24,25-Dihydroxyvitamin D$_3$ and Vitamin D Status of Community-Dwelling Black and White Americans

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

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 study participants (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 suggest that alternative measures such as VMR should be considered.

© 2015 American Association for Clinical Chemistry

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)[8] 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 than whites (15–18).

This paradox was partially reconciled by recent findings from the Healthy Aging in Neighborhoods of Diversity Across the Life Span (HANDLS) study (19). Although black Americans have significantly lower mean total 25D concentrations than 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-dihydroxyvitamin D [24,25(OH)$_2$D] *(21, 22)*. 24,25(OH)$_2$D is the major product of catabolism of 25D, and because enzymatic synthesis of 24,25(OH)$_2$D 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)$_2$D is regulated in part by vitamin D receptor activity *(24, 25)*. Because production of 24,25(OH)$_2$D depends upon both concentrations of 25D and on vitamin D-regulated expression of CYP24A1, concentrations of 24,25(OH)$_2$D 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)$_2$D and 25D serum concentrations [hereinafter referred to as the vitamin D metabolite ratio (VMR)] *(21, 22, 26)*. 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 *(21–23, 27, 28)*. Low VMR also may be predictive of responsiveness to vitamin D supplementation *(21, 28)*, and it has been demonstrated that patients with chronic kidney disease do not increase VMR concentrations in response to vitamin D supplementation as much as controls, consistent with the model that defective kidney production of 1,25(OH$_2$)D results in a persistent decrease in 24,25(OH)$_2$D 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 than whites but show no signs of vitamin D deficiency *(19, 29–32)*. 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)$_2$D *(33, 34)*, and we would predict that effects of DBP binding on vitamin metabolites would affect 25D and 24,25(OH)$_2$D equally, and thus both 25D and 24,25(OH)$_2$D may be lower in African Americans than 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 24,25(OH)$_2$D concentrations 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) *(35)*. Study participants were 30–64 years of age, living in Baltimore, MD, and recruited from 13 contiguous US Census tracts. Participants from the original HANDLS cohort were randomly sampled from within age, race, sex, and socioeconomic status strata, excluding those who did not self-identify as black or white. Participants selected for this ancillary study included all patients for whom 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 0915 and 1030 Only participants who completed the examination were included in the study.

