ORIGINAL ARTICLE

Vitamin D–Binding Protein and Vitamin D Status of Black Americans and White Americans

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ABSTRACT

BACKGROUND

Low levels of total 25-hydroxyvitamin D are common among black Americans. Vitamin D–binding protein has not been considered in the assessment of vitamin D deficiency.

METHODS

In the Healthy Aging in Neighborhoods of Diversity across the Life Span cohort of blacks and whites (2085 participants), we measured levels of total 25-hydroxyvitamin D, vitamin D–binding protein, and parathyroid hormone as well as bone mineral density (BMD). We genotyped study participants for two common polymorphisms in the vitamin D–binding protein gene (rs7041 and rs4588). We estimated levels of bioavailable 25-hydroxyvitamin D in homozygous participants.

RESULTS

Mean (±SE) levels of both total 25-hydroxyvitamin D and vitamin D–binding protein were lower in blacks than in whites (total 25-hydroxyvitamin D, 15.6±0.2 ng per milliliter vs. 25.8±0.4 ng per milliliter, P<0.001; vitamin D–binding protein, 168±3 μ g per milliliter vs. 337±5 μ g per milliliter, P<0.001). Genetic polymorphisms independently appeared to explain 79.4% and 9.9% of the variation in levels of vitamin D–binding protein and total 25-hydroxyvitamin D, respectively. BMD was higher in blacks than in whites (1.05±0.01 g per square centimeter vs. 0.94±0.01 g per square centimeter, P<0.001). Levels of parathyroid hormone increased with decreasing levels of total or bioavailable 25-hydroxyvitamin D (P<0.001 for both relationships), yet within each quintile of parathyroid hormone concentration, blacks had significantly lower levels of total 25-hydroxyvitamin D than whites. Among homozygous participants, blacks and whites had similar levels of bioavailable 25-hydroxyvitamin D overall (2.9±0.1 ng per milliliter and 3.1±0.1 ng per milliliter, respectively; P=0.71) and within quintiles of parathyroid hormone concentration.

CONCLUSIONS

Community-dwelling black Americans, as compared with whites, had low levels of total 25-hydroxyvitamin D and vitamin D–binding protein, resulting in similar concentrations of estimated bioavailable 25-hydroxyvitamin D. Racial differences in the prevalence of common genetic polymorphisms provide a likely explanation for this observation. (Funded by the National Institute on Aging and others.)

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OW LEVELS OF TOTAL 25-HYDROXYVITAmin D, which are more common in black Americans than in white Americans, are associated with negative health outcomes in epidemiologic studies.¹⁻⁴ Such studies are responsible for the routine clinical practice of screening for vitamin D deficiency. Among the possible effects of vitamin D deficiency, the strongest evidence is for a role in skeletal disorders,^{5,6} but clinical investigations of vitamin D supplementation to decrease the risk of fracture have been inconclusive.⁷⁻¹⁰

Because blacks consistently have lower levels of total 25-hydroxyvitamin D than whites, they are frequently given a diagnosis of vitamin D deficiency.¹¹⁻¹³ Yet, as compared with whites, blacks have higher bone mineral density (BMD) and a lower risk of fragility fracture.¹⁴⁻¹⁶ Elevated levels of parathyroid hormone, often considered a sensitive marker of vitamin D deficiency, are more common in blacks than in whites.¹⁷ However, the relation between levels of parathyroid hormone and total 25-hydroxyvitamin D may differ in blacks and whites.¹⁸

Vitamin D-binding protein is the primary vitamin D carrier protein, binding 85 to 90% of total circulating 25-hydroxyvitamin D.19 The nonvitamin D-binding protein fraction (bioavailable 25-hydroxyvitamin D) consists primarily of albumin-bound 25-hydroxyvitamin D (10 to 15% of total 25-hydroxyvitamin D), with less than 1% of total 25-hydroxyvitamin D in the free form. Vitamin D-binding protein appears to inhibit some actions of vitamin D, because the bound fraction may be unavailable to act on target cells.^{20,21} Common genetic polymorphisms in the vitamin Dbinding protein gene produce variant proteins that differ in their affinity for vitamin D.^{22,23} The prevalence of these polymorphisms differs between racial groups.24,25 Clinical assays measure the level of total 25-hydroxyvitamin D without distinguishing fractions bound to carrier proteins.

We conducted a study to determine whether vitamin D-binding protein genotypes and concentrations of circulating vitamin D-binding protein differ between black Americans and white Americans, possibly accounting for observed racial differences in manifestations of vitamin D deficiency.

METHODS

STUDY POPULATION

Healthy Aging in Neighborhoods of Diversity across the Life Span (HANDLS) is a populationbased cohort study involving 3720 participants that is supported by the Intramural Research Program of the National Institute on Aging.²⁶ Study participants, who were 30 to 64 years of age and living in Baltimore at the time of enrollment, were recruited from 13 contiguous U.S. Census tracts. Participants were randomly selected within strata based on age, race, sex, and socioeconomic status; those who did not identify themselves as black or white were excluded. The institutional review board of the National Institute of Environmental Health Sciences, National Institutes of Health, approved the protocol. The Partners HealthCare Human Research Committee exempted the present study from the requirement for review. The first and last authors vouch for the accuracy of the data and analyses.

DATA COLLECTION

We used cross-sectional data from the HANDLS study that were collected between 2004 and 2008. After providing written informed consent and being interviewed, participants underwent an examination on a mobile research vehicle in which blood was sampled, height and weight were measured, and bone densitometry was performed. Dietary intake of calcium and vitamin D were determined by means of the U.S. Department of Agriculture Automated Multiple-Pass Method. Only participants who completed the examination, including bone densitometry (performed with the use of the Lunar DPX-IQ densitometer [Lunar] and restricted to participants weighing <122.5 kg [270 lb]), and who had sufficient blood samples available were included in the present study (2085 participants) (Fig. S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). BMD at the femoral neck was used in this study, given its relevance as a risk factor for hip fracture.²⁷

LABORATORY ANALYSES

Blood samples drawn at the examination were stored at -80° C. Levels of total 25-hydroxyvitamin D (D₂ and D₃) were measured with the use of

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tandem mass spectrometry (interassay coefficient of variation, 8.6%). Levels of vitamin Dbinding protein were measured by means of a commercial enzyme-linked immunosorbent assay (R&D Systems) that uses two monoclonal antibodies in a sandwich format (interassay coefficient of variation, 7.2%). Levels of intact parathyroid hormone were measured with the use of the Elecsys Parathyroid Hormone Immunoassay (Modular Analytics E170, Roche Diagnostics) (interassay coefficient of variation, 2.5%). Calcium levels were corrected for the participant's albumin level as follows: corrected calcium = (measured calcium in mg per deciliter) + [0.8 × (4.0 – serum albumin in g per deciliter)].

GENOTYPING

DNA samples from the participants were genotyped for two common single-nucleotide polymorphisms (SNPs) in the coding region of the vitamin D-binding protein gene (rs4588 and rs7041) (see the Methods section in the Supplementary Appendix). These polymorphisms were chosen because of their high prevalence in the general population, their association with race, and their known link with vitamin D-binding protein function.²²⁻²⁵ We successfully genotyped samples from 1981 participants (95.0%).

CALCULATION OF BIOAVAILABLE 25-HYDROXYVITAMIN D

Bioavailable 25-hydroxyvitamin D was defined as circulating 25-hydroxyvitamin D not bound to vitamin D-binding protein, which is analogous to the definition of bioavailable testosterone.28 Concentrations of bioavailable 25-hydroxyvitamin D were calculated in 1025 homozygotes, for whom we could use a single genotype-specific binding affinity constant on the basis of the presence of a single vitamin D-binding protein variant (see the Methods section in the Supplementary Appendix).²² Calculated concentrations of bioavailable 25-hydroxyvitamin D were validated by direct measurement in a subgroup of homozygous participants with the use of a competitive radioligand-binding assay (see the Methods section and Fig. S2 and S3 in the Supplementary Appendix). Measured and calculated 25-hydroxyvitamin D concentrations were correlated (Pearson's r=0.81 in 32 Gc1F homozygotes and 0.90 in 13 Gc1S homozygotes; P<0.001 for both relationships) (Fig. S4 in the Supplementary Appendix).

