

Vitamin D status of black and white Americans and changes in vitamin D metabolites after varied doses of vitamin D supplementation^{1,2}

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ABSTRACT

Background: Controversy exists over the disparate circulating 25-hydroxyvitamin D [25(OH)D] concentrations between black and white Americans.

Objective: We sought to determine whether there are differences in total and directly measured free 25(OH)D concentrations between black and white American adults and how daily supplementation with cholecalciferol changes these concentrations.

Design: Cross-sectional and longitudinal analyses were conducted with the use of data from 2 placebo-controlled, randomized trials at 2 academic medical centers in the United States: CaDDM (Calcium and Vitamin D in Type 2 Diabetes) and DDM2 (Vitamin D for Established Type 2 Diabetes). A total of 208 subjects with pre- or well-controlled diabetes with a mean age of 59.1 y and mean body mass index (BMI; in kg/m²) of 31.6 were randomly assigned to receive daily cholecalciferol supplementation at 1 of 2 doses (2000 or 4000 IU) or a matching placebo for 16 wk. We measured serum total 25(OH)D, vitamin D-binding protein (DBP) by 2 different immunoassays (with the use of monoclonal or polyclonal antibodies), parathyroid hormone, and albumin. Free 25(OH)D concentration was directly measured and calculated.

Results: Blacks had lower total 25(OH)D concentrations than whites [adjusted median: 20.3 ng/mL (95% CI: 16.2, 24.5 ng/mL) compared with 26.7 ng/mL (95% CI: 25.2, 28.1 ng/mL), respectively; *P* = 0.026], and a higher proportion of blacks had total 25(OH)D concentrations <20 ng/mL (46% compared with 19%, respectively; *P* < 0.001). Directly measured free 25(OH)D concentrations were lower in blacks than in whites [adjusted median: 4.5 ng/mL (95% CI: 3.7, 5.4 ng/mL) compared with 5.7 ng/mL (95% CI: 5.4, 5.9 ng/mL), respectively; *P* = 0.044] and were strongly correlated with total 25(OH)D without an effect of race. DBP was lower in blacks when measured by the monoclonal but not the polyclonal antibody immunoassay. Cholecalciferol supplementation increased total and measured free 25(OH)D concentrations proportionally to the dose and without a difference between races.

Conclusions: The relation between free and total 25(OH)D did not vary systematically by race in this multiracial population with pre- or well-controlled diabetes. The results need to be replicated in additional cohorts before concluding that the clinical assessment of vitamin D status in blacks and whites should follow a single standard. The CaDDM and DDM2 trials were registered at clinicaltrials.gov as NCT00436475 and NCT01736865, respectively. *Am J Clin Nutr* 2016;104:205–14.

Keywords: vitamin D, free vitamin D, vitamin D-binding protein, clinical trial, African American, white, supplementation

INTRODUCTION

Observational studies have reported associations between low concentrations of circulating 25-hydroxyvitamin D [25(OH)D]⁸ and increased risk of many common medical conditions, including osteoporosis, diabetes, and cardiovascular disease (1, 2). However, despite inconclusive results from randomized controlled trials (3, 4) and disagreement among experts regarding what constitutes “optimal” vitamin D concentrations (5, 6), supplementation with vitamin D to reach and maintain elusive 25(OH)D targets has become increasingly widespread in clinical practice.

Vitamin D status is assessed by measuring total 25(OH)D, which includes the fraction that is bound to vitamin D-binding protein (DBP) or albumin (~90% and ~10%, respectively) and the free nonprotein-bound form (~0.05%) (7). Consistent with the concept of the free hormone hypothesis (8), it has been postulated that circulating free 25(OH)D might be a better marker of vitamin D status than total 25(OH)D. This possibility is of particular importance because of some paradoxical clinical correlates of vitamin D in relation to race. Specifically, it has been consistently reported that black Americans have lower

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² Supplemental Figure 1 and Supplemental Tables 1 and 2 are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at <http://ajcn.nutrition.org>.

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⁸ Abbreviations used: CaDDM, Calcium and Vitamin D in Type 2 Diabetes; DBP, vitamin D-binding protein; DDM2, Vitamin D for Established Type 2 Diabetes; *m*-DBP, vitamin D-binding protein measured by the monoclonal antibody method; *p*-DBP, vitamin D-binding protein measured by the polyclonal antibody method; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.

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concentrations of total 25(OH)D than white Americans (6, 9) largely because of reduced cutaneous biosynthesis (10). This observation has led to blacks being diagnosed with vitamin D deficiency at higher rates despite having a higher bone mineral density and lower risk of fracture than whites, contrary to what would be expected for low 25(OH)D concentrations (11–14). Moreover, several associations between total 25(OH)D concentration and favorable nonskeletal clinical outcomes reported in whites, such as diabetes and cardiovascular disease, were not observed in blacks (2, 15, 16).

These findings suggest that measuring free 25(OH)D might improve our understanding of the physiological and clinical importance of vitamin D and any potential racial differences in vitamin D status. In a large cross-sectional study, Powe et al. (17) reported that despite having lower concentrations of total 25(OH)D than whites, blacks had similar bioavailable (albumin-bound plus free) 25(OH)D concentrations, which was attributed to reduced DBP concentrations in blacks. However, these results have been questioned because bioavailable 25(OH)D was not directly measured and because the formula used to calculate bioavailable 25(OH)D depended on an assay of DBP that may give falsely low values in blacks (18–21). In a smaller case-control study by Aloia et al. (22), directly measured free 25(OH)D was similar in black and white postmenopausal women despite lower concentrations of total 25(OH)D in black women. In a post hoc analysis by the same research group, measured free 25(OH)D reflected changes in total 25(OH)D after cholecalciferol supplementation in a cohort of predominantly postmenopausal white women (23).

The objectives of this study were to determine whether there are differences in concentrations of total and directly measured free 25(OH)D between black and white American adults and to assess the degree to which daily supplementation with 2 different doses of cholecalciferol changes these concentrations.

METHODS

Study population and interventions

Data used for this analysis were from 2 randomized controlled trials: DDM2 (Vitamin D for Established Type 2 Diabetes; NCT01736865) (24) and CaDDM (Calcium and Vitamin D in Type 2 Diabetes; NCT00436475) (25). Because both trials had a nearly identical study design with similar eligibility criteria, similar populations [e.g., age, race, BMI, baseline 25(OH)D], and both used cholecalciferol, we combined the results in certain analyses. DDM2 was a 2-site randomized controlled trial (2013–2015) that tested how 52 wk of cholecalciferol supplementation (4000 IU/d) affected glycemic outcomes in overweight adults aged 25–75 y and with a BMI (in kg/m²) of 25–40 with well-controlled type 2 diabetes (with or without metformin). Data from the baseline and 16-wk visits of DDM2 are included in our analyses. CaDDM was a single-site randomized controlled trial (2007–2009) that tested how 16 wk of cholecalciferol (2000 IU/d) and calcium (800 mg/d) supplementation in a 2 × 2 factorial design affected glycemic outcomes in overweight adults (age, ≥40 y; BMI, 25–40) at risk of type 2 diabetes as defined by glycemic criteria. Because calcium supplementation does not affect the 25(OH)D response to vitamin D supplementation (26), the 2 vitamin D groups from each trial were pooled and compared with the no-vitamin D groups. Key exclusion criteria for both trials were

the use of supplements containing vitamin D at doses higher than recommended by the Institute of Medicine (6), hyperparathyroidism, hypercalcemia, nephrolithiasis, chronic kidney disease, conditions that may affect vitamin D metabolism (e.g., sarcoidosis, antiepileptic medications, glucocorticoids), and regular visits to tanning booths. Vitamin D intake at baseline was self-reported. Participants who did not identify themselves as black or white were excluded. Both trials were approved by the Tufts Medical Center and Tufts University Health Sciences Institutional Review Boards. The DDM2 trial was also approved by the University of Cincinnati Institutional Review Board. All participants provided written informed consent.