LABORATORY ANALYSIS

Blood samples were drawn at the examination into serum separator tubes without anticoagulant and centrifuged at 1430*g* for 15 min, and then 1.8 mL of serum was transferred to Nunc Cryotubes and stored at −80 °C for future analysis. We mixed 100 μL of serum with 25D$_3$-[^{2}H$_6$] and 24R,25-(OH)$_2$D$_3$–[^{2}H$_6$] isotopic internal standards dissolved in 5% BSA (IsoSciences). 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), and eluted with 1 mL ethyl acetate containing 0.1 g/L 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 reversed-phase chromatography coupled to tandem mass spectrometry in multiple reaction monitoring (MRM) mode [intraassay CV 1.1% and 3.5% for 25D$_3$ and 24,25(OH)$_2$D$_3$, respectively]. Assays were calibrated using 25D$_3$ and 24R,25-(OH)$_2$D$_3$ commercial standards (Cerilliant). Intact parathyroid hormone
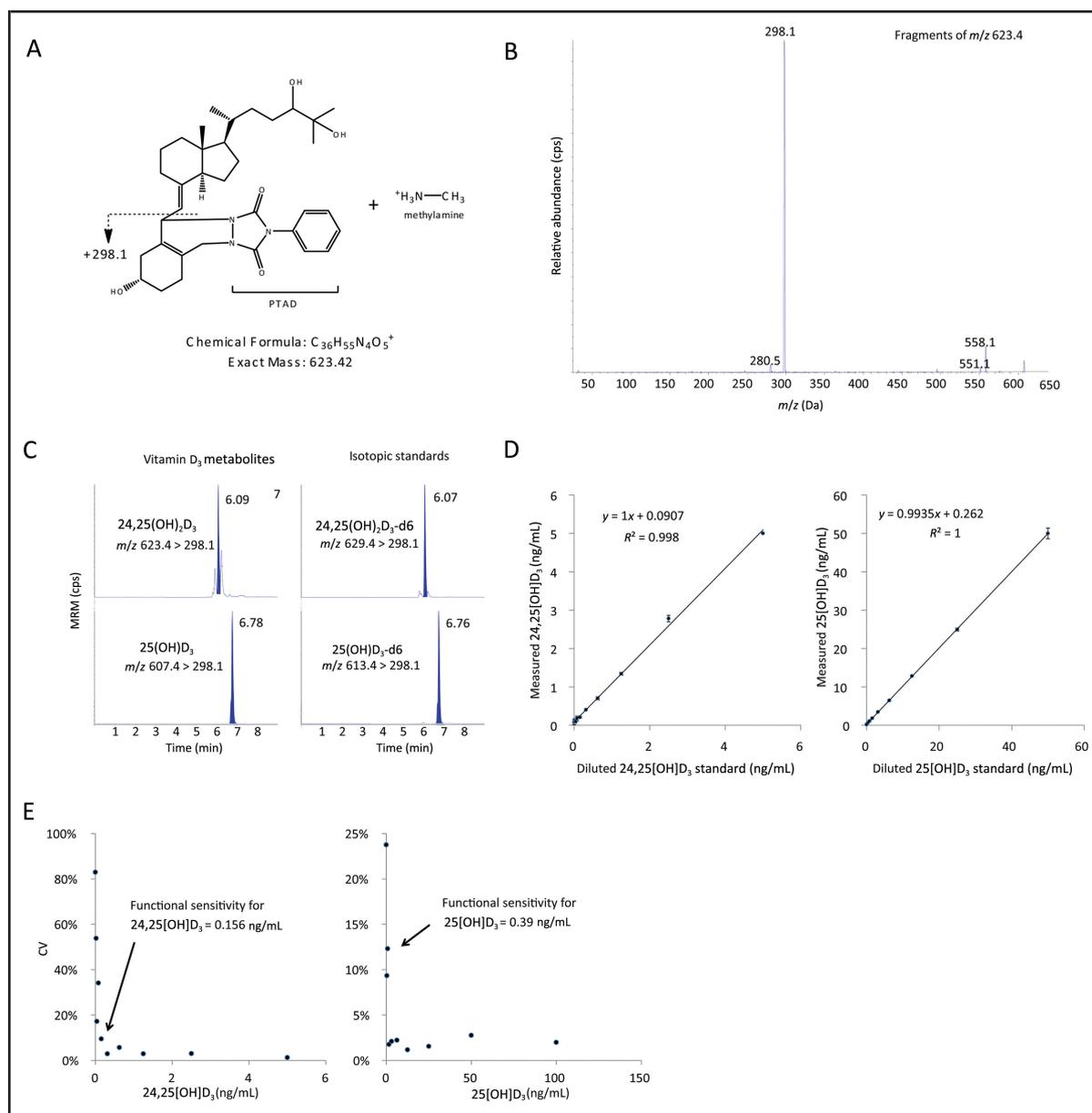
Fig. 1. Assay validation experiments.

(A), Chemical structure and predicted molecular weight of the methylamine adduct of PTAD-derivatized 24,25(OH)$_2$D$_3$. (B), Tandem mass spectrometry fragmentation spectra of the 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.

(PTH) levels were measured using the Cobas electrochemiluminescense immunoassay on the Modular Analytics E170 automated analyzer (Roche Diagnostics; interassay CV, 2.5%). Additional method details are described in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol61/issue6.

