assay demonstrated adequate linearity and functional sensitivity, reaching sub-nanomolar concentrations (Figure 1).

Subject characteristics

Our recently reported study of DBP and vitamin D in HANDLS subjects included 1181 black and 904 white participants (19). In the present study, we analyzed samples from 376 randomly selected subjects from this cohort who had sufficient remaining serum for analysis, including 212 blacks and 164 whites. Baseline characteristics of included black and white subjects were similar in age, sex, body mass index, menopausal status, and renal function (Table 1). There were no statistically significant differences in baseline characteristics of the included subjects compared to non-included HANDLS subjects (data not shown). Significantly more blacks had household incomes <125% of the poverty line than whites (49.5% vs. 32.9%, p=0.001). Blacks were less likely to have a diagnosis of osteoporosis than whites (1.4% vs. 5.5%, p=0.04) and had higher femoral neck bone mineral density (1.04 g/cm^2 vs.. 0.95 g/cm^2), despite comparable dietary intake of vitamin D and calcium.

Vitamin D concentrations

Serum concentrations of $25D_3$ were significantly lower in blacks than whites (17.0±7.8 vs. 27.5±11.3 ng/mL (42.4±19.5 vs. 68.6±28.2 nmol/L), p<0.001), as were the mean $24,25(OH)_2D_3$ concentrations (2.1±1.3 vs. 3.6±2.0 ng/mL (5.1±3.1 vs. 8.7±4.8 nmol/L)), p<0.001) (Table 1). Concentrations of $25D_3$ highly correlated with $24,25(OH)_2D_3$ (Figure 2), both in the overall population (r=0.91, P<0.001) and when examined by race (in blacks, r=0.86, p<0.001; in whites, r=0.90, p<0.001). The ratio of $24,25(OH)_2D_3$ to $25D_3$ (i.e., the VMR) in blacks and whites was similar (11.9±4.0 vs. 12.5±3.4, p=0.16). Although VMR values were similar between blacks and whites, when subjects' VMR values were plotted against $25D_3$ concentrations (Figure 3), there was a linear association between VMR and $25D_3$ concentrations in both blacks and whites (β (95% CI) for 25D in Blacks was 0.14 (0.09, 0.19) and for whites was 0.11 (0.08, 0.15). Interestingly, the interaction term in the linear regression model for $25D_3$*race was p<0.001, indicating that the difference between the two linear models for blacks and whites was statistically significant, and that at any given concentration of $25D_3$ blacks had higher average VMR values.

Associations between Vitamin D parameters and PTH

Median PTH concentrations were slightly higher in blacks than in whites (38 pg/mL vs. 34 pg/mL, p=0.01). The VMR was negatively associated with PTH to a similar degree in both blacks (r_s=−0.26, p=0.01) and whites (r_s=−0.25, p<0.01) (Figure 4). The overall correlation between VMR and PTH amongst all subjects was r_s=−0.26, p=0.01; in comparison, the correlation between was r_s=XXX, p=. Grouping subjects by PTH levels divided into tertiles (i.e., Tertile 1 [low] <32 pg/mL, Tertile 2 [mid] 32 pg/mL 43 pg/mL, Tertile 3 [high] >43 pg/mL), we observed the expected inverse relationship between PTH and all vitamin D parameters (Figure 5). Both $25D_3$ and $24,25(OH)_2D_3$ concentrations were significantly lower in blacks than in whites within each PTH tertile (p<0.001 for all comparisons), but VMRs were nearly indistinguishable by race (p=0.96 for Tertile 1; p=0.29 for Tertile 2; p=0.56 for Tertile 3).