Laboratory measurements

Frozen aliquots stored at –80°C and not previously thawed were used for the assays. Measurements were made in a masked fashion with paired samples (before and after intervention) included in the same analytic run to eliminate interassay variability. Total 25(OH)D was measured by liquid chromatography–mass spectrometry and certified through the National Institute of Standards and Technology vitamin D quality assurance program (interassay CV, 7%) (27). Albumin was measured by a Bromocresol green dye-binding procedure (Abbott Diagnostics). Intact parathyroid hormone (PTH) was measured with the use of a 2-site sandwich chemiluminescent microparticle immunoassay with an interassay CV of 3–6.4% (ARCHITECT; Abbott Diagnostics). DBP was measured with the use of 2 different commercial enzyme-linked immunoassays. One used a monoclonal antibody for capture and detection (R&D Systems; interassay CV, 5.1–7.4%); the other used polyclonal antibodies in a similar format (DRG Instruments; interassay CV, 12.7%). Direct measurement of free 25(OH)D was made at Future Diagnostics (interassay CV, 5.6–6.9%) and was based on a 2-step immunoassay procedure performed in a microtiter plate as previously described (22, 23, 28). According to the manufacturer, the evidence that the assay measures free vitamin D was obtained from enriching samples with either DBP or albumin, and in both cases the measured concentration of free vitamin D decreased. We used a method described by Bikle et al. (7) to calculate free 25(OH)D. The relative concentration of measured free 25(OH)D to total 25(OH)D was obtained by dividing the free 25(OH)D (ng/mL) by total 25(OH)D (ng/mL) and expressed as a percentage of total 25(OH)D.

Statistical analyses

Descriptive statistics were used to determine the distribution of data and to identify outliers. Baseline characteristics are expressed as means ± SDs for normally distributed variables; medians (IQRs) for skewed, nonnormally distributed variables; and frequency for categorical variables. Group comparisons at baseline used independent sample *t* tests for means, nonparametric Kruskal-Wallis tests for medians, and chi-square tests for frequencies. Bivariate correlations were examined with scatter plots and correlation coefficients (Pearson for linear associations and Spearman rank for nonlinear associations).

To adjust for potential confounding, group comparisons of baseline vitamin D metabolites between blacks and whites were assessed with the use of quantile regression methods to account

for nonnormally distributed data (29). Adjusted medians and 95% CIs were estimated by fitting separate median regression models for each group (30). Regression models contained terms for age, sex, BMI, season at study entry, vitamin D intake, and study (CaDDM or DDM2). Because recent vitamin D intake may confound results, cross-sectional analyses also adjusted for vitamin D intake. Group differences in adjusted medians were assessed with *P* values calculated from 2-sample *z* tests. We performed sensitivity analyses to examine 1) the potential impact of glucose intolerance on study outcomes by adjusting for baseline glycated hemoglobin and 2) the impact of potentially influential observations by excluding participants with very high total 25(OH)D concentrations (≥ 50 ng/mL) at baseline.

In the longitudinal analyses of the change in vitamin D metabolites after supplementation, mean differences and 95% CIs between the vitamin D and placebo arms were estimated with the use of linear regression models. Adjusted differences controlled for baseline values, age, sex, BMI, season (31), and study (CaDDM or DDM2). Model residuals were examined, and there were no violations of regression assumptions. Comparisons of the treatment effect were assessed with *P* values calculated from 2-sample *z* tests.

Statistical analyses were performed with the use of SAS version 9.4 (SAS Institute). Two-sided *P* values < 0.05 were considered to indicate statistical significance. Because the study tested post hoc hypotheses, analyses did not correct for a false discovery rate, and longitudinal analyses were among participants with postbaseline data available.

RESULTS

Participant characteristics

Participant flow through the trials is depicted in **Supplemental Figure 1**. The 208 participants analyzed had a mean age of 59.1 y, BMI of 31.6, and median total 25(OH)D concentration of 26.0 ng/mL (**Tables 1** and **2**). Twenty-six percent had a total 25(OH)D concentration < 20 ng/mL (termed vitamin D insufficiency) (6). Self-reported median vitamin D intake was 300 IU/d (approximately

half from supplements). Within each trial, the placebo and vitamin D groups were well balanced at baseline (**Supplemental Table 1**). Specific differences between the 2 trials (e.g., age, sex) reflect differences in some inclusion criteria and site location, whereas others (e.g., season of study entry) are likely caused by chance and were adjusted in the analyses.

Comparison at baseline

Compared with whites, black participants (27% of the cohort) were younger, and this group also had a different sex distribution and season at study entry (Table 1). Self-reported vitamin D intake was similar between races. Unadjusted median total 25(OH)D concentration was lower in blacks than in whites [22.0 ng/mL (IQR: 14.0–32.1 ng/mL) compared with 26.8 ng/mL (IQR: 22.0–31.0 ng/mL), respectively; $P = 0.011$], and a higher proportion of blacks had vitamin D insufficiency (46% compared with 19%, in whites; $P < 0.001$) (Table 2). Racial differences in total 25(OH)D concentration persisted after adjustment [adjusted median: 20.3 ng/mL (95% CI: 16.2, 24.5 ng/mL) in blacks compared with 26.7 ng/mL (95% CI: 25.2, 28.1 ng/mL) in whites; $P = 0.026$]. Unadjusted concentrations of directly measured free 25(OH)D were also lower in blacks than in whites, and racial differences persisted after adjustment [median: 4.54 ng/mL (95% CI: 3.72, 5.37 ng/mL) in blacks compared with 5.66 ng/mL (95% CI: 5.40, 5.92 ng/mL) in whites; $P = 0.044$] (Table 2). Directly measured free 25(OH)D was strongly correlated with total 25(OH)D in the entire cohort ($r = 0.82$; $P < 0.001$) and among blacks ($r = 0.87$; $P < 0.001$) and whites ($r = 0.77$; $P < 0.001$) (**Figure 1**). Measured free 25(OH)D as a fraction of the total 25(OH)D concentration was similar between races (Table 2).