STATISTICAL ANALYSIS

The characteristics of the study participants are presented as means (SD) or numbers and percentages and were compared according to race with the use of t-tests or χ^2 tests. Because PTH was nonnormally distributed, it is presented as a median (quartile 1, quartile 3) and compared with a Mann–Whitney U-test between races. The

Table 1. Characteristics of subset of HANDLS study participants overall and by race.[a]

Characteristic	Overall (n = 376)	Whites[b] (n = 164)	Blacks (n = 212)	P value
Age, years, mean (SD)	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, mean (SD)	29.6 (7.6)	30.1 (7.4)	29.3 (7.8)	0.31
Household income <125% 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 hormone replacement therapy, % 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[c] <60 mL · min^{-1} · (1.73 m^2)$^{-1}$, %	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, mean (SD)	154 (181)	164 (223)	146 (141)	0.37
Dietary calcium intake, mg/day, mean (SD)	745 (568)	761 (601)	732 (542)	0.62
Femoral neck bone mineral density, g/cm^2, mean (SD)	1.00 (0.19)	0.95 (0.16)	1.04 (0.19)	<0.001
Serum calcium, mg/dL, mean (SD)	9.3 (0.4)	9.3 (0.4)	9.3 (0.4)	0.63
PTH, pg/mL[b]	36 (29, 47)	34 (27, 45)	38 (30, 48)	0.01
25D$_3$, ng/mL, mean (SD)	21.6 (10.8)	27.5 (11.3)	17.0 (7.8)	<0.001
24,25D$_3$, ng/mL, mean (SD)	2.7 (1.8)	3.6 (2.0)	2.1 (1.3)	<0.001
VMR × 100, mean (SD)	12.1 (3.7)	12.5 (3.4)	11.9 (4.0)	0.16

[a] Values are mean (SD), median (quartile 1, quartile 3), or number (percentage).
[b] There were no statistically significant differences between any of the characteristics of white HANDLS individuals included in this study compared to nonincluded whites, and no differences between included and nonincluded black HANDLS individuals.
[c] eGFR, estimated glomerular filtration rate.

relationship between 25D$_3$ and 24,25D$_3$ was summarized with the use of Pearson product–moment correlation, and Spearman's rank correlation coefficient (r_s) 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 were used to examine interactions between PTH and race in predicting 25D$_3$, 24,25(OH)$_2$D$_3$, or the VMR. Additionally, the association between the ratio of the VMR and 25D$_3$ was examined with the use of general linear models, including an interaction term between race and 25D$_3$. Because of a possible inflection point, associations between 25D$_3$ and 24,25(OH)$_2$D$_3$ were examined after stratifying by 25D$_3$ concentrations less than and more than 12 ng/mL within each race. Results of general linear models are presented as means (95% CIs). Statistical analyses were conducted with the use of SAS software, version 9.4 (SAS Institute). Two-tailed P values of <0.05 were considered to indicate statistical significance.

Results

ASSAY VALIDATION

To sensitively measure both 25D$_3$ and 24,25(OH)$_2$D$_3$, we developed an isotope-dilutional tandem mass spectrometric assay adapted from recently described methods *(23)*. The assay uses derivatization with 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 subnanomolar concentrations (Fig. 1).

STUDY PARTICIPANT CHARACTERISTICS

Our recently reported study of DBP and vitamin D in HANDLS study participants included 1181 black and 904 white participants *(19)*. In the present study, we analyzed samples from 376 randomly selected patients from this cohort who had sufficient remaining serum for analysis, including 212 blacks and 164 whites. Baseline
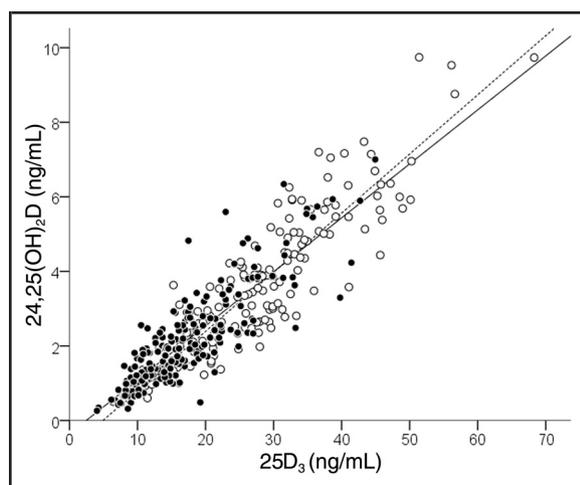
Fig. 2. Association between $25D_3$ and $24,25(OH)_2D_3$.
In the overall population, $25D_3$ and $24,25(OH)_2D_3$ were strongly associated ($r = 0.91$, $P < 0.001$). In blacks (closed circles, solid line) and whites (open circles, dashed line), this association remained highly significant when examined separately by race (blacks: $r = 0.86$, $P < 0.001$; whites: $r = 0.90$, $P < 0.001$).