STATISTICAL ANALYSIS

The characteristics of the study participants were compared according to race with the use of t-tests or chi-square tests and are presented as means ±SE or numbers and percentages. Non-normally distributed variables were natural log-transformed for parametric testing. Adjusted means were derived from multivariable linear regression models containing terms for age, sex, body-mass index (BMI), status with respect to poverty (defined as self-reported household income of <125% of the federal poverty level in 2003), season, smoking status, and calcium intake. Microalbuminuria (defined as a urinary microalbumin-tocreatinine ratio >30 μ g of microalbumin per milligram of creatinine) was included as a covariate in models predicting levels of vitamin D-binding protein and total 25-hydroxyvitamin D. Squared semi-partial correlation coefficients (expressed as percentages) are presented for multivariable linear regression models exploring variation in total 25-hydroxyvitamin D and vitamin D-binding protein. Total r² values are presented for unadjusted models and for the overall variance explained in multivariable models.

Chi-square tests were used to compare allele frequencies according to race. Race-stratified linear regression models were used to summarize associations of levels of vitamin D-binding protein and total 25-hydroxyvitamin D with the two SNPs of interest (rs7041 and rs4588). For a subgroup of 774 samples from black participants with complete data from genomewide association studies, we created two models, one including 10 principal components from a discriminant analysis of racial groups and a second with only one covariate, percent African ancestry. Adjustment for population substructure had little effect on the model. Thus, these covariates are not included in reported results.

Participants were divided into quintiles to examine relationships between 25-hydroxyvitamin D measures and markers of vitamin D status (parathyroid hormone level, calcium level, and BMD).

Statistical analyses were conducted with the use of SAS software, version 9.2 (SAS Institute). Two-tailed P values of less than 0.05 were con-

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sidered to indicate statistical significance, with the exception of the genotype analysis, in which the significance threshold was adjusted for the presence of two SNPs, with P values of less than 0.025 considered to indicate statistical significance.

RESULTS

CHARACTERISTICS OF THE PARTICIPANTS

Blacks (1181 participants) and whites (904 participants) were similar in terms of age, sex, BMI, and menopausal status (Table 1). Blacks were more likely than whites to be impoverished, to be active smokers, and to have microalbuminuria. Blacks were less likely than whites to have received a diagnosis of osteoporosis or to have

been prescribed osteoporosis therapies. Use of hormone-replacement therapy and medications that affect vitamin D metabolism (e.g., antiepileptic agents and glucocorticoids) was uncommon (Table 1).

TOTAL 25-HYDROXYVITAMIN D, VITAMIN D-BINDING PROTEIN, AND MARKERS OF VITAMIN D STATUS

Unadjusted levels of total 25-hydroxyvitamin D were lower in blacks than in whites (15.6 ± 0.2 ng per milliliter vs. 25.8 ± 0.4 ng per milliliter, P<0.001) (Fig. 1A). Racial differences in total 25-hydroxyvitamin D levels persisted after multivariable adjustment (17.3 ± 0.3 ng per milliliter in blacks vs. 25.5 ± 0.4 ng per milliliter in whites, P<0.001). There were seasonal differences in 25-hydroxyvitamin D levels (Table S1 in the Supplementary

Table 1. Characteristics of the Study Participants Overall and According to Race.*							
Characteristic	Overall (N = 2085)	Blacks (N=1181)	Whites (N = 904)	P Value			
Age — yr	48.3±0.2	48.3±0.3	48.3±0.3	0.92			
Male sex — no. (%)	921 (44.2)	523 (44.3)	398 (44.0)	0.91			
BMI†	29.6±0.2	29.4±0.2	29.8±0.2	0.14			
Household income <125% of poverty line — no. (%)	850 (40.8)	573 (48.5)	277 (30.6)	<0.001			
Score on Houston Activity Scale‡	2.4±0.1	2.2±0.1	2.8±0.1	<0.001			
Current smoker — no./total no. (%)	930/1938 (48.0)	552/1091 (50.6)	378/847 (44.6)	0.01			
Diagnosis of osteoporosis — no./total no. (%)	51/1730 (2.9)	19/946 (2.0)	32/784 (4.1)	0.01			
Prescribed osteoporosis therapies — no. (%) ${ m J}$	29 (1.4)	10 (0.8)	19 (2.1)	0.02			
Postmenopausal — no. of women/total no. (%)	623/1104 (56.4)	345/626 (55.1)	278/478 (58.2)	0.42			
Prescribed HRT — no. of women/total no. (%)	27/1105 (2.4)	10/618 (1.6)	17/487 (3.5)	0.045			
Microalbuminuria — no./total no. (%)¶	37/1383 (2.7)	27/710 (3.8)	10/673 (1.5)	0.01			
Estimated GFR of <60 ml/min/1.73 m² — no./total no. (%)∥	114/2039 (5.6)	67/1141 (5.9)	47/898 (5.2)	0.53			
Prescribed antiepileptic agents — no. (%)**	14 (0.7)	6 (0.5)	8 (0.9)	0.30			
Prescribed glucocorticoids — no. (%)††	28 (1.3)	20 (1.7)	8 (0.9)	0.11			
Dietary vitamin D intake — IU/day	152±4	149±5	157±6	0.38			
Dietary calcium intake — mg/day	731±11	720±14	744±17	0.10			

* Race was self-reported. Plus-minus values are means ±SE. P values of less than 0.05 were considered to indicate statistical significance. HRT denotes hormone-replacement therapy.

 \dagger The body-mass index (BMI) is the weight in kilograms divided by the square of the height in meters.

Scores on the Houston Activity Scale range from 0 to 7, with higher scores indicating greater habitual physical activity. Data were missing for 785 black participants (66.5%) and 672 white participants (74.3%).

Steoporosis therapies included pamidronate, neridronic acid, olpadronate, alendronate, ibandronate, risedronate, zoledronate, denosumab, teriparatide, and raloxifene.

¶ Microalbuminuria was defined as a urinary microalbumin-to-creatinine ratio of more than 30 μg of microalbumin per milligram of creatinine.

The estimated glomerular filtration rate (GFR) was calculated with the use of the Chronic Kidney Disease Epidemiology Collaboration equation.

** Antiepileptic agents included phenobarbital, carbamazepine, phenytoin, and primidone.

†† Glucocorticoids included prednisone, hydrocortisone, methylprednisolone, prednisolone, and dexamethasone.

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Appendix). Race explained 22.7% of the variation in total 25-hydroxyvitamin D levels in an unadjusted model.

Unadjusted levels of vitamin D-binding protein were lower in blacks than in whites (168±3 μ g per milliliter vs. 337±5 μ g per milliliter, P<0.001) (Fig. 1B). Racial differences in vitamin D-binding protein levels persisted after multivariable adjustment (169±5 μ g per milliliter in blacks vs. 339±5 μ g per milliliter in whites, P<0.001). There were seasonal differences in vitamin Dbinding protein levels; they appeared to explain 0.5% of the variation in vitamin D-binding protein levels (Table S1 in the Supplementary Appendix). Race explained 30.5% of the variation in vitamin D-binding protein levels in an unadjusted model.

Adjusted mean BMD at the femoral neck was greater in blacks than in whites $(1.05\pm0.01 \text{ g per} \text{ square centimeter vs. } 0.94\pm0.01 \text{ g per square centimeter}$, P<0.001), as were adjusted mean calcium levels (9.11±0.01 mg per deciliter vs. 8.99 ± 0.01 mg per deciliter, P<0.001). Adjusted mean levels of parathyroid hormone were higher in blacks than in whites (39±1 pg per milliliter vs. 34 ± 1 pg per milliliter, P<0.001). When we excluded participants with measurable 25-hydroxyvitamin D₂, our findings did not change appreciably (data not shown).

GENETIC POLYMORPHISMS, VITAMIN D-BINDING PROTEIN, AND TOTAL 25-HYDROXYVITAMIN D

Blacks were more likely than whites to have the T allele at rs7041, whereas whites were more likely than blacks to have the G allele at this location (P<0.001 for both comparisons); blacks were less likely to have the A allele at rs4588 (P<0.001) (Table 2).

The T allele at rs7041 was associated with decreased levels of vitamin D-binding protein in both blacks and whites (Table 2). The A allele at rs4588 was associated with higher vitamin D-binding protein levels in both blacks and whites after we accounted for the allele at rs7041. The polymorphisms at rs7041 and rs4588 had additive effects on vitamin D-binding protein concentrations (Table 2). Genetic variants independently appeared to explain 79.4% of the variation in vitamin D-binding protein levels after we accounted for other factors. After genetic variants were taken into account, race appeared to explain less than 0.1% of the variation in vitamin D-binding protein levels.



Figure 1. Levels of Total 25-Hydroxyvitamin D and Vitamin D–Binding Protein in Community-Dwelling White and Black Study Participants.

Histograms representing stacked distributions are shown. Mean (±SE) levels of total 25-hydroxyvitamin D were significantly lower in blacks than in whites (15.6±0.2 ng per milliliter vs. 25.8±0.4 ng per milliliter, P<0.001) (Panel A), as were levels of vitamin D-binding protein (168±3 μ g per milliliter vs. 337±5 μ g per milliliter, P<0.001) (Panel B).

