Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Powe CE, Evans MK, Wenger J, et al. Vitamin D–binding protein and vitamin D status of black Americans and white Americans. N Engl J Med 2013;369:1991-2000. DOI: 10.1056/NEJMoa1306357

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

Powe CE et al.

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 Amp-erase 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.

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]

DBP_2 = 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 D-binding 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 = $[D_{DBP}] + [D_{Alb}] + [D_{Free}]$

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

K_{alb} = affinity constant between 25-hydroxyvitamin D and albumin = $6 \times 10^5\ M^{-1}$

$KDBP_{generic}$ = genotype-nonspecific affinity constant between 25-hydroxyvitamin D and DBP = $0.7 \times 10^9\ M^{-1}$

$KDBP_{1S}$ = 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} = $1.12 \times 10^9\ M^{-1}$

$KDBP_2$ = affinity constant between 25-hydroxyvitamin D and DBP_2 = $0.36 \times 10^9\ M^{-1}$

*Affinity constants are taken from Arnaud et al.[9]

EQUATIONS (Adapted from Vermuelen et al.)[7]

Total 25-Hydroxyvitamin D

[Total D] = 25-hydroxyvitamin D_2 + 25-hydroxyvitamin D_3 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

$[D_{Free}] + [Alb] \leftrightarrow [D_{Alb}]$

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 $K_{DBP} = [D_{DBP}] \div ([DBP] \cdot [D_{Free}])$

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:

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

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):

$[Bio D] = [D_{Free}] + [D_{Alb}] = (K_{alb}\cdot [Alb] + 1) \cdot [D_{Free}]$ (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 25-hydroxyvitamin D can be calculated using previously measured binding affinities for the three variants (reference (34) in main manuscript):

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

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

For subjects homozygous for Gc2 variant, $K_{DBP} = 0.36 \times 10^9\, M^{-1}$

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 µg/mL = 4.3×10^{-6}mol/L

Total serum albumin = [Alb] = 4.3 g/dL = 6.4×10^{-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

25-hydroxyvitamin D affinity adsorption plates 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 µg of D-binding protein diluted into 100 µL of water and allowed to adsorb at 4°C overnight. Wells were then washed with water and blocked with 500 µL 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.

Vitamin D binding protein calibrators 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).

Radiolabeled 25-hydroxyvitamin D_3 ligand was purchased from Perkin Elmer (Hydroxyvitamin D_3, 25-[26,27-^{3}H]-, 5µCi(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 µL (~1 nCi) of radioligand was diluted into 100 µLof 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 D_3 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 + non-adsorbed] (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 D_3radioligand measurements in subjects' samples into their corresponding calculated % bioavailable 25-hydroxyvitamin D_3 values (Fig. S3). The absolute concentration of bioavailable 25-hydroxyvitamin D_3 in each subject's plasma is obtained by multiplying the total concentration of 25-hydroxyvitamin D_3 by the % bioavailable.

Assay Procedure

Three µL of each subject's plasma was diluted into 15 mL of phosphate buffered saline (1:5000). 100 µL 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 µL 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 µL 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 µL 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 D_3using the calibrator standard curves (Fig. S3). The concentration of bioavailable 25-hydroxyvitamin D_3 (in ng/mL) in subject samples was obtained by multiplying their measured total 25-hydroxyvitamin D_3 concentrations by the calculated % bioavailable25-hydroxyvitamin D_3.

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.