Fig. 3. Association between VMR [$24,25(OH)_2D_3/25D_3$ ratio] and $25D_3$.
Associations between VMR vs $25D_3$ values for blacks (solid circles) and whites (open circles). For blacks, $r_s = 0.26$, $P < 0.001$. For whites, $r_s = 0.41$, $P < 0.001$.

characteristics of included black and white study participants 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 patients compared to nonincluded HANDLS participants (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/cm² vs 0.95 g/cm²), 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 $24,25(OH)_2D_3$ (Fig. 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 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 patients' VMR values were plotted against $25D_3$ concentra-

tions (Fig. 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 2 linear models for blacks and whites was statistically significant, and that at any given concentration of $25D_3$ blacks had higher mean 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 ($r_s = -0.25$, $P < 0.01$) (Fig. 4). The overall correlation between VMR and PTH among all study participants was $r_s = -0.26$, $P = 0.01$. Grouping patients by PTH levels divided into tertiles [i.e., tertile 1 (low), <32 pg/mL; tertile 2 (mid), ≥32 pg/mL to ≤43 pg/mL; tertile 3 (high) >43 pg/mL], we observed the expected inverse relationship between PTH and all vitamin D parameters (Fig. 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).
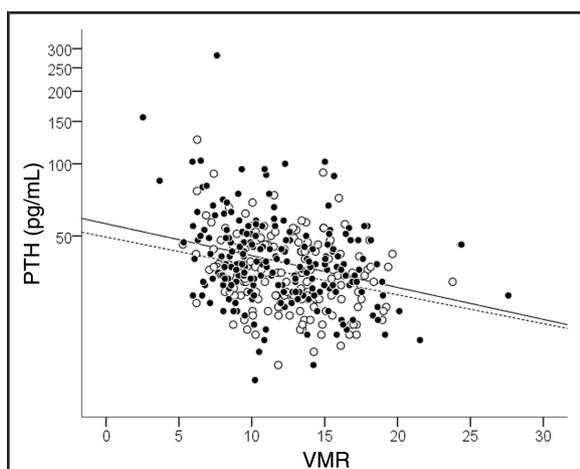
Fig. 4. Association between VMR [24,25(OH)₂D₃/25D₃ ratio] and PTH concentrations.

Associations between VMR values and PTH concentrations 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 multiplied by 100.