The T allele at rs7041 was associated with decreased levels of total 25-hydroxyvitamin D among blacks. In whites, the A allele at rs4588 was associated with decreased levels of total 25-hydroxyvi-

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Table 2. Influence of Genetic Polymorphisms on Levels of Vitamin D-Binding Protein and Total 25-Hydroxyvitamin D.*								
SNP	Reference Allele	Change in Vitamin D– Binding Protein Variant Level per Variant erence Variant Allele Allele Copy lele Allele Frequency (95% CI) P Valu		P Value	Change in Total 25-Hydroxyvitamin D Level per Variant Allele Copy (95% Cl)) P Value		
				µg/ml		ng/ml		
Blacks								
rs7041	G	Т	0.83	-189.4 (-195.7 to -183.1)	<0.001	-2.0 (-2.9 to -1.1)	<0.001	
rs4588	С	А	0.10	57.0 (49.2 to 64.7)	<0.001	-0.5 (-1.7 to 0.6)	0.33	
Whites								
rs7041	G	Т	0.42	-189.1 (-201.0 to -177.3)	<0.001	0.2 (-1.3 to 1.7)	0.77	
rs4588	С	А	0.28	48.9 (36.0 to 61.8)	<0.001	-2.5 (-4.1 to -0.9)	0.003	

* Data on 1126 blacks and 855 whites were included in the analysis. CI denotes confidence interval, and SNP single-nucleotide polymorphism. P values of less than 0.025 were considered to indicate statistical significance (adjusted for two SNPs).

tamin D (Table 2). These genetic polymorphisms appeared to explain 9.9% of the variation in total 25-hydroxyvitamin D levels after other factors were taken into account. In the same model, season and race appeared to explain 10.5% and 7.3% of the variation in total 25-hydroxyvitamin D levels, respectively, whereas sex, age, smoking, calcium intake, BMI, poverty, and microalbuminuria each appeared to account for less than 2.0% of the variation. Overall, 31.2% of the variation in total 25-hydroxyvitamin D levels appeared to be explained in a model containing the aforementioned variables. The concentration of vitamin Dbinding protein and the genotype of vitamin Dbinding protein appeared to explain a similar amount of variation.

FINDINGS IN HOMOZYGOUS PARTICIPANTS

Vitamin D–Binding Protein Phenotypes and Bioavailable 25-Hydroxyvitamin D

Figure 2A shows the percentage of homozygous participants in each racial group with each variant vitamin D-binding protein (resulting from unique combinations of rs7041 and rs4588). Vitamin D-binding protein levels were lowest in Gc1F homozygous participants, highest in Gc1S homozygous participants, and intermediate in Gc2 homozygous participants (P<0.001 for all comparisons) (Fig. 2B). Among all 1025 homozygous participants, calculated levels of bioavailable 25-hydroxyvitamin D were similar in blacks and whites (2.9 ± 0.1 ng per milliliter and 3.1 ± 0.1 ng per milliliter, respectively; P=0.71) (Fig. 2C).

Markers of Vitamin D Status and 25-Hydroxyvitamin D

BMD was not associated with levels of bioavailable or total 25-hydroxyvitamin D in black homozygous participants; however, in white homozygous participants, BMD generally increased with increasing levels of total or bioavailable 25-hydroxyvitamin D (Table S2 in the Supplementary Appendix). Calcium levels increased with increasing levels of total 25-hvdroxyvitamin D in blacks only (Table S2 in the Supplementary Appendix). Lower levels of total or bioavailable 25-hydroxyvitamin D were associated with higher levels of parathyroid hormone in homozygotes of both races (P<0.001 for all relationships) (Table S2 in the Supplementary Appendix). As compared with white homozygotes with similar parathyroid hormone levels, black homozygotes had significantly lower levels of total 25-hydroxyvitamin D (Fig. 3A). In contrast, homozygous blacks and whites with similar parathyroid hormone levels had similar levels of bioavailable 25-hydroxyvitamin D (Fig. 3B). Relationships between total 25-hydroxyvitamin D levels and markers of vitamin D status in homozygotes were similar to those in the overall study population.

DISCUSSION

Because levels of total 25-hydroxyvitamin D are consistently lower in black Americans than in white Americans, blacks are frequently classified as being vitamin D–deficient.¹¹⁻¹³ In our study

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As shown in Panel A, unique combinations of the rs7041 and rs4588 polymorphisms produce amino acid changes resulting in variant vitamin D-binding proteins (left side of panel; Asp denotes aspartic acid, Glu glutamic acid, Lys lysine, and Thr threonine). The Gc1F phenotype was most common in black homozygotes, whereas the Gc1S phenotype was most common in white homozygotes (right side of panel). As shown in Panel B, levels of vitamin D-binding protein were lowest in Gc1F/Gc1F homozygotes (632 participants, $93\pm2 \mu g$ per milliliter), highest in Gc1S/Gc1S homozygotes (313 participants, $468\pm6 \mu g$ per milliliter), and intermediate in Gc2/Gc2 homozygotes (80 participants, $190\pm4 \mu g$ per milliliter). Plasma vitamin D-binding protein concentrations in Gc1F/Gc1S heterozygotes (413 participants, $285\pm4 \mu g$ per milliliter) were intermediate between those of Gc1F/Gc1F homozygotes and Gc1S/Gc1S homozygotes. These differences were significant (P<0.001 for all comparisons). Panel C shows a histogram representing stacked distributions. Among homozygous participants, levels of bioavailable 25-hydroxyvitamin D were similar in blacks and whites (2.9\pm0.1 ng per milliliter in blacks and 3.1\pm0.1 ng per milliliter in whites, P=0.71).

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Figure 3. Total and Bioavailable 25-Hydroxyvitamin D Levels among Homozygous Blacks and Whites with Similar Parathyroid Hormone Levels.

Within quintiles of parathyroid hormone values, blacks generally had lower levels of total 25-hydroxyvitamin D levels than whites (Panel A) but similar levels of bioavailable 25-hydroxyvitamin D (Panel B). I bars indicate standard errors. One asterisk denotes P<0.01 for the comparisons between blacks and whites within the quintile, two asterisks P<0.01 for the comparison with the highest quintile among whites, and three asterisks P<0.01 for the comparison with the highest quintile among blacks.

involving community-dwelling adults, we found that levels of vitamin D–binding protein are also lower in blacks, probably because of the high prevalence of a common genetic variant. Lower levels of vitamin D–binding protein in blacks appear to result in levels of bioavailable 25-hydroxyvitamin D that are equivalent to those in whites. These data, combined with previous data from our group,²⁹ suggest that low total 25-hydroxyvitamin D levels do not uniformly indicate vitamin D deficiency and call into question routine supplementation in persons with low levels of both total 25-hydroxyvitamin D and vitamin Dbinding protein who lack other traditional manifestations of this condition.

Thresholds for vitamin D sufficiency have been based on total 25-hydroxyvitamin D levels at which calcium absorption declines or parathyroid hormone levels increase.30,31 Because experimental data are inconclusive, controversy surrounds the precise level of total 25-hydroxyvitamin D at which these changes occur.^{30,31} We studied a community-dwelling population, in which overt vitamin D deficiency was rare; in fact, few participants had parathyroid hormone levels outside the normal range. Still, on the basis of the current guidelines (suggesting a threshold for sufficiency of 20 or 30 ng per milliliter), 77 to 96% of our black participants would be classified as vitamin D-deficient.32,33 Labeling the majority of the black participants as vitamin D-deficient would be inconsistent with the observation that they had higher BMD, higher calcium levels, and only slightly higher parathyroid hormone levels than their white counterparts.

Low levels of vitamin D-binding protein in blacks may provide protection against the manifestations of vitamin D deficiency despite low levels of total 25-hydroxyvitamin D. The bioavailability of other lipophilic hormones, such as thyroid hormone, is known to be influenced by the concentration of carrier proteins. When the concentration of the thyroxine-binding globulin is low or undetectable, there is a lower total thyroid hormone requirement for sufficiency.34 Analogously, mice that lack vitamin D-binding protein have low levels of total 25-hydroxyvitamin D but do not show signs of vitamin D deficiency.20 Therefore, low levels of total 25-hydroxyvitamin D probably do not indicate true vitamin D deficiency when levels of vitamin D-binding protein are also low, as in many black Americans. Bioavailable 25-hydroxyvitamin D may be a more appropriate cross-racial marker of vitamin D sufficiency; however, investigations in populations with overt vitamin D deficiency are required before routine clinical use is warranted.