DISCUSSION

In this study we calculated VMR values from measured concentrations of $25D_3$ and $24,25D_3$ in a randomly selected subset of black and white Americans in order to assess how this new indicator reflects vitamin D status, and whether it this marker can be informative independent of race. We observed that although concentrations of $25D_3$ and $24,25D_3$ strongly correlated with each other and were both lower in black Americans compared to whites, blacks and whites had equivalent median VMR values. Although there were no differences in serum calcium concentrations and no differences in calcium and vitamin D intake between blacks and whites, black Americans in this cohort had significantly higher median bone mineral density compared to whites, despite their lower concentrations of $25D_3$ and $24,25D_3$. When blacks and whites were analyzed separately and stratified according to their PTH concentrations, there was a significant association between high PTH and lower median VMR values, as well as lower $25D_3$ and $24,25D_3$ concentrations, corroborating the independent associations of each of these measures of vitamin D sufficiency with calcium homeostasis. Perhaps even more relevant, however, was the observation that blacks with similar PTH concentrations as whites had lower median $25D_3$ and $24,25D_3$ concentrations but equivalent VMR values. Lastly, there were statistically significant correlations between VMR values and serum PTH concentrations in both blacks and whites; furthermore, these scatterplots appeared largely overlapping, suggesting that the association between low VMR and rising PTH concentrations may be equivalent between races. Thus it may be possible to interpret VMR values using universal clinical thresholds for all patients if future studies confirm VMR to be an accurate indicator of vitamin D sufficiency.

Because both $25D_3$ and $24,25(OH)_2D_3$ are bound by serum DBP, differences in concentrations of DBP may influence concentrations of total $24,25(OH)_2D$ in the same way as they do $25D_3$, independent of vitamin D sufficiency (19, 31). As a result, measurements of $24,25(OH)_2D_3$ would be predicted to be lower in blacks (as observed in this study), and interpretation of $24,25(OH)_2D_3$ concentrations in the evaluation of vitamin D status in African Americans will be subject to the same caveats as $25D_3$. In contrast, VMR values should be less influenced by racial differences in DBP concentrations and vitamin D binding affinity characteristics since these differences will influence both the numerator and denominator of the VMR ratio similarly and should cancel each other out. Importantly, the organic extraction methods used in this study to measure VMR extract $24,25(OH)_2D_3$ and $25D_3$ away from DBP, and thus VMR measurements will not be influenced by DBP concentrations, as has been observed in immunoassay methods of $25D_3$ measurements (35).

There are currently no automated immunoassays available for measurement of $24,25(OH)_2D$, and thus measurement of $24,25(OH)_2D$ and VMR values for now will only be available to clinical laboratories with LC-MS capabilities. Synthesis of $1,25(OH)_2D$ by the kidneys is induced by vitamin D deficiency (36), and thus it is possible that the ratio of $1,25(OH)_2D : 25D$ may be altered in patients with vitamin deficiencies in the same manner as $24,25(OH)_2D$ VMR values are. We believe that a sensitive and robust multiplex LC-MS/MS assay for simultaneous measurement of $1,25(OH)_2D$, $24,25(OH)_2D$, and $25D$ will enhance future investigations regarding the optimal combination of analytes for the assessment of vitamin D sufficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

LIST OF ABBREVIATIONS

$24,25(OH)_2D$	24,25-dihydroxyvitamin D
25D	25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 combined
$25D_3$	25-hydroxyvitamin D_3
VMR	Vitamin D Metabolite Ratio
HANDLS	Healthy Aging in Neighborhoods of Diversity Across the Lifespan study
DBP	Vitamin D binding protein
CYP24A1	24-hydroxylase enzyme
PTAD	4-Phenyl-1,2,4-triazole-3,5-dione