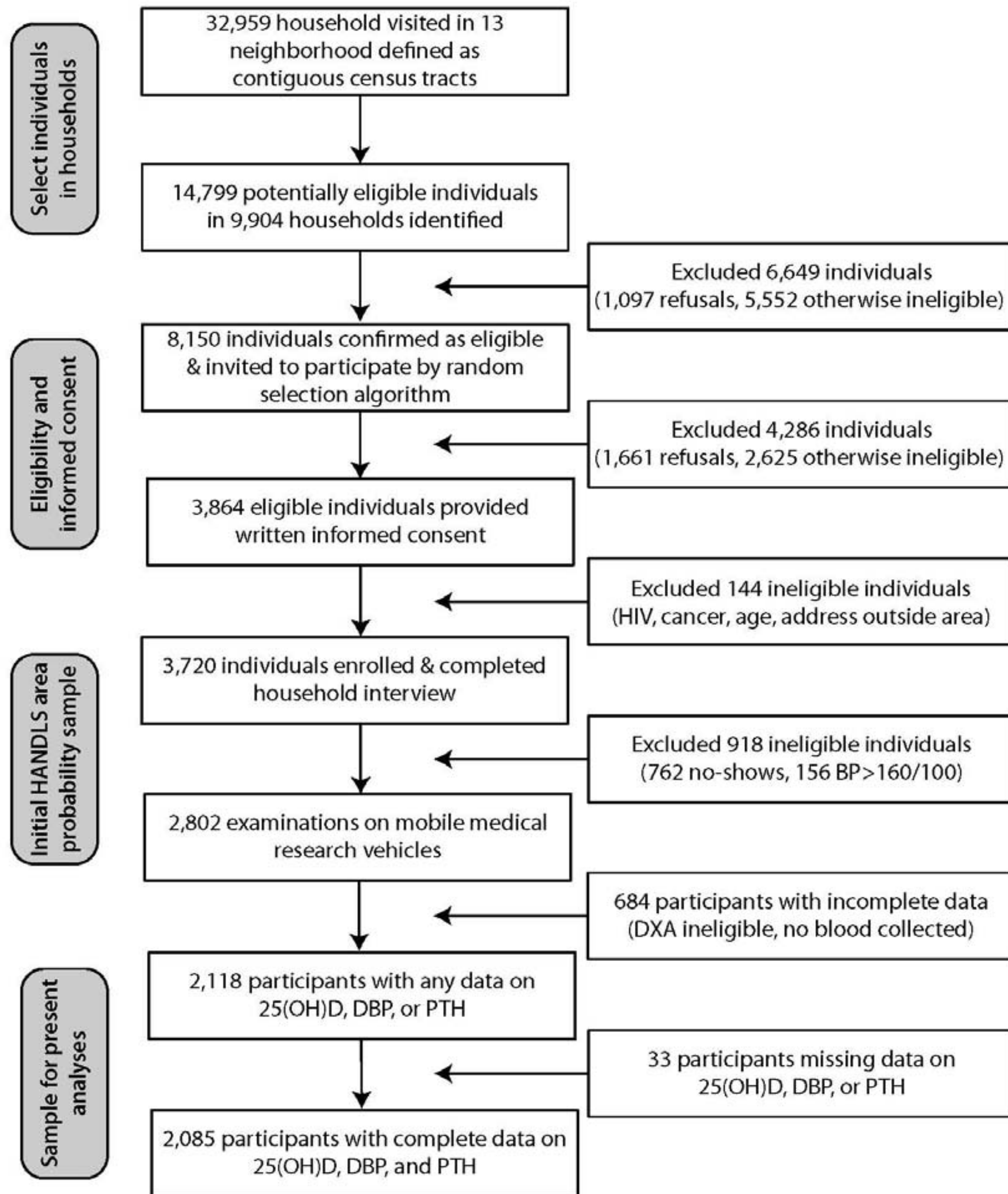


Figure S1. HANDLS Enrollment and Inclusions.

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.