Levels of total 25-hydroxyvitamin D are, in part, genetically determined.^{24,35,36} In our study, genetic polymorphisms in vitamin D–binding protein appeared to account for a greater proportion of the variation in total 25-hydroxyvitamin D levels than most factors known to be associated with 25-hydroxyvitamin D levels.³⁵⁻³⁷ The

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effect of vitamin D-binding protein polymorphisms on total 25-hydroxyvitamin D concentrations appeared to be mediated by the concentration of vitamin D-binding protein, an observation that is consistent with the findings in a previous study.38 Data in genetically modified mice suggest that the function of vitamin D-binding protein is to prolong the half-life of 25-hydroxyvitamin D, supporting this hypothesis.²⁰ Although mice lacking vitamin D-binding protein do not have manifestations of overt deficiency at baseline, they are more susceptible to deficiency than normal mice when deprived of vitamin D.20 Vitamin D-binding protein prolongs the half-life of 25-hydroxyvitamin D by serving as a reservoir and aiding in the reabsorption of filtered vitamin D through megalin in the kidney.^{20,39} We speculate that low levels of vitamin D-binding protein may confer a predisposition to inadequate 25-hydroxyvitamin D levels when vitamin D sources are scarce. Levels of vitamin D-binding protein only partially explained racial differences in levels of total 25-hydroxyvitamin D; other factors, including skin pigmentation and other polymorphisms, probably contribute to low levels of total 25-hydroxyvitamin D in blacks.1,40,41

Our study has certain limitations. First, given the cross-sectional and observational nature of the study, we were unable to predict the effects of vitamin D-binding protein levels on the risk of fracture. Second, measurement of bone-turnover markers, levels of 1,25-dihydroxyvitamin D, and urinary calcium excretion might have provided additional insight into the effect of vitamin Dbinding protein on mineral metabolism. Third, we did not have data on the use of vitamin D supplements. However, when we excluded participants with measurable 25-hydroxyvitamin D₂, which suggests exogenous supplementation with vitamin D derived from plants or fungi, our findings did not change. Further investigation is needed to determine the effects of supplementation on total and bioavailable 25-hydroxyvitamin D levels in persons with different vitamin D-binding protein genotypes. Finally, we relied predominantly on calculation of bioavailable 25-hydroxyvitamin D rather than direct measurement. Among homozygous participants, however, direct measurement of bioavailable 25-hydroxyvitamin D was well correlated with calculated levels.

There is an alternative commercially available assay for measuring vitamin D–binding protein

levels; the results of that assay are inconsistent with those of the assay used in this study (Fig. S5 and S6 in the Supplementary Appendix). The vitamin D-binding protein levels we report correlate inversely with the percentage of bioavailable 25-hydroxyvitamin D measured directly. Given the lack of genotype-specific standards in our direct assay format, we could not accurately report absolute concentrations. Our data should provide an impetus for the development of assays that directly measure bioavailable 25-hydroxyvitamin D.

Vitamin D deficiency is certainly present in persons with very low levels of total 25-hydroxyvitamin D accompanied by hyperparathyroidism, hypocalcemia, or low BMD. However, communitydwelling blacks with total 25-hydroxyvitamin D levels below the threshold used to define vitamin D deficiency typically lack the accompanying characteristic alterations. The high prevalence among blacks of a polymorphism in the vitamin D-binding protein gene that is associated with low levels of vitamin D-binding protein results in levels of bioavailable 25-hydroxyvitamin D that are similar to those in whites. despite lower levels of total 25-hydroxyvitamin D. Alterations in vitamin D-binding protein levels may therefore be responsible for observed racial differences in total 25-hydroxyvitamin D levels and manifestations of vitamin D deficiency. To improve the determination of vitamin D status in diverse populations, the measurement of vitamin D-binding protein will most likely need to be incorporated into the assessment.

Drs. Berg, Bhan, Karumanchi, and Thadhani report being coinventors on a patent pending on the use of bioavailable vitamin D for the assessment of vitamin D status. No other potential conflict of interest relevant to this article was reported.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Vitamin D Binding Protein and Vitamin D Status of Black and White Americans

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SUPPLEMENTARYAPPENDIX

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SUPPLEMENTARY METHODS

1. Genotyping

Vitamin D Binding Protein Single Nucleotide Polymorphism Genotyping

Samples were genotyped for two common single nucleotide polymorphisms (SNPs) in the vitamin D binding protein (D-binding protein) gene (rs4588 and rs7041). All samples were genotyped using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), in 384-well format. The 5' nuclease assay (TaqMan®) was used to distinguish the 2 alleles of a gene. PCR amplification was carried out on 5-20ng DNA using 1 X TaqMan® universal PCR master mix (No Amperase UNG) in a 5µl reaction volume. Amplification conditions on an AB 9700 dual plate thermal cycler (Applied Biosystems, Foster City, CA) were as follows: 1 cycle of 95°C for 10min, followed by 50 cycles of 92°C for 15s and 60°C for 1 min. TaqMan® assays were ordered using the ABI Assays-on-Demand service. The success rate for genotyping was 95%. Although the TaqMan® assays report the antisense genomic DNA sequences for these SNPs, we have reported our results using the coding strand, consistent with the NCBI SNP database (http://www.ncbi.nlm.nih.gov/snp).

HANDLS Whole Genome Sequencing and Ancestry Estimates

Participants were successfully genotyped to 907763 SNPs at the equivalent of Illumina 1M SNP coverage (709 samples using Illumina 1M and 1Mduo arrays, the remainder using a combination of 550K, 370K, 510S and 240S to equate the million SNP level of coverage), passing inclusion criteria into the genetic component of the study. Initial inclusion criteria for genetic data in HANDLS includes concordance between self-reported sex and sex estimated from X chromosome heterogeneity, > 95% call rate per participant (across all equivalent arrays), concordance between self-reported African ancestry and ancestry confirmed by analyses of genotyped SNPs, and no cryptic relatedness to any other samples at a level of proportional sharing of genotypes > 15% (effectively excluding 1st cousins and closer relatives from the set of probands used in the analyses). In addition, SNPs were filtered for HWE p-value > 1e-7, missing by haplotype p-values > 1e-7, minor allele frequency > 0.01, and call rate > 95%. Basic genotype quality control and data management were conducted using PLINKv1.06.¹ Cryptic relatedness was estimated via pairwise identity by descent analyses in PLINK and confirmed using RELPAIR.²

Ancestry estimates were assessed using both STRUCTUREv2.3and the multidimensional scaling (MDS) function in PLINKv1.06.³⁻⁵ In the MDS analysis, HANDLS participants were clustered with data made available from HapMap Phase 3 for the YRI, ASW, CEU, TSI, JPT and CHB populations, using a set of 36892 linkage-disequilibrium-pruned SNPs common to each population. HANDLS participants with component vector estimates consistent with the HapMap ASW samples for the first 4 component vectors were included. In addition, the 1024 quality controlled HANDLS samples were later clustered among themselves using MDS to generate 10 component vectors estimating internal population structure within the HANDLS study. Of the SNPs utilized for MDS clustering, the 2000 SNPs with the most divergent allele frequency estimates between African populations (frequency estimates based on YRI samples) and European populations (frequency estimates based on combined CEU and TSI samples) were utilized as ancestry informative markers (AIMs). These 2000 AIMs were associated with frequency differences on the level of p-values < 1e-3 based on chi-squared tests. A two population model in STRUCTURE was used to estimate percent African and percent European ancestry in the HANDLS samples, for a 10000 iteration burn-in period, and a10000 iteration follow-up of the Markov Chain Monte Carlo model utilized by STRUCTURE. The ancestry estimates from STRUCTURE were highly concordant with the first component vector of the MDS clustering of HANDLS samples, with an r^2 > 0.82.

HANDLS participant genotypes were imputed using MACH and miniMac

(http://www.sph.umich.edu/csg/abecasis/mach/) based on combined haplotype data for HapMap Phase 2 YRI and CEU samples that includes monomorphic SNPs in either of the two constituent populations (release 22, build 36.3). This process followed two stages, first estimating recombination and crossover events in a random sample of 200 participants, then based on this data and the reference haplotypes, 200 iterations of the maximum likelihood model were used to estimate genotype dosages for imputed SNPs. After filtering based on a minimum imputation quality of 0.30, indicated by the RSQR estimate in MACH, with a total yield of 2939993 SNPs.

