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Original Contribution

Ancestry-Adjusted Vitamin D Metabolite Concentrations in Association With Cytochrome P450 3A Polymorphisms

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We investigated the association between genetic polymorphisms in cytochrome P450 (*CYP2R1*, *CYP24A1*, and the CYP3A family) with nonsummer plasma concentrations of vitamin D metabolites (25-hydroxyvitamin D_3 (25(OH)D_3) and proportion 24,25-dihydroxyvitamin D_3 (24,25(OH)$_2D_3$)) among healthy individuals of sub-Saharan African and European ancestry, matched on age (within 5 years; $n = 188$ in each ancestral group), in central suburban Pennsylvania (2006–2009). Vitamin D metabolites were measured using high-performance liquid chromatography with tandem mass spectrometry. Paired multiple regression and adjusted least-squares mean analyses were used to test for associations between genotype and log-transformed metabolite concentrations, adjusted for age, sex, proportion of West-African genetic ancestry, body mass index, oral contraceptive (OC) use, tanning bed use, vitamin D intake, days from summer solstice, time of day of blood draw, and isoforms of the vitamin D receptor (*VDR*) and vitamin D binding protein. Polymorphisms in *CYP2R1*, *CYP3A43*, vitamin D binding protein, and genetic ancestry proportion remained associated with plasma 25(OH)D_3 after adjustment. Only *CYP3A43* and *VDR* polymorphisms were associated with proportion 24,25(OH)$_2D_3$. Magnitudes of association with 25(OH)D_3 were similar for *CYP3A43*, tanning bed use, and OC use. Significant least-squares mean interactions (*CYP2R1*/OC use ($P = 0.030$) and *CYP3A43*/*VDR* ($P = 0.013$)) were identified. A *CYP3A43* genotype, previously implicated in cancer, is strongly associated with biomarkers of vitamin D metabolism. Interactive associations should be further investigated.

25(OH)D_2; 25(OH)D_3; *CYP2R1*; *CYP3A43*; group-specific component; proportion 24,25(OH)$_2D_3$; vitamin D binding protein; vitamin D receptor

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin D_3; Ala, alanine; Arg, arginine; Asn, asparagine; BMI, body mass index; CYP, cytochrome P450; dbSNP, database of single nucleotide polymorphisms; Gc, vitamin D binding protein; *GC*, group-specific component (vitamin D binding protein); Gln, glutamine; IU, international units; Met, methionine; OC, oral contraceptive; Pro, proline; SIFT, Sorting Intolerant From Tolerant; Thr, threonine; VDR, vitamin D receptor.

Human and in vitro studies have established the cytochrome P450 (CYP) family 2 subfamily R member 1 (CYP2R1) as a vitamin D_3 25-hydroxylase (1), contributing to the production of 25-hydroxyvitamin D_3 (25(OH)D_3). Epidemiologic studies have identified associations between genetic polymorphisms near the *CYP2R1* gene and circulating concentrations of 25-hydroxyvitamin D (25(OH)D) (2–4). While other 25-hydroxylases are known, they are currently considered to play a lesser role in vitamin D metabolism (5).

The 1,25-dihydroxyvitamin D_3 24-hydroxylase enzyme (CYP24A1) is highly inducible by the active form of vitamin D (i.e., 1,25(OH)$_2D_3$), and it produces both 24,25-dihydroxyvitamin D_3 (24,25(OH)$_2D_3$) from 25(OH)D_3 and 1,24,25(OH)$_3D_3$ from the active form (6). Elevated genetic *CYP24A1* expression is implicated in disease-related alterations in circulating vitamin D metabolites, including 25(OH)D_3 and 24,25(OH)$_2D_3$ (7). The role of *CYP24A1* polymorphic variants in humans is not well understood.

Cytochrome P450 family 3 subfamily A (CYP3A) enzymes comprise CYP3A4, CYP3A5, CYP3A7, and CYP3A43, which are heme-containing monooxygenases that catalyze hydroxylation of a wide range of substrates (8). CYP3A4 has been

characterized as a 24- and 25-vitamin D_3 hydroxylase (5). CYP3A4 is the most abundant P450 enzyme in the liver and is involved in xenobiotic metabolism of over half of prescription drugs (8). Pharmacologic induction of *CYP3A4* gene expression in healthy volunteers is associated with alterations in circulating vitamin D metabolites (9). In contrast, CYP3A43, CYP3A5, and CYP3A7 enzymes have no established role in vitamin D metabolism, yet amino acid homology with CYP3A4 is >70% (10, 11).

Polymorphic variants in *CYP2R1*, *CYP24A1* and the CYP3A family are known to vary by race/ethnicity, with the greatest frequency of variants among individuals of African ancestry. In addition, with a difference of approximately 2-fold, circulating concentrations of 25(OH)D among racial/ethnic groups in the United States are known to be lowest among African Americans and highest among white Americans.

The purpose of this study was to determine the association between genetic polymorphisms in the CYP3A family of enzymes (*CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*) and circulating vitamin D metabolites, including plasma $25(OH)D_3$ and the proportion of plasma $24,25(OH)_2D_3$ (relative to circulating $25(OH)D_3$) among individuals of African-American and European ancestry.