DBP measured by the monoclonal antibody method (*m*-DBP) was lower in blacks than in whites in unadjusted analyses, and differences persisted in adjusted analyses [adjusted median: 109 mg/L (95% CI: 90, 129 mg/L) compared with 220 mg/L (95% CI: 207, 232 mg/L); $P < 0.001$] (Table 2). DBP measured by the polyclonal antibody method (*p*-DBP) did not differ by race in unadjusted or adjusted analyses [adjusted median:

TABLE 1
Baseline characteristics of study participants overall and according to race¹

Characteristic	Overall (<i>n</i> = 208)	Blacks (<i>n</i> = 57)	Whites (<i>n</i> = 151)	<i>P</i> value
Age, y	59.1 \pm 8.6 ²	56.3 \pm 7.8	60.2 \pm 8.6	0.004
Women, % (<i>n</i>)	39.4 (82)	50.9 (29)	35.1 (53)	0.038
BMI, kg/m ²	31.6 \pm 3.6	31.2 \pm 3.8	31.7 \pm 3.6	NS
Season at study entry, % (<i>n</i>)				0.035
January–March	26.9 (56)	24.6 (14)	27.8 (42)	
April–June	25.5 (53)	29.8 (17)	23.8 (36)	
July–September	21.6 (45)	31.6 (18)	17.9 (27)	
October–December	26.0 (54)	14.0 (8)	30.5 (46)	
Vitamin D intake, IU/d				
From diet + supplements	300 (149–542) ³	306 (147–533)	297 (149–544)	NS
Diet only	170 (120–278)	210 (106–308)	168 (122–268)	NS
Albumin, g/dL	4.0 (3.8–4.2)	4.0 (3.8–4.1)	4.0 (3.8–4.2)	NS

¹Race was self-reported. *P* values of group differences (blacks compared with whites) for continuous variables were analyzed with the use of independent sample *t* tests for means and nonparametric Kruskal-Wallis tests for medians. Proportions were analyzed with the use of chi-square tests. Percentages may not total 100 because of rounding.

²Mean \pm SD (all such values).

³Median; IQR in parentheses (all such values).

TABLE 2
Vitamin D metabolites at baseline according to race¹

	Unadjusted median (IQR)				Adjusted median (95% CI) ²		
	Overall (n = 208)	Black (n = 57)	White (n = 151)	P ³	Black (n = 57)	White (n = 151)	P ⁴
Total 25(OH)D, ng/mL	26.0 (19.4–31.3)	22.0 (14.0–32.1)	26.8 (22.0–31.0)	0.011	20.3 (16.2, 24.5)	26.7 (25.2, 28.1)	0.026
Total 25(OH)D <20 ng/mL, % (n)	26.0 (54)	45.6 (26)	18.5 (28)	<0.001	—	—	—
Directly measured free 25(OH)D, pg/mL	5.45 (4.17–6.76)	4.88 (3.40–6.26)	5.73 (4.54–6.84)	0.006	4.54 (3.72, 5.37)	5.66 (5.40, 5.92)	0.044
Measured free 25(OH)D as % total 25(OH)D	0.022 (0.019–0.026)	0.023 (0.02–0.026)	0.021 (0.019–0.025)	NS	0.024 (0.021, 0.026)	0.022 (0.021, 0.023)	NS
Calculated free 25(OH)D, ⁵ pg/mL							
Monoclonal assay	9.17 (7.28–12.55)	11.89 (8.57–17.09)	8.84 (7.23–11.22)	<0.001	11.83 (9.05, 14.61)	8.88 (8.36, 9.40)	0.080
Polyclonal assay	9.14 (6.88–11.43)	7.75 (5.99–11.68)	9.52 (7.63–11.41)	0.008	8.32 (6.74, 9.90)	9.41 (8.85, 9.96)	NS
Vitamin D-binding protein, mg/L							
Monoclonal assay	194 (133–247)	105 (82–154)	212 (172–254)	<0.001	109 (90, 129)	220 (207, 232)	<0.001
Polyclonal assay	199 (174–235)	195 (176–234)	199 (173–235)	NS	192 (173, 211)	201 (192, 209)	NS
PTH, pg/mL	54.9 (43.6–69.5)	60.9 (46.3–85.1)	52.5 (42.8–65.3)	0.065	58.8 (47.5, 70.0)	51.2 (47.8, 54.6)	NS

¹CaDDM, Calcium and Vitamin D for Diabetes Mellitus; DDM2, Vitamin D for Established Type 2 Diabetes; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.

²Estimated from race-stratified quantile regression models adjusted for age, sex, BMI, season, vitamin D intake, and study (CaDDM or DDM2).

³P values of group differences (black vs. white) for continuous variables were analyzed with the use of nonparametric Kruskal-Wallis tests for medians. Proportions were analyzed with the use of chi-square tests.

⁴P values of group differences (black vs. white) for continuous variables are based on 2-sample z tests.

⁵Based on the method described by Bikle et al. (7).

192 mg/L (95% CI: 173, 211 mg/L) compared with 201 mg/L (95% CI: 192, 209 mg/L)] (Table 2). There was no correlation between *m*-DBP and *p*-DBP ($r = 0.03$).

Unadjusted calculated free 25(OH)D was higher in blacks than in whites when *m*-DBP was used in the calculation but was lower in blacks than in whites when *p*-DBP was used (Table 2). Regardless of the method used, racial differences in calculated free 25(OH) were not statistically significant after adjustment

(Table 2). Calculated free 25(OH)D when *m*-DBP was used overestimated measured free 25(OH)D concentration, especially in blacks (Figure 2).

Concentrations of PTH tended to be higher in blacks than in whites in unadjusted analyses, but there was no statistical significance after adjustment (Table 2). PTH concentration was inversely correlated with total 25(OH)D in the entire cohort ($r = -0.19$; $P = 0.007$) and among blacks ($r = -0.27$; $P < 0.039$) but not among whites ($r = -0.10$; $P = 0.224$). PTH was inversely correlated with measured free 25(OH)D in the entire cohort ($r = -0.28$; $P < 0.001$), in blacks ($r = -0.26$; $P = 0.052$), and in whites ($r = -0.27$; $P = 0.001$).

The results did not change in sensitivity analyses after adjusting for baseline hemoglobin A1c. Of the 6 participants with a high baseline total 25(OH)D (>50 ng/mL), 4 were black. Self-reported median vitamin D intake among these outliers was >2-fold than that of the entire cohort (630 compared with 300 IU/d). When these 6 participants were excluded, results were essentially unchanged, but statistical significance was strengthened for all analyses (results not shown).

Effects of vitamin D supplementation

Participant retention was 96% for CaDDM and 92% for DDM2 (Supplemental Figure 1). All participants that withdrew did so for personal reasons. The supplements were well tolerated. One participant in each trial discontinued the study pills for personal reasons.

Supplementation with cholecalciferol increased total and measured free 25(OH)D concentrations proportionally to the dose (Table 3) and without a difference between races (Figure 3). Compared with placebo, total 25(OH)D concentration increased more after supplementation with 4000 than with 2000

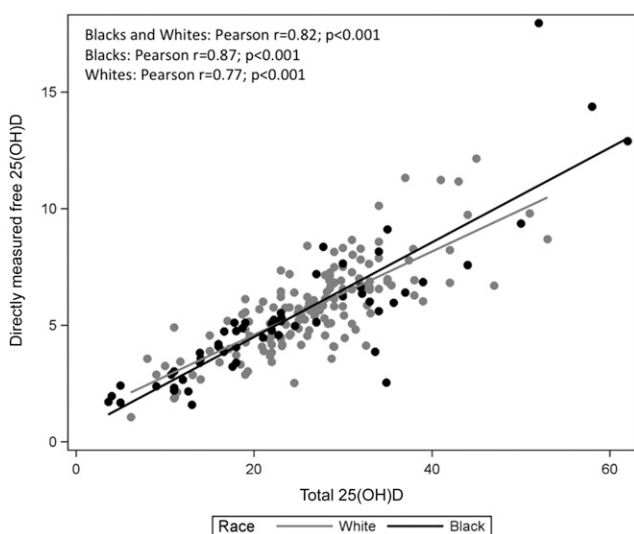


FIGURE 1 Relation between total and directly measured free 25(OH)D according to race. Total 25(OH)D is expressed as ng/mL; free 25(OH)D is expressed as pg/mL. Blacks, $n = 57$; whites, $n = 151$. 25(OH)D, 25-hydroxyvitamin D.