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2. Calculation of Bioavailable 25-Hydroxyvitamin D Concentrations

Bioavailable 25-hydroxyvitamin D is defined as 25-hydroxyvitamin D that is either bound to albumin or free (i.e.: not bound to D-binding protein). Bioavailable 25-hydroxyvitamin D is 10-15% of total circulating 25-hydroxyvitamin D and is measured in ng per mL in contrast to free 25-hydroxyvitamin D, which is less than 1% of total circulating 25-hydroxyvitamin D and is measured in pg per mL. Studies of other lipophilic hormones suggest that albumin-bound hormone is available to exert biologic actions. ^{6, 7}

DEFINITIONS

Total D = 25-hydroxyvitamin D_3 + 25-hydroxyvitamin D_2 in mol/L

Alb = Albumin

- DBP_{1F} = Gc1F variant of the D-binding protein, as encoded by the D-binding protein gene, containing the ancestral alleles for both rs7041 (c.1296T, p.Asp416) and rs4588 (c.1307C; p.Thr420) single nucleotide polymorphisms. These alleles encode for aspartic acid and threonine at positions 416 and 420, respectively, of the D-binding proteinpolypeptide.⁸
- DBP_{1S} = Gc1S variant of the D-binding protein, as encoded by the D-binding protein gene, containing the rs7041 single nucleotide polymorphism (c.1296T>G; p.Asp416Glu). This polymorphism results in substitution of aspartic acid with glutamic acid at residue 416 of the D-binding protein polypeptide. The site of the rs4588 single nucleotide polymorphism within the Gc1S allele encodes for the ancestral threonine at position 420 (c.1307C; p.Thr420).⁸
- DBP₂ = Gc2 variant of the D-binding protein, as encoded by the D-binding protein gene, containing the rs4588 single nucleotide polymorphism (c.1307C>A; p.Thr420Lys).This polymorphism results in substitution of threonine with lysine at residue 420 of the Dbinding protein. The site of the rs7041 single nucleotide polymorphism within the Gc2 allele encodes for the ancestral aspartic acid residue at position 416.⁸

[D_{Alb}] = concentration of albumin-bound 25-hydroxyvitamin D

 $[D_{DBP}]$ = concentration of D-binding protein-bound 25-hydroxyvitamin D

[D_{Free}] = concentration of free (unbound) 25-hydroxyvitamin D

[Total D] = concentration of total 25-hydroxyvitamin D = $[D_{DBP}] + [D_{AIb}] + [D_{Free}]$

[Bio D] = concentration of bioavailable 25-hydroxyvitamin D = $[D_{Free}] + [D_{Alb}]$

 K_{alb} = affinity constant between 25-hydroxyvitamin D and albumin = 6 x 10⁵ M⁻¹

KDBP_{generic} = genotype-nonspecific affinity constant between 25-hydroxyvitamin D and DBP = 0.7 x 10⁹ M⁻¹

KDBP_{1S} = affinity constant between 25-hydroxyvitamin D and DBP_{1S} = $0.6 \times 10^{9} M^{-1}$

 $KDBP_{1F}$ = affinity constant between 25-hydroxyvitamin D and DBP_{1F} = 1.12 x 10⁹ M⁻¹

 $KDBP_2$ = affinity constant between 25-hydroxyvitamin D and DBP_2 = 0.36 x 10⁹ M⁻¹

*Affinity constants are taken from Arnaud et al.9

EQUATIONS (Adapted from Vermuelen et al.)⁷

Total 25-Hydroxyvitamin D

[Total D] = 25-hydroxyvitamin D₂ + 25-hydroxyvitamin D₃ concentration in mol/L

Given that $[Total D] = [D_{Free}] + [D_{Alb}] + [D_{DBP}]$

thus $[D_{DBP}] = [Total D] - [D_{Alb}] - [D_{Free}]$ (Eq. 1)

Albumin

[Alb] = serum albumin concentration in g/L ÷ 66,430 g/mole= albumin concentration in mol/L

 $[\mathsf{D}_{\mathsf{Free}}] + [\mathsf{Alb}] \leftrightarrow [\mathsf{D}_{\mathsf{Alb}}]$

Albumin association constant $K_{alb} = [D_{Alb}] \div ([D_{Free}] \cdot [Alb])$

Thus
$$[D_{Alb}] = K_{alb} \cdot [Alb] \cdot [D_{Free}]$$
 (Eq. 2)

(NB: [Alb] in this example denotes the concentration of free (non-vitamin D-bound) albumin.
However, given the low affinity between albumin and 25-hydroxyvitamin D, the concentrations of total albumin and unbound albumin are effectively equivalent ([Total Albumin] ≈ [Alb]). As a result,
[Alb] may be estimated accurately by measurements of total serum albumin.)

D-binding protein (DBP)

[Total DBP] = concentration of serum DBP in g/L ÷ 58,000 g/mole= DBP concentration in mol/L

[DBP] = free, unbound DBP

[D_{DBP}] = vitamin-bound DBP

Given that	$[D_{Free}] + [DBP] \leftrightarrow [D_{DBP}]$	
And	DBP association constant K_{DBP} = [D _{DBP}] ÷ ([DBP] · [D _{Free}])	
Thus	$[D_{Free}] = [D_{DBP}] \div K_{DBP} \div [DBP]$	(Eq. 3)
Since	[Total DBP] = sum of bound and unbound DBP = [DBP] + $[D_{DBP}]$	
Therefore	[DBP] = [Total DBP] – [D _{DBP}]	(Eq. 4)

Solving for Free 25-hydroxyvitamin D

From Eqs. 3 and 4 we see that:

 $[D_{Free}] = [D_{DBP}] \div K_{DBP} \div ([Total DBP] - [D_{DBP}])$ (Eq. 5)

If we substitute Eq. 1 into Eq. 2, we find that:

 $[D_{DBP}] = [Total D] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{Free}]$ (Eq. 6)

Substituting Eq. 6 into Eq. 5 produces the following:

$$[D_{Free}] = ([Total D] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{Free}]) + K_{DBP} + ([Total DBP] - ([Total D] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{Free}]))$$

Multiply both sides of the equation by the denominator of the right side of equation:

 $[D_{Free}] \cdot \{([Total DBP] - ([Total D] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{Free}])) \cdot K_{DBP}\} = ([Total D] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{Free}])$

Propagate the products and rearrange so that all components are on the left hand side: $\{K_{DBP} \cdot K_{alb} \cdot [Alb] + K_{DBP}\} \cdot [D_{Free}]^2 + \{K_{DBP} \cdot [Total DBP] - K_{DBP} \cdot [Total D] + K_{alb} \cdot [Alb] + 1\} \cdot [D_{Free}] + [Total D] = 0$

The equation is now limited to known constants (K_{DBP} and K_{alb}), measured values ([Total DBP], [Alb], and [Total D]) and the dependent variable for free vitamin D [D_{Free}]. The equation now fits the form of a second-degree polynomial:

$$ax^{2} + bx + c = 0$$
Where $x = [D_{Free}] =$ the concentration of free 25-hydroxyvitamin D
$$a = K_{DBP} \cdot K_{alb} \cdot [Alb] + K_{DBP}$$

$$b = K_{DBP} \cdot [Total DBP] - K_{DBP} \cdot [Total D] + K_{alb} \cdot [Alb] + 1$$

$$c = - [Total D]$$

This polynomial may be solved for $[D_{Free}]$ using the quadratic equation:

$$[DFree] = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

After solving for free 25-hydroxyvitamin D, we may then use Eq. 2 to calculate the concentration of bioavailable (non-DBP bound vitamin):

$$[\text{Bio D}] = [D_{\text{Free}}] + [D_{\text{Alb}}] = (K_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [D_{\text{Free}}]$$
(Eq. 7)

Furthermore, if the DBP genotype for an individual subject is known, for subjects who are homozygous for Gc1S/Gc1S, Gc1F/Gc1F, or Gc2/Gc2, the genotype-adjusted free and bioavailable fractions of 25hydroxyvitamin D can be calculated using previously measured binding affinities for the three variants (reference (34) in main manuscript):

For subjects homozygous for Gc1F variant, $K_{DBP} = 1.12 \times 10^9 \text{ M}^{-1}$

For subjects homozygous for Gc1S variant, $K_{DBP} = 0.60 \times 10^9 \text{ M}^{-1}$

For subjects homozygous for Gc2 variant, K_{DBP} = 0.36 x 10⁹ M⁻¹

EXAMPLE CALCULATIONS

For a subject with a known DBP genotype indicating homozygosity for Gc1F/Gc1F:

Total 25-hydroxyvitamin D = [Total D] = 40 ng/mL = 1.0×10^{-7} mol/L Total serum DBP = [Total DBP] = $250 \mu g/mL = 4.3 \times 10^{-6}$ mol/L Total serum albumin = [Alb] = $4.3 g/dL = 6.4 \times 10^{-4}$ mol/L $K_{alb} = 6 \times 10^{5} M^{-1}$ $K_{DBP} = 1.12 \times 10^{9} M^{-1}$ $a = 4.36 \times 10^{11}$ b = 5147 $c = -1 \times 10^{-7}$ Calculated concentration of free 25-hydroxyvitamin D = 1.94×10^{-11} mol/L = 7.8 pg/mL

Calculated concentration of bioavailable 25-hydroxyvitamin D = 7.54×10^{-9} mol/L = 3.0 ng/mL

3. 25-hydroxyvitamin D radioligand competitive binding assay (VRCBA) to measure bioavailable 25-Hydroxyvitamin D concentrations

Materials

<u>25-hydroxyvitamin D affinity adsorption plates</u> were made using Costar 96-well flat bottom EIA plates. Wells were first coated with purified D-binding protein (Gc globulin from human plasma, >90% pure and enriched in Gc1S variant, Sigma Aldrich). Each well was treated with 1 μ g of D-binding protein diluted into 100 μ L of water and allowed to adsorb at 4°C overnight. Wells were then washed with water and blocked with 500 μ L human serum albumin diluted in water (1% w/v). Plates were stored at 4°C until use, at which time blocking solution was washed away with water.