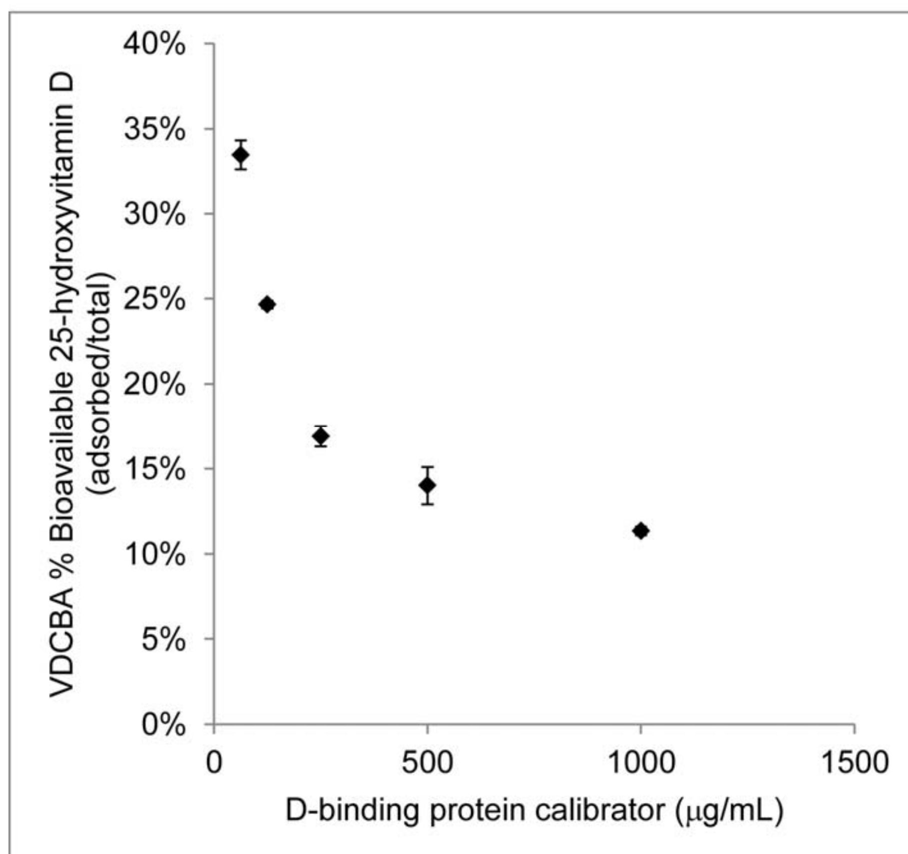


Figure S2. *Direct measurement of % bioavailable 25*-hydroxyvitamin *D in presence of increasing concentrations of purified D-binding protein calibrator*. Reactions contained fixed amount of 25-hydroxyvitamin D radioligand, 5% serum albumin, and increasing concentrations of purified D-binding protein 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.

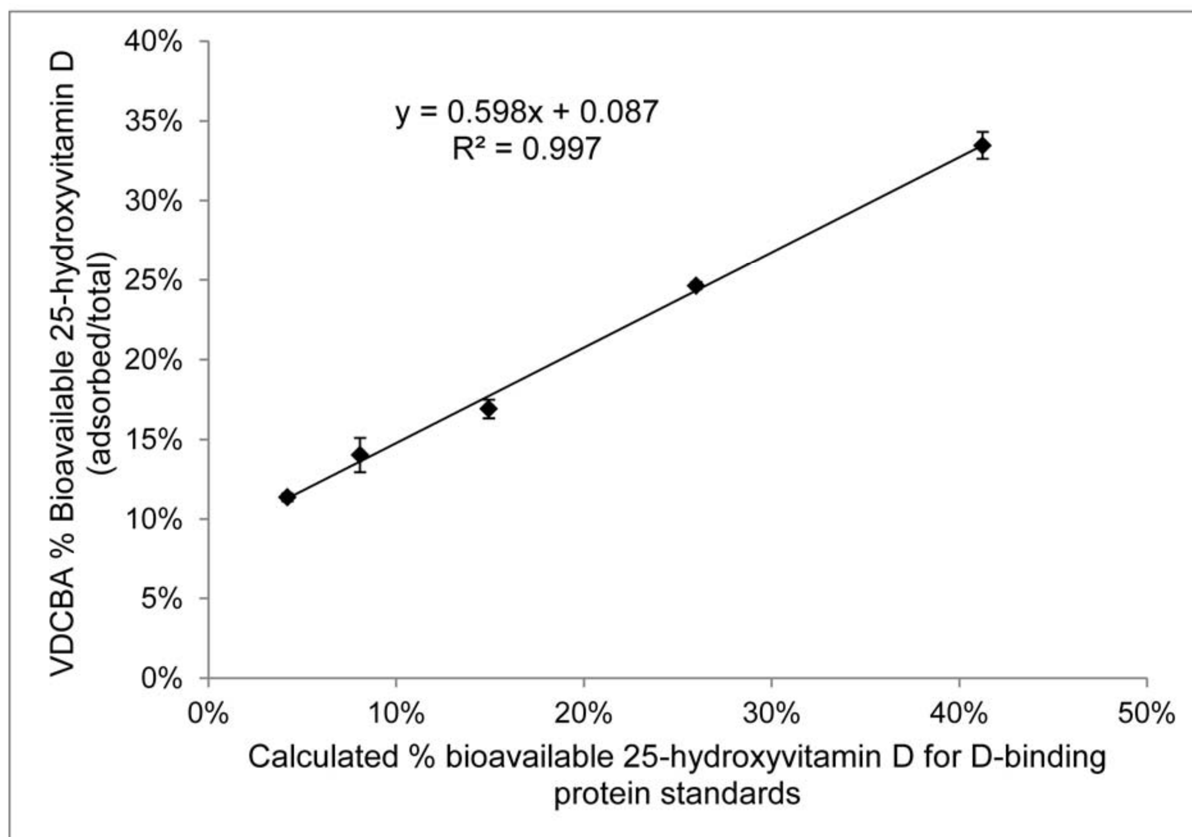


Figure S3. 25-hydroxy*vitamin D radioligand competitive binding assay standard curve for conversion of radioligand binding measurements into equivalent calculated bioavailable 25-* hydroxyvitamin *D values*. % bioavailable 25-hydroxyvitamin D values for the D-binding protein calibrator mixtures shown 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.