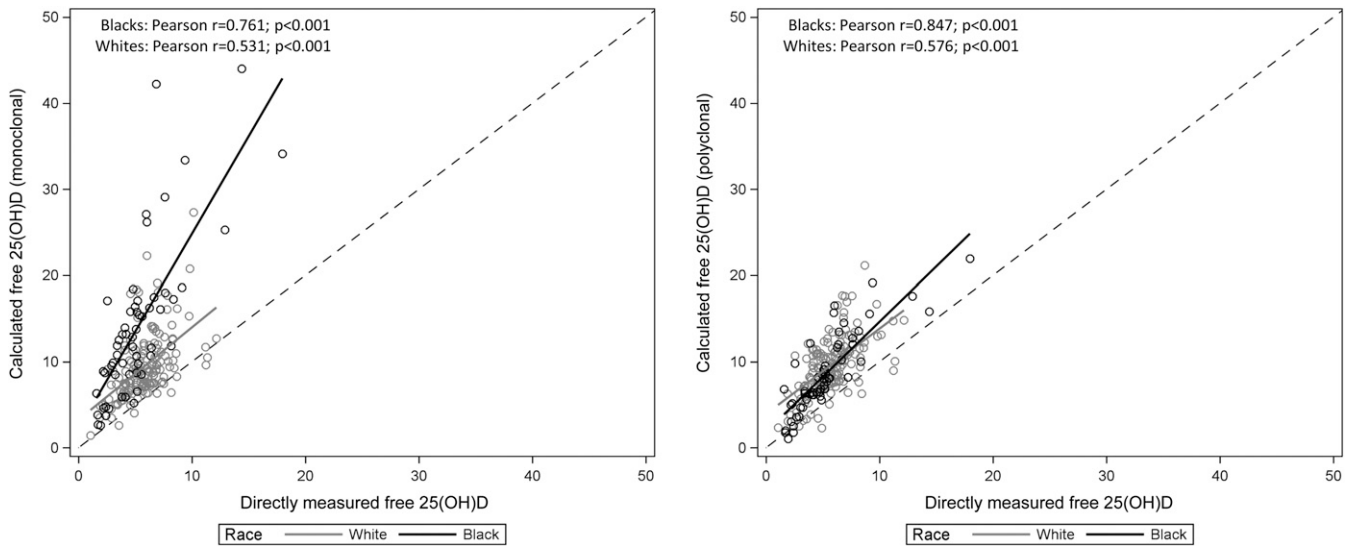


FIGURE 2 Relation between calculated and directly measured free 25(OH)D according to race and whether the monoclonal (left panel) or polyclonal (right panel) assay for vitamin D-binding globulin was used to calculate free 25(OH)D. Free 25(OH)D is expressed as pg/mL. Blacks, $n = 57$; whites, $n = 151$. 25(OH)D, 25-hydroxyvitamin D.

IU cholecalciferol [adjusted mean change: 23.0 ng/mL (95% CI: 19.6, 26.4 ng/mL) compared with 12.9 ng/mL (95% CI: 10.2, 15.6 ng/mL), respectively; $P < 0.001$ for comparison between doses] (Figure 3, Table 3). Measured free 25(OH)D also increased more with the higher dose [6.04 pg/mL (95% CI: 5.14, 6.93 pg/mL) for 4000 IU compared with 3.48 pg/mL (95% CI: 2.90, 4.06 pg/mL) for 2000 IU; $P < 0.001$]. The change in measured free 25(OH)D was strongly correlated ($r \geq 0.79$; $P < 0.001$) with the change in total 25(OH)D in both races at each dose (Figure 4). After cholecalciferol supplementation, the relative concentration of measured free 25(OH)D to total 25(OH)D did not change (Table 3).

Changes in calculated free 25(OH)D after vitamin D supplementation, whether estimated via *m*-DBP or *p*-DBP, paralleled the changes in measured free 25(OH)D (Supplemental Table 2). DBP, regardless of the immune assay used, did not change after supplementation (Supplemental Table 2).

PTH declined after cholecalciferol supplementation, but the change was not statistically significant (Table 3). The change in PTH was inversely proportional to the change in total 25(OH)D ($r = -0.22$; $P = 0.002$) and in measured free 25(OH)D ($r = -0.17$; $P = 0.017$). There was no significant effect of race on these correlations.

DISCUSSION

In this analysis of samples from 2 clinical trials, concentrations of directly measured free 25(OH)D were lower in black than in white Americans in direct proportion to total 25(OH)D concentration, whereas calculated free 25(OH)D concentrations in blacks compared with whites were disparate depending on the type of immunoassay used to measure DBP. Daily supplementation with cholecalciferol increased measured free 25(OH)D in proportion to the dose given, in parallel to the change in total 25(OH)D, and without any difference by race.

Nearly all of 25(OH)D circulates bound to DBP ($\sim 90\%$) and other serum proteins (10%), with only a small fraction ($\sim 0.05\%$)

circulating in the free (unbound) state (7). The role of DBP is presumed to be the maintenance of circulating vitamin D and modulation of the bioavailability, activation, and end-organ responsiveness of the hormone and its metabolites (32). Variations in the blood concentration and binding characteristics of DBP resulting from conditions such as weight change, pregnancy, or liver disease can affect the free fraction of 25(OH)D (7, 28, 33, 34). In addition, common polymorphisms in the *DBP* gene produce variant proteins with different binding affinities for vitamin D, and the prevalence of these polymorphisms varies by race (20, 35–37). Blacks tend to carry the higher-affinity form of DBP, whereas whites more frequently have the lower-affinity DBP forms. Our finding of a lower DBP concentration in blacks than in whites when using the monoclonal antibody immunoassay is consistent with other studies (17, 20, 22, 38). However, this assay has been criticized as giving falsely lower values in blacks because the monoclonal antibody differentiates against a DBP variant (Gc1F) that is more common in blacks (18–20, 39). Therefore, an immunoassay that uses polyclonal antibodies may be preferred when assessing differences in DBP by race. With the use of such an assay, *p*-DBP concentrations did not differ between the 2 races. These results are supported by a recent study (20) that showed that concentrations of DBP determined by liquid chromatography–tandem mass spectrometry were similar to those obtained with the polyclonal immunoassay. Discordant DBP measures, dependent on the type of immunoassay used, have been noted previously (22).