<u>Vitamin D binding protein calibrators</u> were made by diluting varying concentrations of D binding protein (Gc globulin from human plasma, >90% pure and enriched in Gc1S variant, Sigma Aldrich, catalog. no. G8764) into a matrix consisting of 125 mM sodium chloride, 25 mM sodium bicarbonate (pH 7.4), and human serum albumin (5% w/v).

<u>Radiolabeled 25-hydroxyvitamin D₃ ligand</u> was purchased from Perkin Elmer (<u>Hydroxyvitamin D₃, 25-</u> [26,27-³H]-, 5 μ Ci(185kBq),Product number: NET349005UC).Radioligand shipped in toluene was dried under a stream of argon and re-dissolved in acetonitrile. For each binding assay 1 μ L (~1 nCi) of radioligand was diluted into 100 μ Lof binding assay buffer (5% human serum albumin diluted 1:5000 in phosphate buffered saline).

Scintillation counting was performed by mixing all 200 µLof acetonitrile-extracted adsorbed radioligand or 200 µl of non-adsorbed radioligand into 3 mL of Ultima Gold scintillation fluid (Perkin Elmer). Radioactivity was quantified by measuring scintillation decays per minute (DPM) on a Packard TriCarb scintillation counter.

Assay Principle

In this microtiter plate-based competitive binding assay, radiolabeled 25-hydroxyvitamin D₃ partitions between D-binding protein adsorbed to the sides of the microtiter plate wells and the D-binding proteinwithin subjects' diluted plasma. After binding equilibrium is achieved, the soluble ligand within the reaction buffer is removed and the bound ligand is extracted using acetonitrile. The proportions of ligand that are soluble versus adsorbed are then quantified by scintillation counting. The proportion of adsorbed

ligand relative to total ligand is representative of the amount of vitamin D that is bioavailable (i.e. the fraction not bound to subjects' D-binding protein; see Figure S2). Thus:

VRCBA % bioavailable 25-hydroxyvitamin D_3 = adsorbed radiolabel ÷ [adsorbed radiolabel + nonadsorbed] (Eq. 8)

In order to relate the proportions of adsorbed vs. soluble radioligand to the concentrations obtained using our calculated bioavailable assay method, we used purified D-binding protein diluted at various concentrations into a fixed concentration of human serum albumin as a D-binding protein calibrator solution. Using these calibrator solutions, we generated calibration curves and used these to transform VRCBA % bioavailable 25-hydroxyvitamin D₃radioligand measurements in subjects' samples into their corresponding calculated % bioavailable 25-hydroxyvitamin D₃ in each subject's plasma is obtained by multiplying the total concentration of 25-hydroxyvitamin D₃ by the % bioavailable.

Assay Procedure

Three μ L of each subject's plasma was diluted into 15 mL of phosphate buffered saline (1:5000). 100 μ L of diluted plasma was added to each well. Plates were chilled on ice in a refrigerated room kept at 4°C for 15 minutes prior to adding radioligand. 100 μ L of radioligand diluted in binding assay buffer was added to each well and plates were kept refrigerated at 4°C for 8 hours for the binding reaction to reach equilibrium. Soluble radioligand (bound to serum D-binding protein and albumin from test samples) was separated from adsorbed ligand by pipetting all 200 μ L of the reaction volume from the well without leaving any visible amounts. This fraction was added directly to 3 mL of scintillation fluid. Adsorbed radioligand (bound to purified D-binding protein and albumin coating microtiter wells) was extracted by adding 200 μ L of acetonitrile. Plates were tipped back and forth to extract any ligand on upper sides of well, and acetonitrile extract was removed and mixed in 3 mL of scintillation fluid. Scintillation vials were, capped, mixed thoroughly, and counted for 2 minutes each. All samples and assay calibrator standards were measured in triplicate. Subjects' sample measurements were performed in two experiments; each experiment included measurement of five assay standards containing D-binding protein calibrators at

1000, 500, 250, 125, and 62.5µgper µL. Subjects' sample measurements of % bioavailable radioligand (defined by the ratio of adsorbed radioligand divided by total radioligand) were converted into their respective calculated % bioavailable 25-hydroxyvitamin D₃using the calibrator standard curves (Fig. S3). The concentration of bioavailable 25-hydroxyvitamin D₃ (in ng/mL) in subject samples was obtained by multiplying their measured total 25-hydroxyvitamin D₃ concentrations by the calculated % bioavailable25-hydroxyvitamin D₃.

4. ALPCO Vitamin D binding Protein Immunoassay

This immunodiagnostic kit, intended for the quantitative determination of free, non-actin bound D-binding protein in human plasma or serum was obtained commercially (ALPCO Diagnostics, Salem, NH). Reported inter-assay CV for this assay was 12.7%. This alternative assay has been previously used in a study which found no differences in D-binding protein concentrations between black and white women.¹⁰ Using manufacturer's instructions, we performed serum measurements of D-binding protein in a subset of HANDLS cohort samples (n=44, one sample from the original 45 was depleted) from our study with bioavailable 25-hydroxyvitamin D values measured by our direct assay format.





Of 3720 participants enrolled in the HANDLS study, 2085 participants were included in the present study. 25(OH)D=25-hydroxyvitamin D, DBP=D-binding protein, PTH=parathyroid hormone, DXA=Dual energy X-ray Absorptiometry.



Figure S2. *Direct measurement of % bioavailable 25*-hydroxyvitamin *D in presence of increasing concentrations of purified D-binding protein calibrator*. Reactions contained fixed amount of 25-hydroxyvitamin D radioligand, 5% serum albumin, and increasing concentrations of purified D-binding protein calibrator (as indicated on x-axis). Y-axis shows % bioavailable 25-hydroxyvitamin D calculated from amount of adsorbed radioligand as a percentage of the total radioligand added to reaction. Each data point represents the average of triplicate measurements; error bars indicate standard deviation of replicates.



Figure S3. 25-hydroxy*vitamin D radioligand competitive binding assay standard curve for conversion of radioligand binding measurements into equivalent calculated bioavailable 25-hydroxyvitamin D values. % bioavailable 25-hydroxyvitamin D values for the D-binding protein calibrator mixtures shown in Fig. S2 were calculated based upon these solutions' known concentrations of serum albumin, 25-hydroxyvitamin D radioligand, and purified D-binding protein. Calculated % bioavailable 25-hydroxyvitamin D values were plotted against the directly measured % bioavailable 25-hydroxyvitamin D values shown in Fig. S2. Each data point represents the average of triplicate measurements; error bars indicate standard deviation of replicates.*



Figure S4. Correlations between calculated bioavailable 25-hydroxyvitamin D concentrations in homozygous subjects compared to measurements by radioligand competitive binding assay. Direct measurement of % bioavailable 25-hydroxyvitamin D concentrations were performed using radioligand binding assay on a subset of 45 HANDLS subjects homozygous for Gc1F or Gc1S. Direct measurements were transformed into their calculated bioavailable 25-hydroxyvitamin D equivalents using the calibrator curve obtained in Fig. S2 (y = 0.598x + 0.087). Absolute concentrations of bioavailable 25-hydroxyvitamin D (in ng/mL) were obtained by multiplying % bioavailable 25-hydroxyvitamin D values by the subjects' LC-MS/MS measured serum total 25-hydroxyvitamin D concentrations. The directly measured bioavailable 25-hydroxyvitamin D concentrations (y-axis) were then plotted against their corresponding calculated bioavailable 25-hydroxyvitamin D values (x-axis). Our results demonstrate significant correlation between measured and calculated bioavailable 25hydroxyvitamin D for both genotypes. The D-binding protein purified from pooled serum that was used as both the calibrator and adsorbed affinity binding reagent was enriched in Gc1S variant. This competitive assay format results in asymmetric competition for binding between the Gc1S adsorbed to the plate and whichever DBP variant is present in the patients' serum. Based upon these findings, this direct assay may not be valid for making comparisons of absolute levels of bioavailable 25-hydroxyvitamin D in patients with different D-binding protein variants unless genotype-specific calibrators are used.