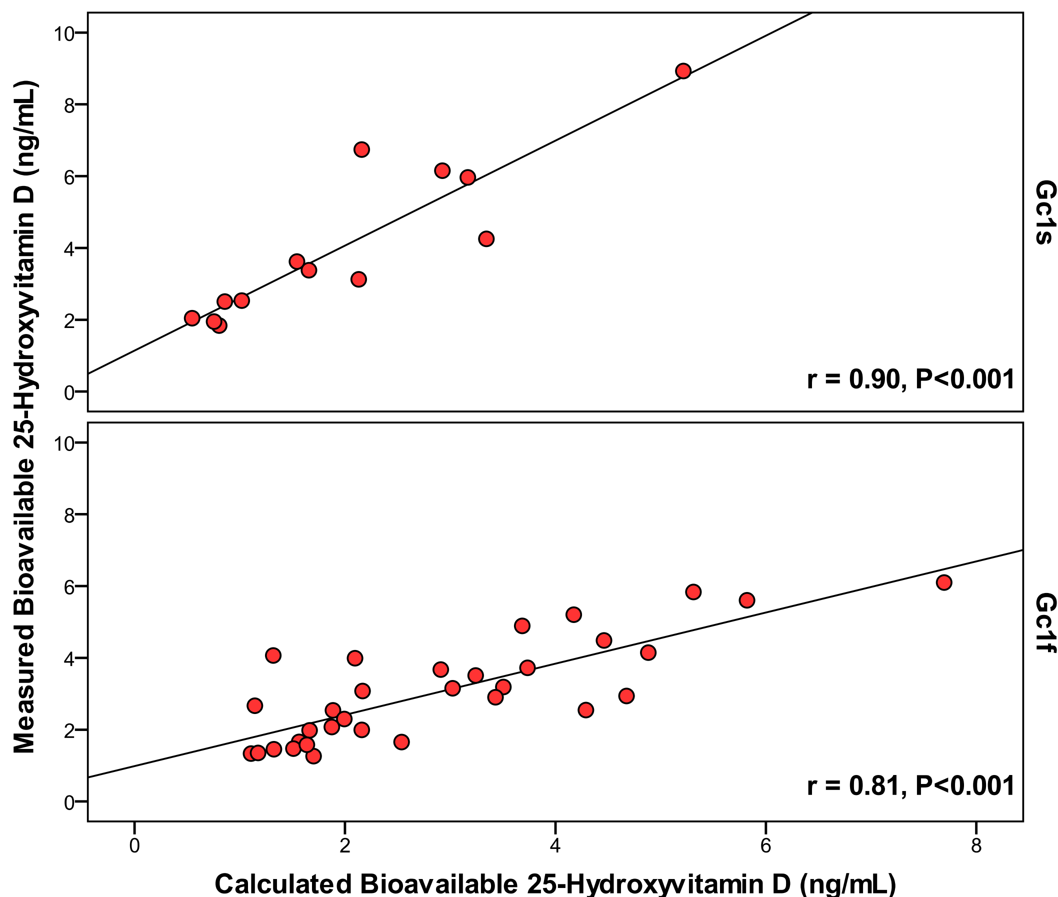