Calculated free of bioavailable (albumin-bound plus free) 25(OH)D has been reported not to differ between blacks and whites despite total 25(OH)D being lower in blacks (17, 22, 38). This observation has been attributed to the lower DBP concentration but higher-affinity DBP genotype in blacks. This mechanism has been proposed as being protective against the manifestations of vitamin D deficiency in black Americans and, therefore, that assessing free 25(OH)D might be a better cross-racial biomarker of vitamin D status than total 25(OH)D (17, 22). In the study by Powe et al. (17), which reported lower bioavailable 25(OH)D in

TABLE 3
Effects of cholecalciferol on vitamin D metabolites¹

	CaDDM		DDM2		P value ²
	Placebo (n = 45)	2000 IU/d cholecalciferol (n = 46)	Placebo (n = 59)	4000 IU/d cholecalciferol (n = 58)	
Total 25(OH)D, ng/mL					
Baseline	24.8 ± 7.2 ³	24.4 ± 7.7	27.8 ± 12.1	26.2 ± 10.5	
Week 16	18.1 ± 7	30.7 ± 8.1	27.7 ± 11.5	49.1 ± 12.1	
Change from baseline	-6.8 ± 5.6	6.3 ± 9.4	-0.3 ± 6.7	22.7 ± 11.8	
Unadjusted difference from placebo		13.1 (9.8, 16.4) ⁴		23 (19.4, 26.6)	
Adjusted difference from placebo ⁵		12.9 (10.2, 15.6)		23 (19.6, 26.4)	<0.001
P value ⁶		<0.001		<0.001	
Measured free 25(OH)D, pg/mL					
Baseline	5.25 ± 1.59	4.82 ± 1.57	6.45 ± 3.07	5.79 ± 2.02	
Week 16	4.54 ± 1.64	7.63 ± 1.79	6.17 ± 2.54	11.71 ± 3.17	
Change from baseline	-0.79 ± 1.07	2.83 ± 1.78	-0.4 ± 1.95	5.86 ± 3.06	
Unadjusted difference from placebo		3.62 (2.99, 4.25)		6.26 (5.29, 7.24)	
Adjusted difference from placebo ⁵		3.48 (2.9, 4.06)		6.04 (5.14, 6.93)	<0.001
P value ⁶		<0.001		<0.001	
Measured free total 25(OH)D, %					
Baseline	0.021 ± 0.004	0.021 ± 0.006	0.024 ± 0.006	0.023 ± 0.006	
Week 16	0.026 ± 0.008	0.026 ± 0.006	0.023 ± 0.004	0.024 ± 0.005	
Change from baseline	0.005 ± 0.007	0.005 ± 0.006	-0.001 ± 0.006	0 ± 0.007	
Unadjusted difference from placebo		0.001 (-0.002, 0.003)		0.002 (-0.001, 0.004)	
Adjusted difference from placebo ⁵		0 (-0.002, 0.003)		0.001 (0, 0.003)	NS
P value ⁶		NS		NS	
Intact PTH, pg/mL					
Baseline	61.5 ± 24.8	66.7 ± 27.1	57.8 ± 24.9	53 ± 20.9	
Week 16	62.1 ± 22.7	59 ± 23.6	56.9 ± 22.6	47.2 ± 18.3	
Change from baseline	1.5 ± 20.8	-8.1 ± 19.7	-2.1 ± 15.1	-5.1 ± 16.7	
Unadjusted difference from placebo		-9.7 (-18.3, -1.1)		-3 (-9.0, 3.1)	
Adjusted difference from placebo ⁵		-7.2 (-14.9, 0.6)		-4.4 (-9.8, 1.1)	NS
P value ⁶		0.068		NS	

¹Participants without postbaseline values (4 in the CaDDM trial and 9 in the DDM2) were excluded from the analyses. In the CaDDM trial, 5 additional participants did not have serum albumin measured; therefore, free 25(OH)D could not be calculated. CaDDM, Calcium and Vitamin D for Diabetes Mellitus; DDM2, Vitamin D for Established Type 2 Diabetes; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D.

²Based on 2-sample z tests for the difference in adjusted mean differences between trials.

³Mean ± SD (all such values).

⁴Mean; 95% CI in parentheses (all such values).

⁵Results are from linear regression models adjusted for age, sex, race, BMI, season of blood draw, and baseline value of the outcome variable.

⁶Based on the adjusted difference in change from baseline between active intervention (each vitamin D dose) and matching placebo.

blacks than whites, the formula used to calculate bioavailable 25(OH)D included a genotype-specific DBP affinity constant and DBP values from the monoclonal immunoassay. Because this assay gives lower values in blacks (20), the study's conclusions have been questioned (18, 19). Two other studies (22, 38) that reported no difference in calculated bioavailable 25(OH)D between blacks and whites also used the monoclonal assay to measure DBP but did not use a genotype-specific affinity constant. Thus, both the assay method and the type of affinity constant used have considerable impacts on the estimates of free vitamin D. Our results demonstrate this starkly in that unadjusted calculated free 25(OH)D, with the use of a nongenotype-specific affinity constant, was higher in blacks when *m*-DBP was used in the calculation but lower than whites when *p*-DBP was used. Consistent with prior studies (22, 28), calculated free 25(OH)D, when *m*-DBP was used, overestimated the directly measured free 25(OH)D concentration, especially in blacks.

The challenge of the disparate, assay-dependent results in DBP concentration and the inherent limitations of the assumptions that enter the indirect estimation of free 25(OH)D

can be circumvented by directly measuring free 25(OH)D. We found measured free 25(OH)D concentrations to be lower in blacks than in whites and to strongly correlate with total 25(OH)D in both races. The proportionality of free to total 25(OH)D indicates a relatively stable free fraction across the population, consistent with the free hormone hypothesis. Our results are in direct contrast with the study by Aloia et al. (22), who used the same assay we used to measure free 25(OH)D and found no difference in free 25(OH)D concentrations between black and white postmenopausal women. However, it is notable that this group also noted a strong correlation between free and total 25(OH)D. There is no obvious explanation for the discordant effects of race on free 25(OH)D in the study by Aloia et al. and ours. One possibility is the difference in populations and study design. Aloia et al. included women only and used a propensity score based only on age and BMI to match the 2 races. Our study included both sexes and conducted analyses that adjusted for additional important covariates, such as season at study entry and vitamin D intake. Another factor might be the heterogeneity of the cohort studied by Aloia et al.,