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 to assess how this new indicator reflects vitamin D status, and whether 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 than in 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 than 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 PTH concentrations similar to those of 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
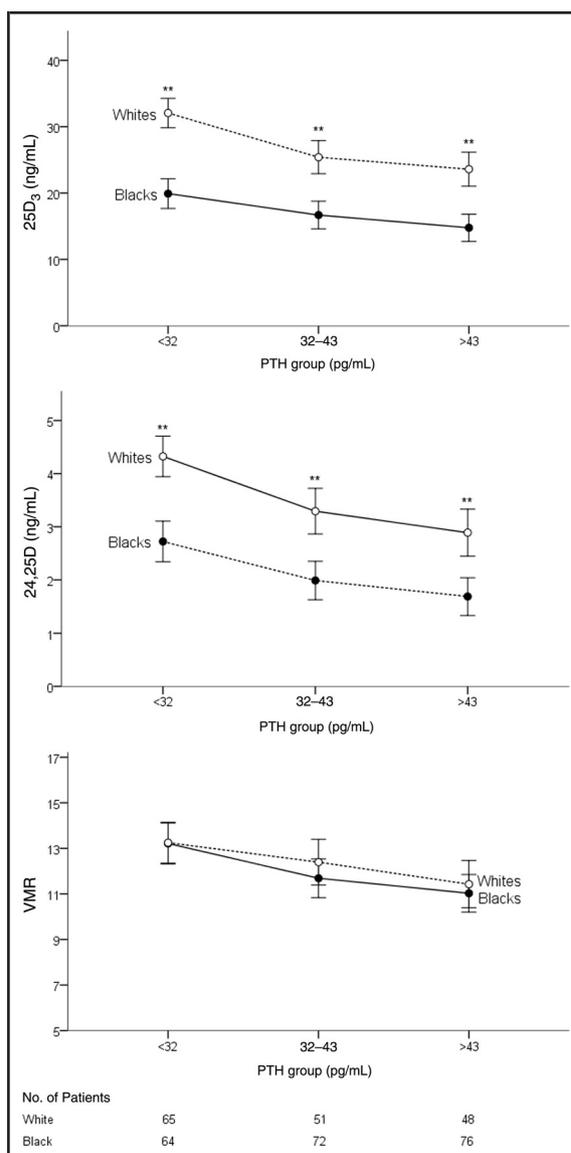
Fig. 5. Vitamin D measures by race among similar PTH concentrations.

Blacks (closed circles, solid line) and whites (open circles, dashed line) for each tertile of PTH concentration. The VMR [24,25(OH)₂D₃/25D₃ ratio] did not differ between races. **Significant at $P < 0.01$.

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, 32)*. 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, because these differences will influence both the numerator and denominator of the VMR 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 *(36)*.

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 be available only to clinical laboratories with LC-MS capabilities. Synthesis of $1,25(OH)_2D$ by the kidneys is induced by vitamin D deficiency *(37)*, and thus it is possible that the VMR 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.