Figures S5. Comparison of the ALPCO Diagnostics D-binding Protein Immunoassay with R&D Systems D-binding Protein Immunoassay.D-binding protein concentrations were measured in 44 serum samples from HANDLS cohort using ELISA kit from ALPCO Diagnostics, Inc. as per manufacturer's instructions. Bland-Altman plot is shown comparing D-binding protein measurements using ALPCO kit with serum measurements of D-binding protein by the R&D Systems ELISA assay. The differences between assay methods are shown plotted on the y-axis compared to the average of both assay measurements on the x-axis. Solid and dotted lines indicate mean difference between the assays ± 2 standard deviations. Our data shows no significant correlation between the two assays.



Figure S6. *Correlations between directly measured serum % bioavailable 25-hydroxyvitamin D using radioligand competitive binding assay and serum D-binding protein concentrations measured by ELISA.* Top panel shows correlation between % bioavailable 25-hydroxyvitamin D and R&D Systems ELISA D-binding protein measurements performed in 44 HANDLS patient samples, bottom panel shows correlation between % bioavailable 25-hydroxyvitamin D and ALPCO Diagnostics ELISA measurements performed in these same samples. Directly measured % bioavailable 25-hydroxyvitamin D refers to the proportion (%) of 25-hydroxyvitamin D radioligand in each reaction that is not bound to D-binding protein in the patient's serum, but instead binds to the D-binding protein adsorbed to the microtiter plate. Pearson correlation coefficients and p-values are shown.

Black Americans	Summer	Fall	Spring	Winter	
no. (%)	182 (15.4)	321 (27.2)	353 (29.9)	325 (27.5)	
Total 25-hydroxyvitamin D (ng/mL)	19.0 ± 0.6**	18.1 ± 0.4**	13.2 ± 0.4	13.8 ± 0.4	
D-binding protein (µg/mL)	173 ± 9	167 ± 6	153 ± 6**	183 ± 6	
Parathyroid Hormone (pg/mL)	35.8 ± 1.0**	36.3 ± 1.0**	40.7 ± 1.0	41.6 ± 1.0	
Calcium (mg/dL)†	9.13 ± 0.03**	9.14 ± 0.2**	9.13 ± 0.02**	9.04 ± 0.02	
White Americans	Summer	Fall	Spring	Winter	
no. (%)	223 (24.7)	278 (30.8)	202 (22.4)	201 (22.2)	
Total 25-hydroxyvitamin D (ng/mL)	30.5 ± 0.7**	27.4 ± 0.6**	22.1 ± 0.7	22.0 ± 0.7	
D-binding protein (µg/mL)	353 ± 9*	324 ± 9	353 ± 10*	322 ± 10	
Parathyroid Hormone (pg/mL)	32.9 ± 1.0	33.7 ± 1.0	34.1 ± 1.0	34.0 ± 1.0	
Calcium (mg/dL)†	9.00 ± 0.02	9.02 ± 0.02*	8.97 ± 0.02	8.94 ± 0.02	

Table S1. Seasonal Differences in 25-Hydroxyvitamin D, D-binding protein, Calcium, and Parathyroid Hormone

*Significantly different from winter P<0.05, **Significantly different from winter at P<0.01, †Corrected for albumin

otal 25-Hydroxyv Quintile (Min-Max)	itamin D	Bone Mineral Density (g/cm²)	Parathyroid Hormone (pg/mL)	Calcium (mg/dL)	Bioavailable 25- Hydroxyvitamin D Quintile (Min-Max)‡		Bone Mineral Density (g/cm²)	Parathyroid Hormone (pg/mL)	Calcium (mg/dL)
Black	Ν	Mean \pm SE [†]	Mean $\pm SE^{\dagger}$	Mean $\pm SE^{\dagger}$	Black	N	Mean \pm SE [†]	Mean $\pm SE^{\dagger}$	Mean ± SE [†]
1 (<10)	184	1.04 ± 0.01	44.0 ± 1.0	9.07 ± 0.03	1 (0.00-1.40)	130	1.03 ± 0.02	44.2 ± 1.0	9.06 ± 0.03
2 (10-14)	182	1.06 ± 0.01	38.3 ± 1.0	9.09 ± 0.03	2 (1.41-1.98)	136	1.05 ± 0.02	41.2 ± 1.0	9.10 ± 0.03
3 (15-20)	137	1.03 ± 0.01	36.3 ± 1.0	9.14 ± 0.03	3 (1.99-2.76)	112	1.05 ± 0.02	38.4 ± 1.0	9.17 ± 0.04
4 (21-28)	106	1.03 ± 0.02	37.3 ± 1.0	9.09 ± 0.04	4 (2.77-4.24)	137	1.03 ± 0.01	36.2 ± 1.0	9.11 ± 0.03
5 (>28)	49	1.02 ± 0.03	32.2 ± 1.1	9.24 ± 0.06	5 (4.25-15.72)	125	1.06 ± 0.02	34.1 ± 1.0	9.11 ± 0.04
P-value for Tre	end	0.40	<0.001*	0.05*	P-value for	Trend	0.54	<0.001*	0.39
White	Ν	Mean \pm SE [†]	Mean $\pm SE^{\dagger}$	Mean $\pm SE^{\dagger}$	White	Ν	Mean \pm SE [†]	Mean $\pm SE^{\dagger}$	Mean ± SE [†]
1 (<10)	14	0.91 ± 0.04	46.2 ± 1.1	8.94 ± 0.10	1 (0.00-1.40)	71	0.88 ± 0.02	39.3 ± 1.0	9.06 ± 0.04
2 (10-14)	36	0.88 ± 0.03	40.6 ± 1.1	9.09 ± 0.06	2 (1.41-1.98)	65	0.92 ± 0.02	35.4 ± 1.0	8.98 ± 0.04
3 (15-20)	74	0.90 ± 0.02	35.7 ± 1.0	9.02 ± 0.04	3 (1.99-2.76)	89	0.97 ± 0.02	32.5 ± 1.0	9.01 ± 0.04
4 (21-28)	95	0.94 ± 0.02	33.0 ± 1.0	8.99 ± 0.04	4 (2.77-4.24)	64	0.97 ± 0.02	29.7 ± 1.0	9.01 ± 0.04
5 (>28)	148	0.98 ± 0.01	29.5 ± 1.0	9.02 ± 0.04	5 (4.25-15.72)	76	0.95 ± 0.02	29.7 ± 1.0	9.02 ± 0.04
P-value for Tre	ənd	<0.001*	<0.001*	0.85	P-value for	Trend	0.006*	<0.001*	0.75

Powe CE et al.

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BMD=Bone Mineral Density, PTH=Parathyroid Hormone

[†]Adjusted for age, season, sex, body mass index, smoking status, socioeconomic status, and calcium intake (except where calcium is the outcome).

[‡]Sample sizes do not sum to total homozygous participants due to missing albumin levels.

*Significant at P<0.05

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Bioavailability of Vitamin D and Its Metabolites in Black and White Adults

Michael F. Holick, M.D., Ph.D.

Vitamin D deficiency has become a global health problem that has been associated not only with metabolic bone disease but also with many chronic illnesses.1 The definition of vitamin D deficiency has been controversial, in part owing to the interpretation of surrogates associated with vitamin D status. The Institute of Medicine concluded that to maximize bone health, the level of 25-hydroxyvitamin D should be higher than 20 ng per milliliter.² Others have relied on parathyroid hormone levels to assess vitamin D status, arguing that such levels reach a nadir and plateau when 25-hydroxyvitamin D levels are 30 to 40 ng per milliliter.³ One study indicated that a 25-hydroxyvitamin D level of more than 30 ng per milliliter ensured the absence of osteomalacia.4 Bone mineral density has a positive correlation with serum 25-hydroxyvitamin D levels,5 and numerous association studies have suggested that the risk of many chronic illnesses is increased with a 25-hydroxyvitamin D level of less than 30 ng per milliliter.1

A perplexing paradox is that blacks have a higher bone mineral density but lower 25-hydroxyvitamin D levels than whites.6 In this issue of the Journal, Powe et al.⁶ report the evaluation of serum levels of calcium, 25-hydroxyvitamin D, and parathyroid hormone as well as bone mineral density in more than 2000 community-dwelling blacks and whites. The investigation confirmed that blacks had higher bone mineral density and lower 25-hydroxyvitamin D levels than whites. However, when the authors compared two common polymorphisms in the vitamin D-binding protein gene in blacks and whites and then measured vitamin D-binding protein levels and calculated free 25-hydroxyvitamin D, they found that blacks had lower vitamin Dbinding protein levels than whites and that their bioavailable 25-hydroxyvitamin D levels were similar to those in whites. The authors concluded that vitamin D deficiency may need to be redefined, to consider not only total but also bioavailable 25-hydroxyvitamin D levels.