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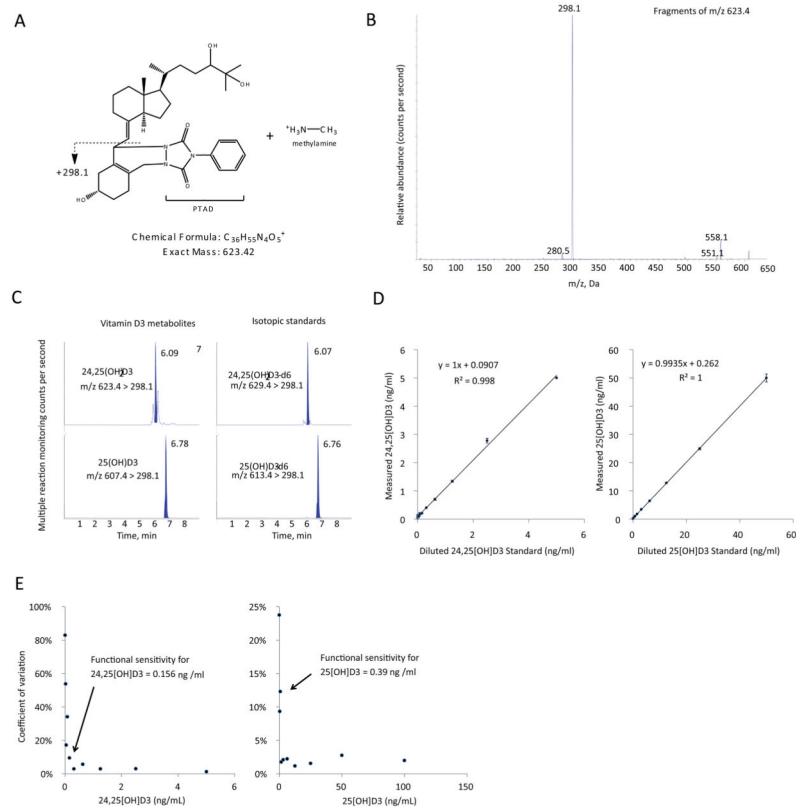
Figure 1. Assay validation experiments
(A) Chemical structure and predicted molecular weight of methylamine adduct of PTAD-derivatized 24,25(OH)$_2$D$_3$. (B) MS/MS fragmentation spectra of methylamine adduct of PTAD-derivatized 24,25(OH)$_2$D$_3$. (C) LC-MRM elution peaks of 24,25(OH)$_2$D$_3$ and 25D$_3$ metabolites and their respective isotopic standards from a representative patient sample run. Mass transitions are shown. (D) Assay linearity of serially diluted samples, n=4 replicates per dilution, standard error bars shown. (E) Coefficients of variance of sample assay measurements plotted against analyte concentration; n=4 replicate measurements per data point.
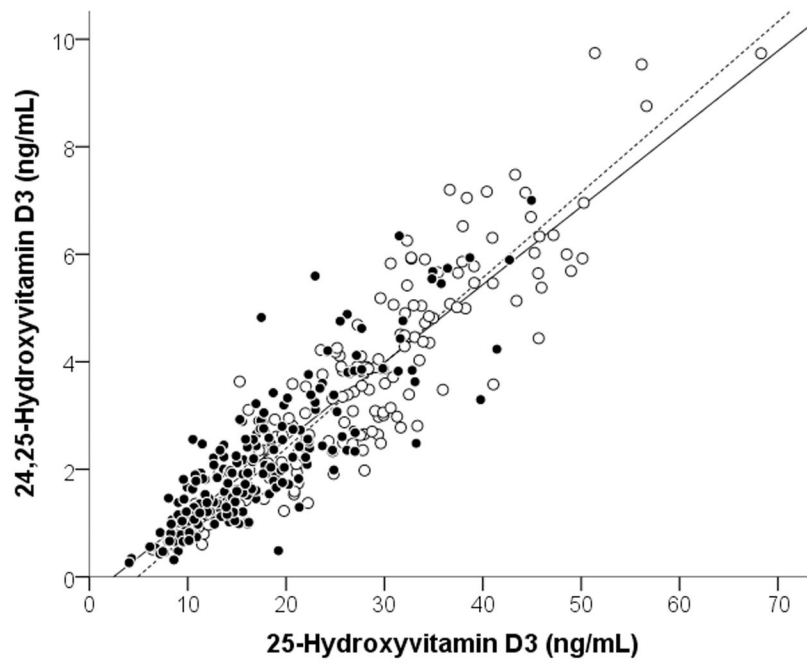
Figure 2. Association between 25D$_3$ and 24,25(OH)$_2$D$_3$
In the overall population, 25D$_3$ and 24,25(OH)$_2$D$_3$ were strongly associated (r = 0.91, p<0.001). In blacks (closed circles, solid line) and whites (open circles, dashed line), this association remained highly significant when examined separately by race (blacks: r = 0.86, P<0.001; whites: r = 0.90, P<0.001).
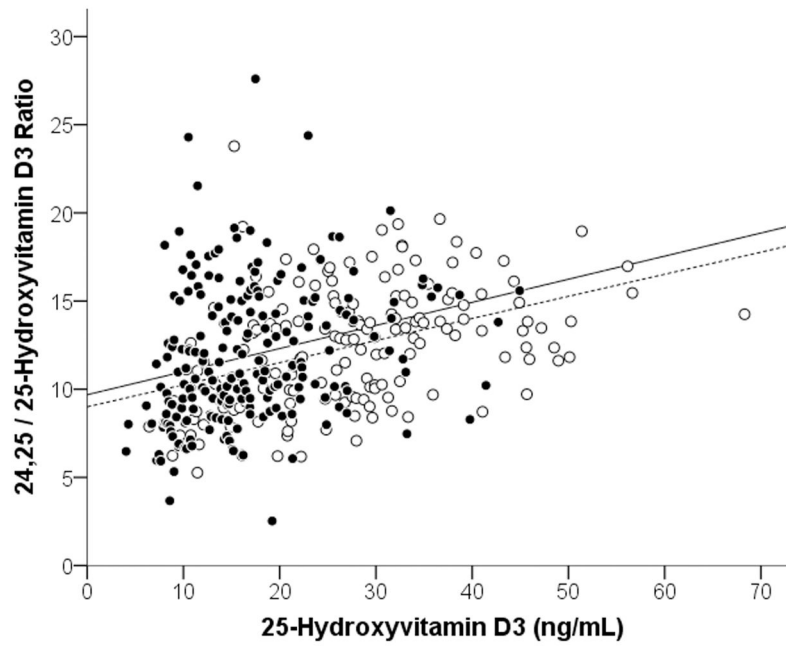
Figure 3. Association between VMR (24,25(OH)$_2$D$_3$/25D$_3$ ratio) and 25D$_3$
Associations between 24,25D$_3$:25D$_3$ ratio values vs 25D$_3$ for blacks (solid circles) and whites (open circles). For blacks, r_s= 0.26, P<0.001. For whites, r_s= 0.41, P<0.001.
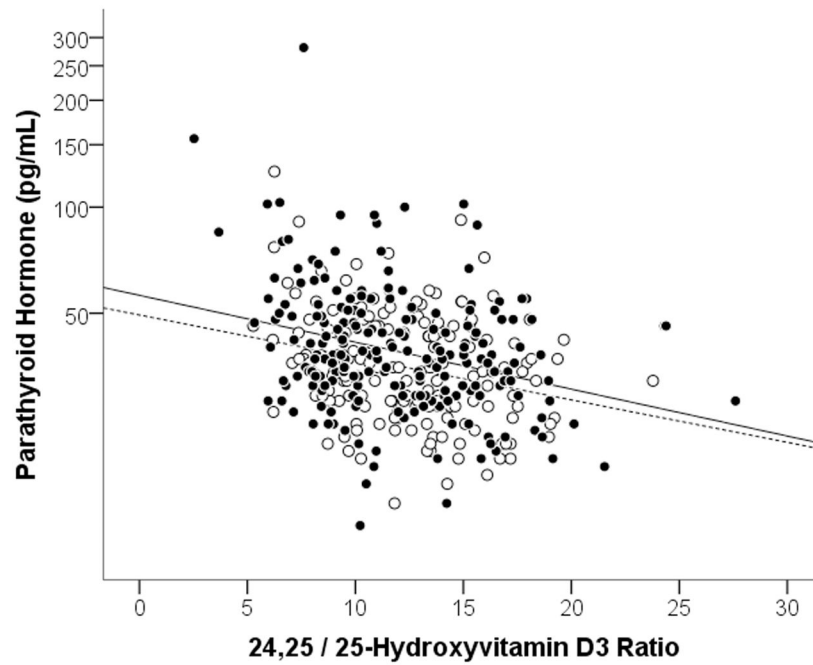
Figure 4. Association between VMR (24,25(OH)$_2$D$_3$/25D$_3$ ratio) and parathyroid hormone (PTH) concentrations