References

1. Melamed ML, Michos ED, Post W, Astor B. 25-hydroxyvitamin D levels and the risk of mortality in the general population. Arch Int Med 2008;168:1629–37.
2. Cauley JA, Lacroix AZ, Wu L, Horwitz M, Danielson ME, Bauer DC, et al. Serum 25-hydroxyvitamin D concentrations and risk for hip fractures. Ann Intern Med 2008; 149:242–50.
3. Ginde AA, Liu MC, Camargo CA Jr. Demographic differences and trends of vitamin D insufficiency in the US population, 1988–2004. Arch Int Med 2009;169: 626–32.
4. Wactawski-Wende J, Kotchen JM, Anderson GL, Assaf AR, Brunner RL, O'Sullivan MJ, et al. Calcium plus vitamin D supplementation and the risk of colorectal cancer. N Engl J Med 2006;354:684–96.
5. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, et al. Vitamin D deficiency and risk of cardiovascular disease. Circulation 2008;117:503–11.
6. Trummer O, Pilz S, Hoffmann MM, Winkelmann BR, Boehm BO, Marz W, et al. Vitamin D and mortality: a Mendelian randomization study. Clin Chem 2013;59: 793-7.
7. Thomas MK, Lloyd-Jones DM, Thadhani RI, Shaw AC, Deraska DJ, Kitch BT, et al. Hypovitaminosis D in medical inpatients. N Engl J Med 1998;338:777–83.
8. Heaney RP, Weaver CM. Calcium and vitamin D. Endocrinol Metab Clin North Am 2003;32:181–94, vii–viii.
9. Villareal DT, Civitelli R, Chines A, Avioli LV. Subclinical vitamin D deficiency in postmenopausal women with low vertebral bone mass. J Clin Endocrinol Metab 1991; 72:628–34.
10. Chapuy MC, Arlot ME, Duboeuf F, Brun J, Crouzet B, Arnaud S, et al. Vitamin D3 and calcium to prevent hip fractures in the elderly women. N Engl J Med 1992; 327:1637–42.
11. Jackson RD, LaCroix AZ, Gass M, Wallace RB, Robbins J, Lewis CE, et al. Calcium plus vitamin D supplementation and the risk of fractures. N Engl J Med 2006;354: 669–83.
12. Ooms ME, Roos JC, Bezemer PD, van der Vijgh WJ, Bouter LM, Lips P. Prevention of bone loss by vitamin D supplementation in elderly women: a randomized double-blind trial. J Clin Endocrinol Metab 1995;80: 1052–8.
13. Mitchell DM, Henao MP, Finkelstein JS, Burnett-Bowie SA. Prevalence and predictors of vitamin D deficiency in healthy adults. Endocr Pract 2012;18:914–23.
14. Gutierrez 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. Osteopor Int 2010;22:1745–53.
15. Hannan MT, Litman HJ, Araujo AB, McLennan CE, McLean RR, McKinlay JB, et al. 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.
16. Bischoff-Ferrari HA, Dietrich T, Orav EJ, Dawson-Hughes B. Positive association between 25-hydroxy vitamin D levels and bone mineral density: a population-based study of younger and older adults. Am J Med 2004; 116:634–9.
17. Cauley JA, Lui LY, Ensrud KE, Zmuda JM, Stone KL, Hochberg MC, Cummings SR. Bone mineral density and the risk of incident nonspinal fractures in black and white women. JAMA 2005;293:2102–8.
18. Cauley JA, Danielson ME, Boudreau R, Barbour KE, Horwitz MJ, Bauer DC, et al. Serum 25-hydroxyvitamin D and clinical fracture risk in a multiethnic cohort of women: the Women's Health Initiative (WHI). J Bone Miner Res 2011;26:2378–88.
19. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med 2013;369:1991–2000.
20. Carter GD, Phinney KW. Assessing vitamin D status: time for a rethink? Clin Chem 2014;60:809–11.
21. Wagner D, Hanwell HE, Schnabl K, Yazdanpanah M, Kimball S, Fu L, et al. The ratio of serum 24,25-dihydroxyvitamin D(3) to 25-hydroxyvitamin D(3) is predictive of 25-hydroxyvitamin D(3) response to vita-

min D(3) supplementation. J Ster Biochem Molec Biol 2011;126:72–7.

22. Bosworth CR, Levin G, Robinson-Cohen C, Hoofnagle AN, Ruzinski J, Young B, et al. The serum 24,25-dihydroxyvitamin D concentration, a marker of vitamin D catabolism, is reduced in chronic kidney disease. Kid Int 2012;82:693–700.

23. de Boer IH, Sachs MC, Chonchol M, Himmelfarb J, Hoofnagle AN, Ix JH, et al. Estimated GFR and circulating 24,25-dihydroxyvitamin D3 concentration: a participant-level analysis of 5 cohort studies and clinical trials. Am J Kidney Dis 2014;64:187–97.

24. Jones G, Prosser DE, Kaufmann M. Cytochrome P450-mediated metabolism of vitamin D. J Lipid Res 2013; 55:13–31.

25. Pike JW, Meyer MB. Regulation of mouse Cyp24a1 expression via promoter-proximal and downstream-distal enhancers highlights new concepts of 1,25-dihydroxyvitamin D(3) action. Arch Biochem Biophys 2011;523:2–8.

26. Cashman KD, Hayes A, Galvin K, Merkel J, Jones G, Kaufmann M, et al. Significance of serum 24,25-dihydroxyvitamin D in the assessment of vitamin D status: a double-edged sword? Clin Chem 2015;61:636–45.

27. Kaufmann M, Gallagher JC, Peacock M, Schlingmann KP, Konrad M, DeLuca HF, et al. Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D by LC-MS/MS involving derivatization with DMEQ-TAD. J Clin Endocrinol Metab 2014;99:2567–74.