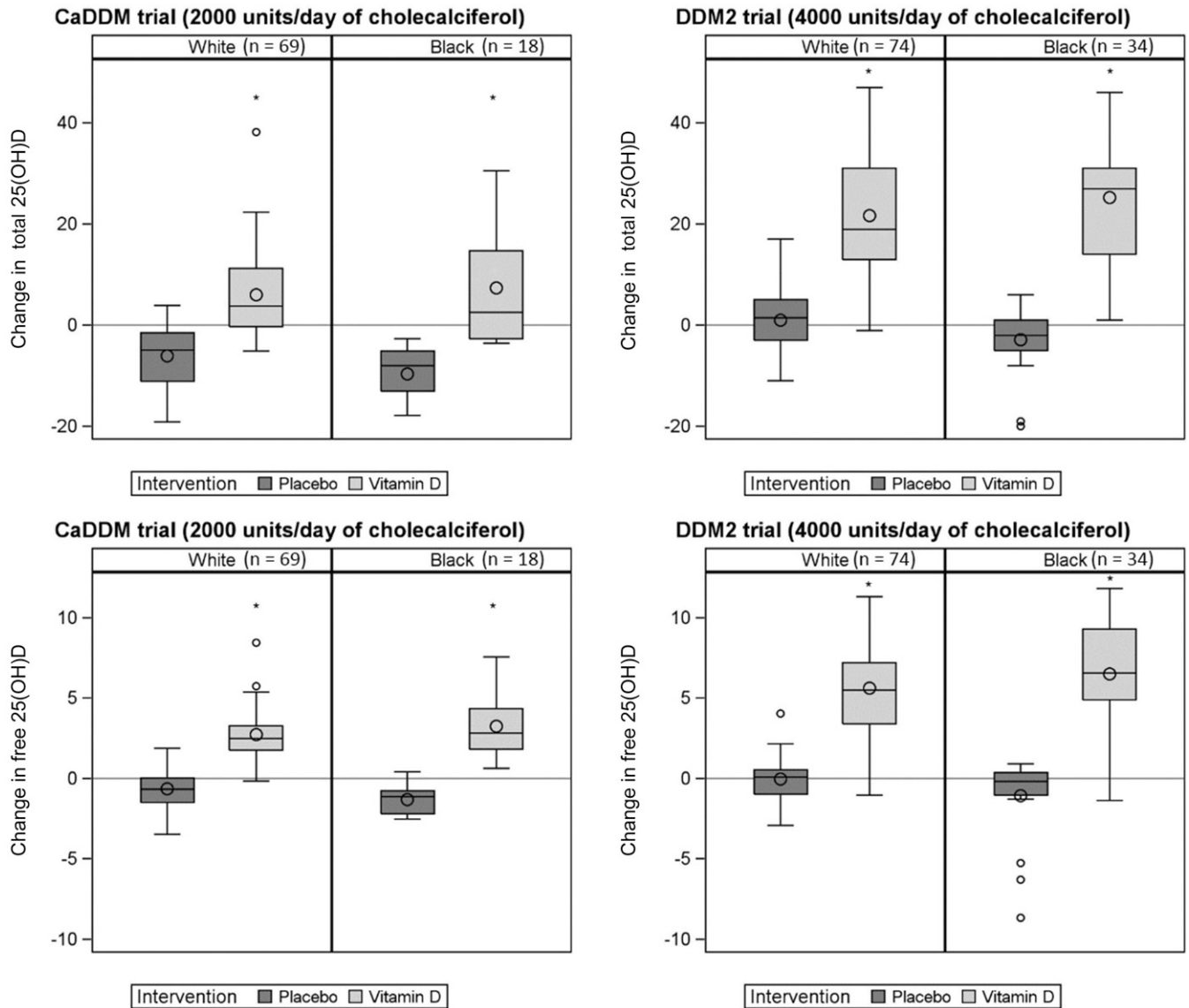


FIGURE 3 Change in total (upper panels) and directly measured free (lower panels) 25(OH)D after supplementation by dose (left panels, 2000 IU/d; right panels, 4000 IU/d) and race (black or white). Total 25(OH)D is expressed as ng/mL; free 25(OH)D is expressed as pg/mL. The *P* values for intervention effect by race were obtained from stratified regression models. The box indicates the IQR. The circle within the box indicates the mean value, and the vertical line indicates the median value. Whiskers indicate the maximum (or minimum) observation below (or above) 1.5 times the IQR. Circles above (or below) whiskers indicate outliers. **P* < 0.01 for comparisons between vitamin D and placebo groups. CaDDM, Calcium and Vitamin D for Diabetes Mellitus; DDM2, Vitamin D for Established Type 2 Diabetes; 25(OH)D, 25-hydroxyvitamin D.

which was composed of participants from 4 different trials (40–42), including 1 with participants who had X-linked hypophosphatemia (40).

There was no change in DBP concentration after 4 mo daily cholecalciferol, as previously reported (43). Cholecalciferol supplementation increased total 25(OH)D concentration in proportion to the dose, consistent with prior studies (23, 31, 44, 45). The change in measured free 25(OH)D was strongly correlated with the change in total 25(OH)D across both doses and races. A recently published study reported similar changes in measured free 25(OH)D after 8 wk cholecalciferol supplementation, but the study included only postmenopausal, predominantly white women (23). The similar effects of vitamin D supplementation on free and total 25(OH)D in blacks and whites in our study demonstrate that there is no systematic racial

difference in the absorption of cholecalciferol or bioavailability of vitamin D metabolites. It is important to note that because DBP concentrations did not change, there appears to be no major differences in the relative balance between free and total 25(OH)D between blacks and whites, as evidenced by the lack of change in the ratio of measured free to total 25(OH)D. Our findings do not support the existence of compensatory mechanisms to protect blacks against the manifestations of vitamin D deficiency (17) and are in line with the hypothesis that black Americans have lower concentrations of 25(OH)D than white Americans because of reduced cutaneous biosynthesis resulting from a dark skin color and decreased sun exposure (10, 46). Furthermore, absorption and metabolism of vitamin D appears to be the same in blacks and whites and, therefore, different cutoffs for 25(OH)D by race are not justified (45).

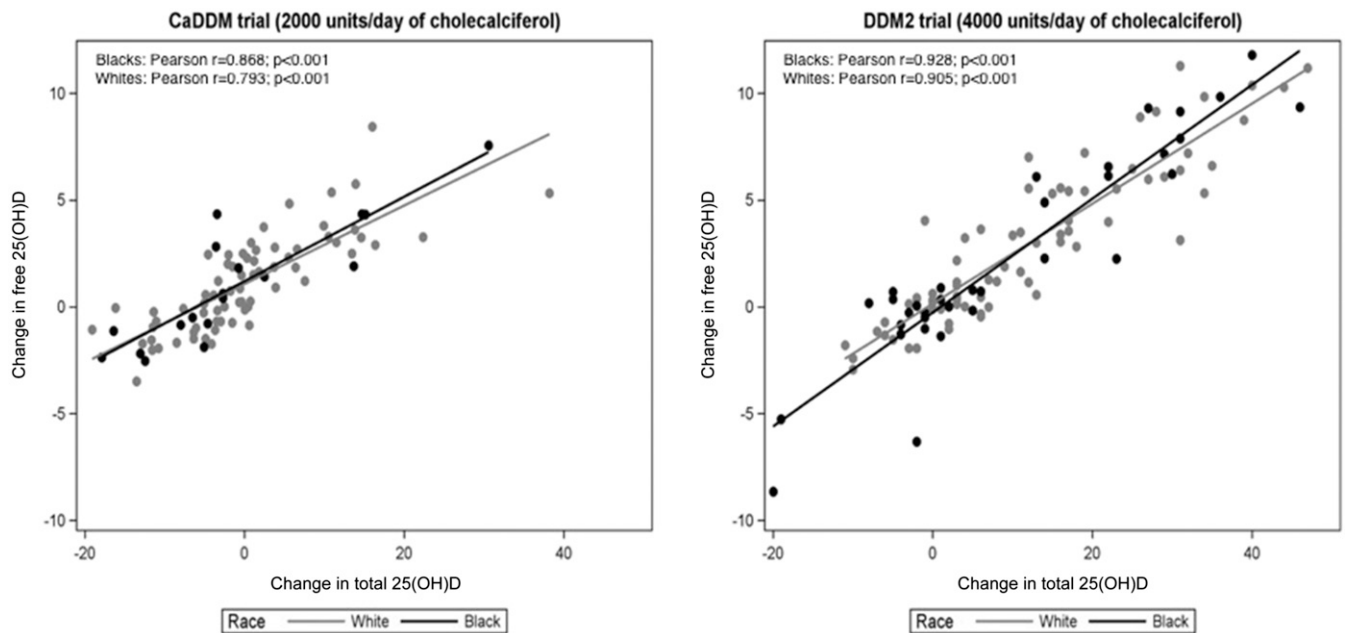


FIGURE 4 Correlation between the change in measured free and total 25(OH)D after cholecalciferol supplementation (left panel, 2000 IU/d; right panel, 4000 IU/d). Total 25(OH)D is expressed as ng/mL; free 25(OH)D is expressed as pg/mL. Blacks, $n = 57$; whites, $n = 151$. CaDDM, Calcium and Vitamin D for Diabetes Mellitus; DDM2, Vitamin D for Established Type 2 Diabetes; 25(OH)D, 25-hydroxyvitamin D.