Associations between VMR values and PTH for Blacks (closed circles, solid line) and whites (open circles, dashed line). For blacks, $r_s = -0.26$, p<0.001. For whites, $r_s = -0.25$, P=0.001). The VMR values are multipled by 100.
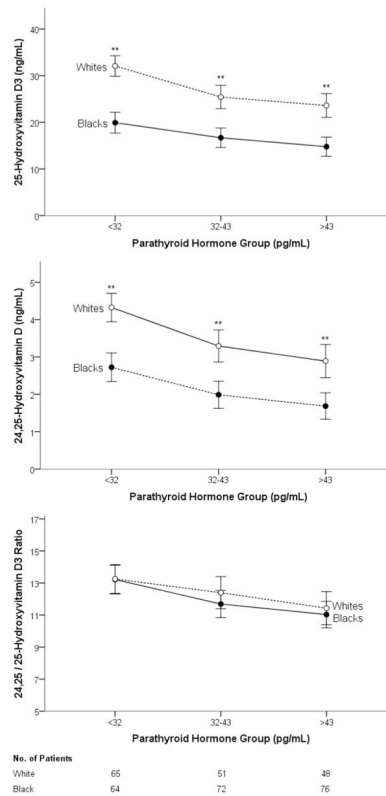
Figure 5. Vitamin D measures by race among similar parathyroid hormone concentrations
Blacks (closed circles, solid line) and whites (open circles, dashed line) for each tertile of PTH concentration. The VMR ($24,25(OH)_2D_3/25D_3$ ratio) did not differ between races.
**Significant at $p < 0.01$.