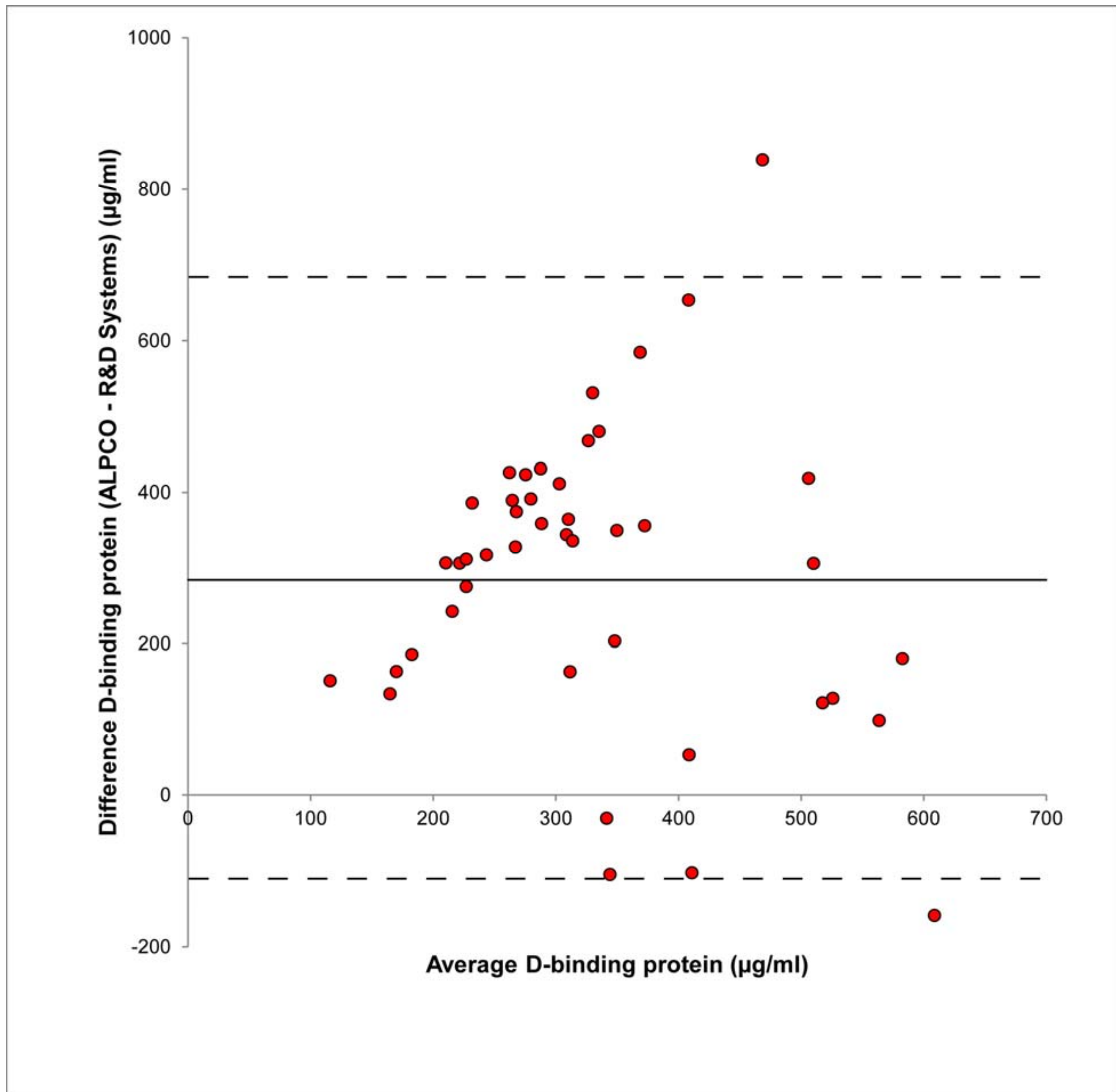