Beyond vitamin D homeostasis, there may also be racial differences in PTH sensitivity and calcium economy. PTH concentrations are generally higher in blacks than in whites (14, 17, 22, 47) and inversely proportional to total 25(OH)D in a nonlinear fashion in both races. In our study, baseline PTH concentrations did not differ by race because few participants had total 25(OH)D concentrations below which PTH starts to rise, which occurs at ~ 15 ng/mL in blacks and ~ 23 ng/mL in whites (14, 47). PTH was inversely correlated with total 25(OH)D and measured free 25(OH)D. These findings are in agreement with Schwartz et al. (28) but contradict 2 studies by Aloia et al. (22, 23) that reported no significant correlation between PTH and measured free 25(OH)D with the use of the same assay to measure free 25(OH)D. Not surprisingly, PTH did not change considerably in response to supplementation because the end-of-study total 25(OH)D concentration, which correlates with PTH, remained relatively high in the placebo groups. The change in PTH did correlate inversely with the change in total 25(OH)D and measured free 25(OH)D. The results suggest that both free and bound fractions of 25(OH)D are relevant for PTH regulation, consistent with both the free hormone hypothesis and the megalin pathway, which allows certain cells to acquire 25(OH)D by receptor-mediated endocytosis of the DBP-25(OH)D complex (48, 49).

This study has several strengths, such as the inclusion of both sexes, information on vitamin D intake, measurement of DBP by 2 different assays, direct measurement of free 25(OH)D, inclusion of 2 different doses of cholecalciferol, and analyses that adjusted for important covariates. We measured DBP and albumin before and after supplementation, which provides improved estimates of change in calculated free 25(OH)D. There are also some limitations. The cohort was composed of overweight people with early-stage well-controlled diabetes or at risk of diabetes; however, glucose intolerance did not affect results. Although total 25(OH)D concentration was lower in blacks than whites, absolute concentration

in blacks was higher than prior reports, and the difference between races was not as large as previously reported (47), which reflects contemporary clinical practice. We calculated free 25(OH)D with the use of a nongenotype-specific binding affinity constant; however, this allowed us to directly compare our results with other studies (22, 28, 38, 43).

In conclusion, in a multiracial population with prediabetes or well-controlled diabetes, directly measured free 25(OH)D concentration was lower in blacks than in whites in direct relation to total 25(OH)D concentration, whereas supplementation increased measured free 25(OH)D concentrations in both races and in direct proportion to changes in total 25(OH)D. These findings, which need to be replicated in additional cohorts, suggest that both total and free 25(OH)D are indicative of vitamin D status, that either could be used to assess the response to supplementation, and that these relations hold independent of race. Our findings reinforce previous cautions about the use of calculated free 25(OH)D because of variability resulting from the method for measuring DBP and assumptions of binding affinity.

The authors' responsibilities were as follows—NSA, BD-H, and AGP: designed the research; NSA and AGP: conducted the research; JN: analyzed the data; NSA, JN, DD, and AGP: wrote the manuscript; AGP: had primary responsibility for the final content; and all authors: read and approved the final manuscript. None of the authors reported a conflict of interest related to this study.

REFERENCES

1. Song Y, Wang L, Pittas AG, Del Gobbo LC, Zhang C, Manson JE, Hu FB. Blood 25-hydroxy vitamin D levels and incident type 2 diabetes: a meta-analysis of prospective studies. *Diabetes Care* 2013;36:1422–8.
2. Robinson-Cohen C, Hoofnagle AN, Ix JH, Sachs MC, Tracy RP, Siscovick DS, Kestenbaum BR, de Boer IH. Racial differences in the association of serum 25-hydroxyvitamin D concentration with coronary heart disease events. *JAMA* 2013;310:179–88.