The vitamin D-binding protein that is also known as GC-globulin is the approximate size

of albumin and has three major polymorphic forms — GC1F, GC1S, and GC2. Vitamin Dbinding protein has the highest affinity for 25hydroxyvitamin D, with 20 times and 100 times less affinity for 1,25-dihydroxyvitamin D and vitamin D₃, respectively. The vitamin D-binding protein variants have different abundances and affinities for vitamin D and these two vitamin D metabolites, with GC1F greater than GC1S and GC1S greater than GC2.7 GC1F is the most abundant form in persons of African ancestry, whereas GC1S is most abundant in European populations. Albumin also binds 25-hydroxyvitamin D with lower affinity than that of the vitamin D-binding protein. Albumin in the circulation is 15 times more abundant than vitamin D-binding protein; approximately 10% of the total 25-hydroxyvitamin D in the circulation is bound to albumin, which may also be an important component of bioavailable 25-hydroxyvitamin D.6 Megalin (LRP2), a transmembrane protein, internalizes 25-hydroxyvitamin D bound to the vitamin D-binding protein and albumin.7 Mice lacking megalin have increased urinary loss of 25-hydroxyvitamin D bound to vitamin Dbinding protein and albumin and have a metabolic bone disease that is more severe than that seen in mice lacking vitamin D-binding protein.7 Thus, the bound 25-hydroxyvitamin D appears to be important for the renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D for calcium and bone metabolism.

It has been hypothesized that during the course of evolution, the most abundant polymorphic form of the vitamin D-binding protein in blacks had an increased affinity for vitamin D_3 and thus was able to transport vitamin D_3 more efficiently from the skin to the liver for its metabolism to 25-hydroxyvitamin D.⁸ However, mice lacking vitamin D-binding protein have been shown to metabolize vitamin D_3 to 25-hydroxyvitamin D with increased efficiency, which suggests that free, not bound, vitamin D_3 is what the liver prefers.⁷⁻⁹

Maasai warriors living near the equator, who are outside daily, have mean blood levels of

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25-hydroxyvitamin D of 44 ng per milliliter.^{1,3} As the people in equatorial Africa began migrating farther north and south, their deeply pigmented skin was less efficient in producing vitamin D₃, resulting in rickets due to severe vitamin D deficiency; for women, the flattened, deformed pelvis caused by rickets complicated giving birth. One may speculate that this might have been the evolutionary driver for the mutation of the melanocyte-stimulating hormone receptor gene, leading to skin with little sunscreening pigment to enhance vitamin D₂ production.¹⁰ Is it possible that this loss of pigment may also have led to lower bone density? The higher blood calcium levels observed in blacks as compared with whites may result from higher bioavailable levels of 1,25-dihydroxyvitamin D due to the observed lower levels of vitamin D-binding protein. Bioavailability may be relative regarding vitamin D and its metabolites. Megalin serves to transport the bioavailable 25-hydroxyvitamin D bound to the vitamin D-binding protein and albumin.7 However, the liver prefers unbound vitamin D₃.9 Immune and other cells lack megalin and thus may be able to use only unbound 25-hydroxyvitamin D. Therefore, more research is needed to fully appreciate what bioavailable versus total vitamin D status means for 25-hydroxyvitamin D as well as 1,25-dihydroxyvitamin D. Full elucidation will be important not only for bone health but also for physiological aspects of vitamin D in health and disease.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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Current practice may over-diagnose vitamin D deficiency Genetic variants in vitamin D-binding protein could explain lack of symptoms in some currently classified as deficient

BOSTON – The current "gold standard" test for measuring vitamin D status may not accurately diagnose vitamin D deficiency in black individuals. In an article in the Nov. 21 *New England Journal of Medicine*, a team of researchers report finding that genetic differences in a vitamin D carrier protein referred to as D-binding protein may explain the discrepancy between the prevalence of diagnosed vitamin D deficiency in black Americans – based on measuring the molecule 25-hydroxyvitamin D (250HD) – and a lack of the usual symptoms of vitamin deficiency.

The essential role of vitamin D in maintaining bone health is well recognized, but while measurement of 25OHD alone consistently classifies from 70 to 90 percent of black Americans as vitamin D deficient, the usual consequences of deficiency – such as low bone density and increased fracture risk – are actually less prevalent among black individuals. That inconsistency led the team led by Ravi Thadhani, MD, MPH, chief of the Division of Nephrology in the Massachusetts General Hospital (MGH) Department of Medicine, to take a closer look at whether current methods accurately determine vitamin D deficiency.

The investigators examined data from more than 2,000 participants in HANDLS (Healthy Aging in Neighborhoods of Diversity Across the Life Span), a larger National Institutes of Health (NIH)-sponsored study, led by Michele K. Evans, MD, co-corresponding author of the current report, and Alan B. Zonderman, PhD, also a co-author. HANDLS is prospective, long-term, epidemiologic study of age-associated health disparities in socioeconomically diverse black and white individuals in the city of Baltimore. Participants – adults ages 30 to 64 – were interviewed and received medical examinations between 2004 and 2009.

For the current study, researchers analyzed levels of 25OHD, levels and genetic variants of D-binding protein, and levels of calcium and parathyroid hormone – another marker of vitamin D deficiency – along with bone density readings in almost 1,200 white and around 900 black participants. The results indicated that black participants had significantly lower levels of both 25OHD and D-binding protein, compared with white participants and also showed that about 80 percent of the difference in D-binding protein levels could be explained by genetic variation. However, bone density and calcium levels were higher in black participants, and while their parathyroid hormone levels also were higher, the difference between black and white participants was slight.

"Black people are frequently treated for vitamin D deficiency, but we may not be measuring the right form of vitamin D to make that diagnosis," says Thadhani, who is senior and co-corresponding author of the *NEJM* report. "While our finding that 80 percent of black participants in this study met criteria for vitamin D deficiency is consistent with previous studies, we were surprised to find no evidence of problems with bone health. Most vitamin D in the bloodstream is tightly bound to D-binding protein and is not active. When we determined the concentrations of circulating non-bound vitamin D, which would be available to cells, we found that levels of this form were equivalent between black and white participants, which suggested to us that these black individuals may not be truly deficient."

He adds, "Although currently there are no commercially available assays that directly measure bioavailable levels of 250HD, these results suggest that such assays would more accurately identify those with true vitamin D deficiency, allowing us to direct treatment toward those who really need it. Additional studies need to be conducted to establish optimal levels of bioavailable 250DH across all racial and ethnic groups." Thadhani is a professor of Medicine at Harvard Medical School.

Evans, who is deputy scientific director and chief of the Health Disparities Research Section at the National Institute on Aging Intramural Research Program (NIA-IRP), notes, "This study confirms the value of addressing clinical questions from a health disparities standpoint that overcomes barriers to inclusion of diverse populations in biomedical research."

Camille Powe, M.D., of Brigham and Women's Hospital is lead author of the *NEJM*. Additional coauthors are Julia Wenger, MPH, Hector Tamez, MD, MPH, and Ishir Bhan, MD, MPH, MGH Nephrology; Michael Nalls, PhD, NIA-IRP; Anders Berg, MD, PhD, Dongsheng Zhang, PhD, and Ananth Karumanchi, MD, Beth Israel Deaconess Medical Center; and Neil Powe, MD, University of California, San Francisco. The study was supported by National Institute of Aging grant AG000513 and National Institutes of Health grants K24 DK094872 and R01 DK094486.

Massachusetts General Hospital (www.massgeneral.org), founded in 1811, is the original and largest teaching hospital of Harvard Medical School. The MGH conducts the largest hospital-based research program in the United States, with an annual research budget of more than \$775 million and major research centers in AIDS, cardiovascular research, cancer, computational and integrative biology, cutaneous biology, human genetics, medical imaging, neurodegenerative disorders, regenerative medicine, reproductive biology, systems biology, transplantation biology and photomedicine .

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