28. Stubbs JR, Zhang S, Friedman PA, Nolin TD. Decreased conversion of 25-hydroxyvitamin D3 to 24,25-dihydroxyvitamin D3 following cholecalciferol therapy in patients with CKD. Clin J Am Soc Nephrol 2014;9:1965–73.

29. Powe CE, Ricciardi C, Berg AH, Erdenesanaa D, Collerone G, Ankers E, et al. Vitamin D-binding protein modifies the vitamin D-bone mineral density relationship. J Bone Miner Res 2011;26:1609–16.

30. 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. Kid Int 2012;82:84–9.

31. Wilson RT, Bortner JD Jr, Roff A, Das A, Battaglioli EJ, Richie JP Jr, et al. Genetic and environmental influences on plasma vitamin D binding protein concentrations. Transl Res 2015;165:667–76.

32. Johnsen MS, Grimnes G, Figenschau Y, Torjesen PA, Almas B, Jorde R. Serum free and bio-available 25-hydroxyvitamin D correlate better with bone density than serum total 25-hydroxyvitamin D. Scand J Clin Lab Invest 2014;74:177–83.

33. Kawakami M, Imawari M, Goodman DS. Quantitative studies of the interaction of cholecalciferol (vitamin D3) and its metabolites with different genetic variants of the serum binding protein for these sterols. Biochem J 1979;179:413–23.

34. Haddad JG Jr. Transport of vitamin D metabolites. Clin Orthop Relat Res 1979;142:249–61.

35. Evans MK, Lepkowski JM, Powe NR, LaVeist T, Kuczmarski MF, Zonderman AB. Healthy aging in neighborhoods of diversity across the life span (HANDLS): overcoming barriers to implementing a longitudinal, epidemiologic, urban study of health, race, and socioeconomic status. Ethn Dis 2010;20:267–75.

36. Heijboer AC, Blankenstein MA, Kema IP, Buijs MM. Accuracy of 6 routine 25-hydroxyvitamin D assays: influence of vitamin D binding protein concentration. Clin Chem 2012;58:543–8.

37. Jones G. Expanding role for vitamin D in chronic kidney disease: importance of blood 25-OH-D levels and extrarenal 1alpha-hydroxylase in the classical and nonclassical actions of 1alpha,25-dihydroxyvitamin D(3). Semin Dial 2007;20:316–24.

Supplementary Materials

Materials

$25D_3–[^2H_6]$ and $24R,25-(OH)_2D_3–[^2H_6]$ isotopic standards were purchased from IsoSciences, Inc. (King of Prussia, PA). Isotopic standards were dissolved at 1 mg/ml in acetonitrile, then further dissolved to their working concentrations (100 ng/mL $25D_3$ and 10 ng/mL $24,25(OH)_2D_3$) in water containing 5% (w/v) charcoal-stripped bovine serum albumin as a carrier protein, aliquoted, snap frozen in liquid nitrogen, and stored at -80°C. The 5% bovine albumin solution used in calibrators and isotopic internal standards was charcoal stripped and assayed for contaminating 25-hydroxyvitamin D3 and D2 before addition of standard materials; no vitamin D metabolites could be detected. Calibrators for $25D_3$ and $24,25(OH)_2D_3$ were purchased from Cerilliant, Inc. (Round Rock, TX), dissolved in acetonitrile, aliquoted and stored at -80°C. Solid phase extraction chromatography was performed using Strata C-18E 96-well SPE plates (Phenomenex, Inc., Torrence, CA). 4-Phenyl-1,2,4-triazole-3,5-dione, bovine serum albumin, and all other chemicals were purchased from Sigma Aldrich, Inc. (St. Louis, MO). There was no detectable 25D3 or 24,25D3 present in the charcoal-stripped 5% bovine serum albumin solution. HPLC was performed using a Kinetex C8 HPLC column (4.6 x 50 mm, 2.6 μm bead size) on a Shimadzu Prominence UPLC with autosampler (Shimadzu Scientific Instruments, Columbia, MD). For metabolite monitoring, the HPLC was coupled to an API 5000 triple quadrupole mass spectrometer with Turbo V electrospray ion source (AB Sciex, Framingham, MA). Intact PTH concentrations were measured using the Cobas electrochemiluminescense immunoassay on the Modular Analytics E170 automated analyzer (Roche Diagnostics, Indianapolis, IN, USA; inter-assay CV 2.5%). Structural illustration of the PTAD-derivatized 25D3 was generated using Cambridgesoft ChemDraw Pro 12.0 (Perkin Elmer, Waltham, PA).