3. Sanders KM, Stuart AL, Williamson EJ, Simpson JA, Kotowicz MA, Young D, Nicholson GC. Annual high-dose oral vitamin D and falls and fractures in older women: a randomized controlled trial. *JAMA* 2010;303:1815–22.
4. Seida JC, Mitri J, Colmers IN, Majumdar SR, Davidson MB, Edwards AL, Hanley DA, Pittas AG, Tjosvold L, Johnson JA. Clinical review: effect of vitamin D3 supplementation on improving glucose homeostasis and preventing diabetes: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 2014;99:3551–60.
5. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2011;96:1911–30.
6. Institute of Medicine. Dietary reference intakes for calcium and vitamin D. Washington (DC): National Academies Press; 2011.
7. Bikle DD, Gee E, Halloran B, Kowalski MA, Ryzan E, Haddad JG. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. *J Clin Endocrinol Metab* 1986;63:954–9.
8. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. *J Steroid Biochem Mol Biol* 2014;144:132–7.
9. Kritchevsky SB, Toozé JA, Neiberg RH, Schwartz GG, Hausman DB, Johnson MA, Bauer DC, Cauley JA, Shea MK, Cawthon PM, et al. 25-hydroxyvitamin D, parathyroid hormone, and mortality in black and white older adults: the health ABC study. *J Clin Endocrinol Metab* 2012;97:4156–65.
10. Clemens TL, Adams JS, Henderson SL, Holick MF. Increased skin pigment reduces the capacity of skin to synthesize vitamin D3. *Lancet* 1982;1:74–6.
11. 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.
12. Aloia JF. African Americans, 25-hydroxyvitamin D, and osteoporosis: a paradox. *Am J Clin Nutr* 2008;88:545S–50S.
13. Bell NH, Epstein S, Greene A, Shary J, Oexmann MJ, Shaw S. Evidence for alteration of the vitamin D-endocrine system in obese subjects. *J Clin Invest* 1985;76:370–3.
14. Gutiérrez 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. *Osteoporos Int* 2011;22:1745–53.
15. Scragg R, Sowers M, Bell C. Serum 25-hydroxyvitamin D, diabetes, and ethnicity in the Third National Health and Nutrition Examination Survey. *Diabetes Care* 2004;27:2813–8.
16. Michos ED, Reis JP, Post WS, Lutsey PL, Gottesman RF, Mosley TH, Sharrett AR, Melamed ML. 25-Hydroxyvitamin D deficiency is associated with fatal stroke among whites but not blacks: the NHANES-III linked mortality files. *Nutrition* 2012;28:367–71.
17. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA, et al. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. *N Engl J Med* 2013;369:1991–2000.
18. Hollis BW, Bikle DD. Vitamin D-binding protein and vitamin D in blacks and whites. *N Engl J Med* 2014;370:879–80.
19. Bouillon R, Jones K, Schoenmakers I. Vitamin D-binding protein and vitamin D in blacks and whites. *N Engl J Med* 2014;370:879.
20. Hoofnagle AN, Eckfeldt JH, Lutsey PL. Vitamin D-binding protein concentrations quantified by mass spectrometry. *N Engl J Med* 2015;373:1480–2.
21. Nielson CM, Jones KS, Chun RF, Jacobs J, Wang Y, Hewison M, Adams JS, Swanson CM, Lee CG, Vanderschueren D, et al. Role of assay type in determining free 25-hydroxyvitamin D levels in diverse populations. *N Engl J Med* 2016 Mar 23 (Epub ahead of print; DOI: 10.1056/NEJMc1513502).
22. Aloia J, Mikhail M, Dhaliwal R, Shieh A, Usera G, Stolberg A, Ragolia L, Islam S. Free 25(OH)D and the vitamin D paradox in African Americans. *J Clin Endocrinol Metab* 2015;100:3356–63.
23. Aloia J, Dhaliwal R, Mikhail M, Shieh A, Stolberg A, Ragolia L, Fazzari M, Abrams SA. Free 25(OH)D and calcium absorption, PTH, and markers of bone turnover. *J Clin Endocrinol Metab* 2015;100:4140–5.
24. Vitamin D for Established Type 2 Diabetes (DDM2). ClinicalTrials.gov identifier: NCT01736865. [cited 2016 Apr 24]. Available from: <https://clinicaltrials.gov/ct2/show/NCT01736865?term=NCT01736865&rank=1>.
25. Mitri J, Dawson-Hughes B, Hu FB, Pittas AG. Effects of vitamin D and calcium supplementation on pancreatic beta cell function, insulin sensitivity, and glycemia in adults at high risk of diabetes: the Calcium and Vitamin D for Diabetes Mellitus (CaDDM) randomized controlled trial. *Am J Clin Nutr* 2011;94:486–94.
26. Goussous R, Song L, Dallal GE, Dawson-Hughes B. Lack of effect of calcium intake on the 25-hydroxyvitamin D response to oral vitamin D3. *J Clin Endocrinol Metab* 2005;90:707–11.
27. National Institute of Standards and Technology [Internet]. [cited 2010 Nov 3]. Available from: <http://www.nist.gov/srm>.
28. Schwartz JB, Lai J, Lizaola B, Kane L, Markova S, Weyland P, Terrault NA, Stotland N, Bikle D. A comparison of measured and calculated free 25(OH) vitamin D levels in clinical populations. *J Clin Endocrinol Metab* 2014;99:1631–7.
29. Koenker R, Bassett GW. Robust tests for heteroscedasticity based on regression quantiles. *Econometrica* 1982;50:43–61.
30. McGreevy KM, Lipsitz SR, Linder JA, Rimm E, Hoel DG. Using median regression to obtain adjusted estimates of central tendency for skewed laboratory and epidemiologic data. *Clin Chem* 2009;55:165–9.
31. Ng K, Scott JB, Drake BF, Chan AT, Hollis BW, Chandler PD, Bennett GG, Giovannucci EL, Gonzalez-Suarez E, Meyerhardt JA, et al. Dose response to vitamin D supplementation in African Americans: results of a 4-arm, randomized, placebo-controlled trial. *Am J Clin Nutr* 2014;99:587–98.
32. Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, Liebhaber SA, Cooke NE. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. *J Clin Invest* 1999;103:239–51.
33. Christakos S, Hewison M, Gardner DG, Wagner CL, Sergeev IN, Rutten E, Pittas AG, Boland R, Ferrucci L, Bikle DD. Vitamin D: beyond bone. *Ann N Y Acad Sci* 2013;1287:45–58.
34. Fu L, Yun F, Oczak M, Wong BY, Vieth R, Cole DE. Common genetic variants of the vitamin D binding protein (DBP) predict differences in response of serum 25-hydroxyvitamin D [25(OH)D] to vitamin D supplementation. *Clin Biochem* 2009;42:1174–7.
35. Kamboh MI, Ferrell RE. Ethnic variation in vitamin D-binding protein (GC): a review of isoelectric focusing studies in human populations. *Hum Genet* 1986;72:281–93.
36. Arnaud J, Constans J. Affinity differences for vitamin D metabolites associated with the genetic isoforms of the human serum carrier protein (DBP). *Hum Genet* 1993;92:183–8.
37. Lauridsen AL, Vestergaard P, Nexø E. Mean serum concentration of vitamin D-binding protein (Gc globulin) is related to the Gc phenotype in women. *Clin Chem* 2001;47:753–6.
38. Ashraf AP, Huisinching C, Alvarez JA, Wang X, Gower BA. Insulin resistance indices are inversely associated with vitamin D binding protein concentrations. *J Clin Endocrinol Metab* 2014;99:178–83.
39. US Department of Health and Human Services, Agency for Healthcare Research and Quality. Methods reference guide for effectiveness and comparative effectiveness reviews, version 1.0, 2007 [Internet]. [cited 2009 Aug 19]. Available from: http://effectivehealthcare.ahrq.gov/repFiles/2007_10DraftMethodsGuide.pdf.
40. Alon US, Levy-Ohmucki R, Moore WV, Stubbs J, Liu S, Quarles LD. Calcimimetics as an adjuvant treatment for familial hypophosphatemic rickets. *Clin J Am Soc Nephrol* 2008;3:658–64.
41. Aloia JF, Patel M, Dimaano R, Li-Ng M, Talwar SA, Mikhail M, Pollack S, Yeh JK. Vitamin D intake to attain a desired serum 25-hydroxyvitamin D concentration. *Am J Clin Nutr* 2008;87:1952–8.
42. Aloia JF, Dhaliwal R, Shieh A, Mikhail M, Fazzari M, Ragolia L, Abrams SA. Vitamin D supplementation increases calcium absorption without a threshold effect. *Am J Clin Nutr* 2014;99:624–31.
43. Ponda MP, McGee D, Breslow JL. Vitamin D-binding protein levels do not influence the effect of vitamin D repletion on serum PTH and calcium: data from a randomized, controlled trial. *J Clin Endocrinol Metab* 2014;99:2494–9.
44. Gallagher JC, Sai A, Templin T 2nd, Smith L. Dose response to vitamin D supplementation in postmenopausal women: a randomized trial. *Ann Intern Med* 2012;156:425–37.
45. Gallagher JC, Peacock M, Yalamanchili V, Smith LM. Effects of vitamin D supplementation in older African American women. *J Clin Endocrinol Metab* 2013;98:1137–46.

46. Durazo-Arvizu RA, Camacho P, Bovet P, Forrester T, Lambert EV, Plange-Rhule J, Hoofnagle AN, Aloia J, Tayo B, Dugas LR, et al. 25-Hydroxyvitamin D in African-origin populations at varying latitudes challenges the construct of a physiologic norm. *Am J Clin Nutr* 2014;100:908–14.
47. Wright NC, Chen L, Niu J, Neogi T, Javadi K, Nevitt MA, Lewis CE, Curtis JR. Defining physiologically “normal” vitamin D in African Americans. *Osteoporos Int* 2012;23:2283–91.
48. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D₃. *Cell* 1999;96:507–15.
49. Rowling MJ, Kemmis CM, Taffany DA, Welsh J. Megalin-mediated endocytosis of vitamin D binding protein correlates with 25-hydroxycholecalciferol actions in human mammary cells. *J Nutr* 2006;136:2754–9.