Figure S4*. Correlations between calculated bioavailable 25*-hydroxyvitamin *D concentrations in homozygous subjects compared to measurements by radioligand competitive binding assay*. Direct measurement of % bioavailable 25-hydroxyvitamin D concentrations were performed using radioligand binding assay 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 25-hydroxyvitamin 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.

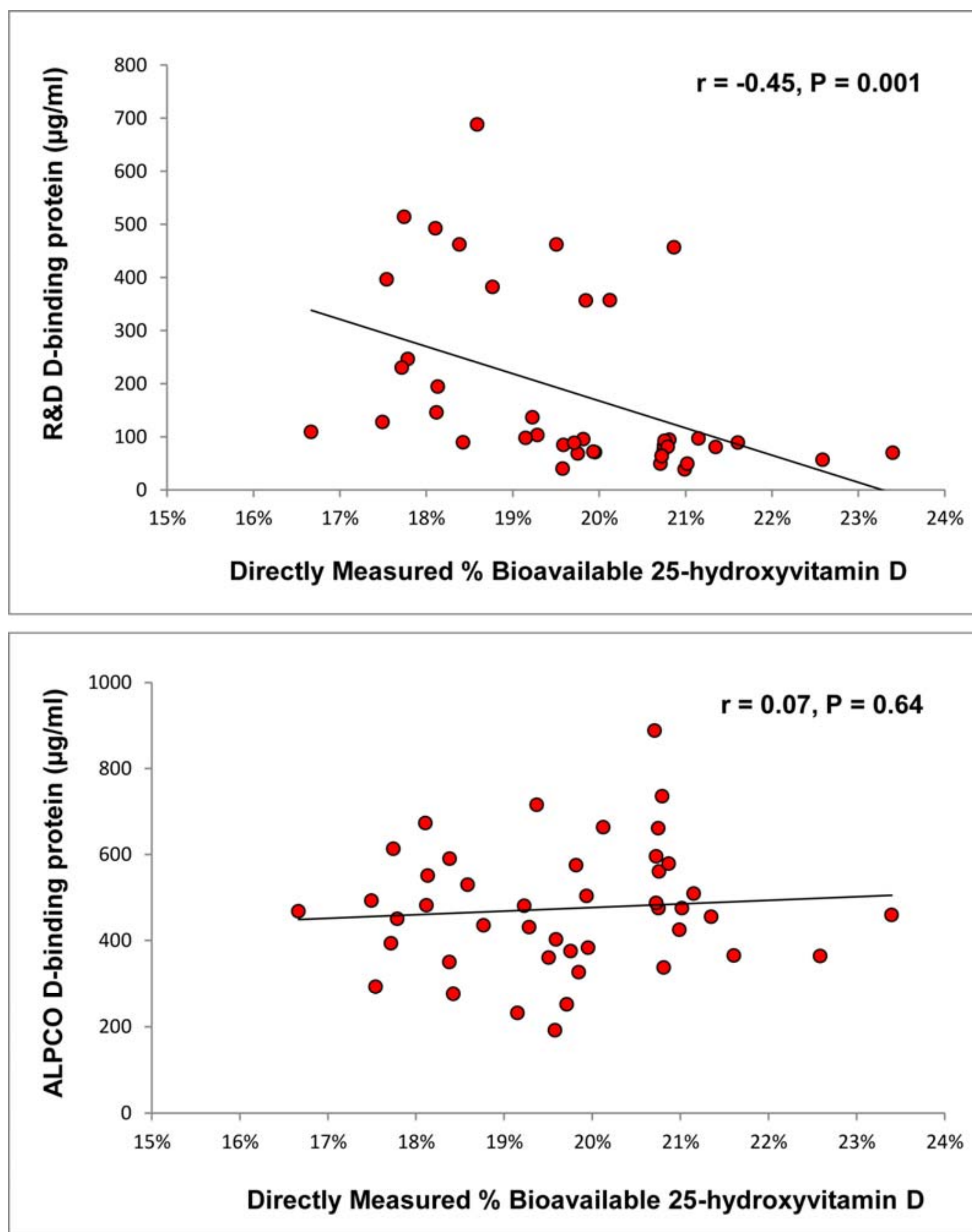


Figure S6. *Correlations between directly measured serum % bioavailable 25-hydroxyvitamin D using radioligand competitive binding assay and serum D-binding protein concentrations measured by ELISA.* Top panel shows correlation between % bioavailable 25-hydroxyvitamin D and R&D Systems ELISA D-binding 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.

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

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

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

Table S2. Total and Bioavailable 25-Hydroxyvitamin D and Markers of Vitamin D Sufficiency in Black and White Homozygous Participants

Total 25-Hydroxyvitamin D Quintile (Min-Max)		Bone Mineral Density (g/cm^2)	Parathyroid Hormone (pg/mL)	Calcium (mg/dL)	Bioavailable 25-Hydroxyvitamin D Quintile (Min-Max)‡		Bone Mineral Density (g/cm^2)	Parathyroid Hormone (pg/mL)	Calcium (mg/dL)
Black	**N**	**Mean ± SE†**	**Mean ± SE†**	**Mean ± SE†**	**Black**	**N**	**Mean ± SE†**	**Mean ± SE†**	**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 Trend		0.40	<0.001*	0.05*	*P-value for Trend*		0.54	<0.001*	0.39
White	**N**	**Mean ± SE†**	**Mean ± SE†**	**Mean ± SE†**	**White**	**N**	**Mean ± SE†**	**Mean ± SE†**	**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 Trend		<0.001*	<0.001*	0.85	*P-value for Trend*		0.006*	<0.001*	0.75

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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