Table 1

Characteristics of Subset of HANDLS Participants Overall and by Race.

Characteristic	Overall (N=376)	Whites[1] (N=164)	Blacks[1] (N=212)	P-value
Age (y)	48.6 ± 9.2	49.3 ± 9.3	48.1 ± 9.1	0.18
Male sex (%)	166 (44.2)	72 (43.9)	94 (44.3)	0.93
Body Mass Index (kg/m^2)	29.6 ± 7.6	30.1 ± 7.4	29.3 ± 7.8	0.31
Household income < 25% poverty line (%)	159 (42.3)	54 (32.9)	105 (49.5)	0.001
Current smoker (%)	176 (46.8)	74 (45.1)	102 (48.1)	0.45
Osteoporosis diagnosis (%)	12 (3.2)	9 (5.5)	3 (1.4)	0.04
Prescribed osteoporosis therapy (%)	10 (2.7)	6 (3.7)	4 (1.9)	0.29
Postmenopausal (% of women)	108 (51.4)	52 (56.5)	56 (47.5)	0.21
Prescribed HRT (% of women)	5 (2.4)	2 (2.2)	3 (2.5)	0.81
Microalbuminuria (%)	4 (1.1)	2 (1.2)	2 (0.9)	0.99
eGFR <60 ml/min/m^2 (%)	26 (6.9)	9 (5.5)	17 (8.0)	0.35
Prescribed antiepileptic agents (%)	3 (0.8)	2 (1.2)	1 (0.5)	0.42
Prescribed glucocorticoids (%)	5 (1.3)	1 (0.6)	4 (1.9)	0.28
Dietary vitamin D intake (IU/day)	154 ± 181	164 ± 223	146 ± 141	0.37
Dietary calcium intake (mg/day)	745 ± 568	761 ± 601	732 ± 542	0.62
Femoral neck bone mineral density (g/cm^2)	1.00 ± 0.19	0.95 ± 0.16	1.04 ± 0.19	<0.001
Serum calcium (mg/dL)	9.3 ± 0.4	9.3 ± 0.4	9.3 ± 0.4	0.63
Parathyroid hormone (pg/mL)[†]	36 (29, 47)	34 (27, 45)	38 (30, 48)	0.01
25D$_3$ (ng/mL)	21.6 ± 10.8	27.5 ± 11.3	17.0 ± 7.8	<0.001
24,25D$_3$ (ng/mL)	2.7 ± 1.8	3.6 ± 2.0	2.1 ± 1.3	<0.001
VMR (24,25(OH)$_2$D$_3$/25D$_3$ ratio) $\times$ 100	12.1 ± 3.7	12.5 ± 3.4	11.9 ± 4.0	0.16

[1]There were no statistically significant differences between any of the characteristics of white HANDLS subjects included in this study compared to non-included whites, and no differences between included and non-included black HANDLS subjects.

HRT = hormone replacement therapy

Table shows means ± standard deviations,

[†]median (quartile 1, quartile 3), or number (percentage).