Methods

For measurement of vitamin D metabolites, 100 µL of serum was mixed with 20 µL isotopic standard (2 ng $25D_3$–[$^2H_6$] and 0.2 ng 24R,25-(OH)$_2$D$_3$–[$^2H_6$] per reaction) and allowed to equilibrate for 30 minutes at room temperature. Total $25D_3$ and $24,25D_3$ and isotopic standards were extracted away from DBP and other serum binding factors by protein precipitation with 250 µL methanol, and cleared by centrifugation at 20,817 × g for 10 minutes. Extract supernatants were diluted with 1 mL of water and vitamin D metabolites were isolated by solid phase extraction chromatography. Diluted samples were applied to SPE wells under vacuum, washed with 2 mL water, dried under a vacuum to remove as much water as possible, followed by elution with 1 mL ethyl acetate containing 0.1 mg/mL 4-Phenyl-1,2,4-triazole-3,5-dione in order to simultaneously elute and derivatize vitamin D metabolites. Samples were lyophilized under vacuum, dissolved with 50 µL ethanol, and diluted with 50 µL water. Samples were then analyzed for vitamin D metabolites by reverse phase chromatography coupled to tandem mass spectrometry in multiple reaction monitoring mode. Briefly, 50 µL of sample was injected by autosampler and vitamin D metabolites were resolved using a gradient of 40% - 100% methanol and 0.1% formic acid and 5 mM methylamine additives at 0.7 mL/min flow rate. Metabolite MRM transitions and mass spectrometer settings are shown in Supplemental Table 1.

Validation experiments

MRM optimization (Fig. 1B, main text): Daughter ion products of collision-induced dissociation of PTAD-derivatized vitamin D metabolites was determined by analyzing calibrator standards and isotopic internal standards using MS/MS fragmentation scanning and MS settings shown in Supplemental Table 1. *Assay linearity (Fig. 1D, main text):* Calibrators were diluted into charcoal-stripped 5% bovine serum albumin starting at a 25D3 concentration of 50 ng/mL and 24,25D3 concentration of 5 ng/mL. These calibrators were then serially diluted 1:1 with

additional 5% bovine serum albumin. 5% bovine albumin without added calibrators was used as the assay blank. Four replicates of each dilution were made, diluted standards were extracted and analyzed using the same methods as patient samples, and measured concentrations were compared to predicted concentrations based upon their dilution factor. *Functional sensitivity* (Fig. 1E, main text): The mean values and standard errors of the four replicate measurements for each of the dilutions made for the linearity study were used to determine the coefficients of variance (%CV) at decreasing concentrations of 25D3 and 24,25D3.

Supplemental Table 1. Metabolite MRM transitions and mass spectrometer settings.

Ion source settings		
Curtain gas: 17	GS1: 40	GS2: 40
Ion spray voltage: +5500V	Temperature: 450°C	Positive ion mode
MS settings		
Declustering potential: 100	Entrance potential: 10	Collision energy: 28
Collision gas: 7	Collision exit potential: 16	
MRM mass transitions (parent ion m/z -> daughter ion m/z)		
Vitamin D metabolite	Endogenous	Isotopic internal standard
25D3	607.4 -> 298.1	613.4 -> 298.1
24,25D3	623.4 -> 298.1	629.4 -> 298.1
25D2	619.4 -> 298.1	