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

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BACKGROUND: 24,25-Dihydroxyvitamin D [24,25(OH)₂D] 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)₂D to 25D [vitamin D metabolite ratio (VMR)] represents a new candidate biomarker for vitamin D status.

METHODS: We measured 24,25(OH)₂D₃ and 25D₃ 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 25D₃ against a physiological indicator of vitamin D deficiency.

RESULTS: Serum 24,25(OH)₂D₃ strongly correlated with 25D₃ 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 25D₃ 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)₂D₃ [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 25D₃ 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 (rₚ = −0.26, P < 0.001, and rₚ = −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.

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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 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)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 (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)₂D 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 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)₂D (33, 34), 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 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)₂D 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 1430g 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 25D₃ [²H₃] and 24R,25-(OH)₂D₃[²H₃] isotopic internal standards dissolved in 5% BSA (IsoSciences). Total 25D₃ and 24,25D₃ were extracted away from DBP and other serum binding factors by protein precipitation with 250 μL of 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 [intra assay CV 1.1% and 3.5% for 25D₃ and 24,25(OH)₂D₃, respectively]. Assays were calibrated using 25D₃ and 24R,25-(OH)₂D₃ commercial standards (Cerilliant). Intact parathyroid hormone
(PTH) levels were measured using the Cobas electrochemiluminescence 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 χ² 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
relationship between 25D₃ and 24,25D₃ was summarized with the use of Pearson product–moment correlation, and Spearman’s rank correlation coefficient (rₛ) was used to summarize the relationship between 24,25D₃ 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 25D₃, 24,25(OH)₂D₃, or the VMR. Additionally, the association between the ratio of the VMR and 25D₃ was examined with the use of general linear models, including an interaction term between race and 25D₃. Because of a possible inflection point, associations between 25D₃ and 24,25(OH)₂D₃ were examined after stratifying by 25D₃ 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 25D₃ and 24,25(OH)₂D₃, 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

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### Table 1. Characteristics of subset of HANDLS study participants overall and by race.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Overall (n = 376)</th>
<th>Whitesb (n = 164)</th>
<th>Blacks (n = 212)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, mean (SD)</td>
<td>48.6 (9.2)</td>
<td>49.3 (9.3)</td>
<td>48.1 (9.1)</td>
<td>0.18</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>166 (44.2)</td>
<td>72 (43.9)</td>
<td>94 (44.3)</td>
<td>0.93</td>
</tr>
<tr>
<td>Body mass index, kg/m², mean (SD)</td>
<td>29.6 (7.6)</td>
<td>30.1 (7.4)</td>
<td>29.3 (7.8)</td>
<td>0.31</td>
</tr>
<tr>
<td>Household income &lt;125% poverty line, %</td>
<td>159 (42.3)</td>
<td>54 (32.9)</td>
<td>105 (49.5)</td>
<td>0.001</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>176 (46.8)</td>
<td>74 (45.1)</td>
<td>102 (48.1)</td>
<td>0.45</td>
</tr>
<tr>
<td>Osteoporosis diagnosis, %</td>
<td>12 (3.2)</td>
<td>9 (5.5)</td>
<td>3 (1.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>Prescribed osteoporosis therapy, %</td>
<td>10 (2.7)</td>
<td>6 (3.7)</td>
<td>4 (1.9)</td>
<td>0.29</td>
</tr>
<tr>
<td>Postmenopausal, % of women</td>
<td>108 (51.4)</td>
<td>52 (56.5)</td>
<td>56 (47.5)</td>
<td>0.21</td>
</tr>
<tr>
<td>Prescribed hormone replacement therapy, % of women</td>
<td>5 (2.4)</td>
<td>2 (2.2)</td>
<td>3 (2.5)</td>
<td>0.81</td>
</tr>
<tr>
<td>Microalbuminuria, %</td>
<td>4 (1.1)</td>
<td>2 (1.2)</td>
<td>2 (0.9)</td>
<td>0.99</td>
</tr>
<tr>
<td>eGFR&lt;60 mL·min⁻¹·(1.73 m²)⁻¹, %</td>
<td>26 (6.9)</td>
<td>9 (5.5)</td>
<td>17 (8.0)</td>
<td>0.35</td>
</tr>
<tr>
<td>Prescribed antiepileptic agents, %</td>
<td>3 (0.8)</td>
<td>2 (1.2)</td>
<td>1 (0.5)</td>
<td>0.42</td>
</tr>
<tr>
<td>Prescribed glucocorticoids, %</td>
<td>5 (1.3)</td>
<td>1 (0.6)</td>
<td>4 (1.9)</td>
<td>0.28</td>
</tr>
<tr>
<td>Dietary vitamin D intake, IU/day, mean (SD)</td>
<td>154 (181)</td>
<td>164 (223)</td>
<td>146 (141)</td>
<td>0.37</td>
</tr>
<tr>
<td>Dietary calcium intake, mg/day, mean (SD)</td>
<td>745 (568)</td>
<td>761 (601)</td>
<td>732 (542)</td>
<td>0.62</td>
</tr>
<tr>
<td>Femoral neck bone mineral density, g/cm², mean (SD)</td>
<td>1.00 (0.19)</td>
<td>0.95 (0.16)</td>
<td>1.04 (0.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum calcium, mg/dL, mean (SD)</td>
<td>9.3 (0.4)</td>
<td>9.3 (0.4)</td>
<td>9.3 (0.4)</td>
<td>0.63</td>
</tr>
<tr>
<td>PTH, pg/mLb</td>
<td>36 (29, 47)</td>
<td>34 (27, 45)</td>
<td>38 (30, 48)</td>
<td>0.01</td>
</tr>
<tr>
<td>25D₃, ng/mL, mean (SD)</td>
<td>21.6 (10.8)</td>
<td>27.5 (11.3)</td>
<td>17.0 (7.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>24,25D₃, ng/mL, mean (SD)</td>
<td>2.7 (1.8)</td>
<td>3.6 (2.0)</td>
<td>2.1 (1.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>VMR × 100, mean (SD)</td>
<td>12.1 (3.7)</td>
<td>12.5 (3.4)</td>
<td>11.9 (4.0)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

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.
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/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)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) (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\(_3\) concentrations (Fig. 3), there was a linear association between VMR and 25D\(_3\) concentrations in both blacks and whites \([\beta (95\% CI) for 25D in blacks was 0.14 (0.09 – 0.19) and for whites was 0.11 (0.08 – 0.15)]\). 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 (\( r_s = 0.26, P = 0.001 \)) 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), \( \geq 32 \) 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)\(_2\)D\(_3\) 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 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 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)$_2$D$_3$ are bound by serum DBP, differences in concentrations of DBP may influence concentrations of total 24,25(OH)$_2$D in
the same way as they do 25D3, independent of vitamin D sufficiency (19, 32). As a result, measurements of 24,25(OH)2D3 would be predicted to be lower in blacks (as observed in this study), and interpretation of 24,25(OH)2D3 concentrations in the evaluation of vitamin D status in African Americans will be subject to the same caveats as 25D3. 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)2D3 and 25D3 away from DBP, and thus VMR measurements will not be influenced by DBP concentrations, as has been observed in immunoassay methods of 25D3 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.

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