METHODS

Subjects

Healthy individuals aged 18–55 years, self-reporting at least 50% European or at least 50% African ancestry and providing written informed consent, were enrolled (PSU IRB No. 36350). These 2 racial/ethnic groups were chosen because of the limited number of studies of vitamin D among African Americans and the large difference in serum 25(OH)D between these groups (approximately 2-fold). Individuals were excluded if they had fever (37.78°C or higher) or severe chronic disease (chronic kidney disease, liver disease, chronic obstructive pulmonary disease, human immunodeficiency virus/acquired immune deficiency syndrome), were unable to provide a blood sample, or were currently undergoing chemotherapy or radiation treatment for cancer. European-American participants were matched in a 1:1 ratio by age (within 5 years) with African-American participants. All but 1 pair was also matched on sex ($n = 188$ in each ancestral group).

Participants completed a questionnaire (including personal medical history, current medication use, age, self-reported race/ethnicity, sex, smoking status, and vitamin D exposure) and had skin melanin index, height, weight, and blood pressure (systolic and diastolic) measured at an in-person visit. All blood samples were collected during nonsummer months in order to minimize seasonal variation (i.e., blood was not collected from May 15th to August 31st). Skin melanin index was measured twice on the upper inner arm by light reflectance (DermaSpectrometer II; CyberDerm Inc., Media, Pennsylvania), as previously described (12).

Sources of vitamin D included current tanning bed use (yes/no), hours spent in a tanning bed per week over the prior month, the number of hours outdoors in the prior month, current outdoor occupation (yes/no), dietary intake, and vitamin D intake through supplements in international units (IU)/day.

Dietary intake

Dietary intake over the past year was estimated using a 24-item calcium and vitamin D screener based on the Block food frequency questionnaire for usual food intake frequency and quantity, as modified by Dr. Hartman (13). Nutrients were calculated using information from the National Cancer Institute's 2009 Diet History Questionnaire Nutrient and Food Group Database made available with Diet*Calc software, a compilation of US Department of Agriculture's Food and Nutrient Database for Dietary Studies, and US Department of Agriculture's MyPyramid Equivalents Database (14).

Biomarker measurement

Three vitamin D metabolites ($25(OH)D_2$, $25(OH)D_3$, and $24,25(OH)_2D_3$) were measured using high-performance liquid chromatography with tandem mass spectrometry, as previously described (12). Plasma samples were prepared by solid phase extraction, spiked with deuterated standards (d_6-$25(OH)D_3$, d_6-$25(OH)D_2$, and d_6-$24,25(OH)_2D_3$ (Chemaphor Inc., Ottawa, Canada)). The percent coefficient of variation, assayed in 10 repeated samples of pooled plasma from healthy individuals on separate days, was 2.7, 12.2, and 6.3 for $25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$, respectively. The limit of quantitation for all 3 metabolites was 10 pg/mL, with a standard curve range of 1–100 ng/mL for $25(OH)D_2$ and $25(OH)D_3$ and 0.1–10 ng/mL for $24,25(OH)_2D_3$. Total serum calcium was measured in duplicate using colorimetry (BioVision, Mountain View, California); percent coefficient of variation = 3.5%.

Genotyping and genetic ancestry

DNA was extracted from whole blood in tubes with ethylenediaminetetraacetic acid using a QIAamp DNA Mini Kit (Qiagen Sciences, Germantown, Maryland) and stored at −80°C. A panel of 112 ancestry-informative markers was used to estimate the proportion of West-African ancestry and proportion of European ancestry, using the STRUCTURE algorithm and frequencies in HapMap YRI (West African) and CEU (European) trios (15, 16). This panel exhibits high agreement with other ancestry panels (concordance correlation coefficient = 0.97) (12).

Genotypes for *CYP24A1* (database of single nucleotide polymorphisms (dbSNP) IDs rs6022990 (Met374Thr), rs16999131 (Thr248Arg), and rs35051736 (Arg157Gln)), *CYP3A4* (dbSNP ID rs2740574 located in the promoter, 208 base pairs from the start site (p-208) and intergenic with *CYP3A43*), *CYP3A43* (dbSNP ID rs680055 (Ala340Pro)), *CYP3A5* (dbSNP ID rs28365083 (Thr398Asn) and rs776746 (located in the promoter, 237 base pairs from the start site (p-237)), and *CYP3A7* (dbSNP ID rs2257401 (Arg409Thr)) were determined using the Illumina BeadXpress assay (Illumina Inc., San Diego, California). Genotypes for *CYP24A1* (dbSNP ID rs2762943 (p-262)), rs111622401 (p-280)), *CYP2R1* (dbSNP ID rs10741657 (p-1127), rs2060793 (p-1559), rs16930609 (p-2157)), and *GC* (rs7041 and rs4588) (the gene encoding for the vitamin D binding protein (Gc)), were determined using TaqMan assays (Applied Biosystems, Foster City, California). Genotyping cluster algorithm outputs were visually inspected, and genotyping

calls were adjudicated by laboratory personnel blinded to the vitamin D status and study characteristics of participants. Samples and assays with >10% unreadable genotyping calls were excluded from the analysis. Genotypes for *CYP3A5* and *CYP3A4* occur proximal to vitamin D response elements (17).

Statistical methods

Because a lower reservoir of 25(OH)D_3 is possibly a function of its downstream metabolite 24,25$(OH)_2D_3$, we calculated the proportion 24,25$(OH)_2D_3$ as a proportion of total 25(OH)D_3 serum concentrations (18). Major circulating Gc-isoform diplotypes were estimated using genotype information for the 2 coding-region polymorphisms (rs4588 and rs7041), as previously described (19). The χ^2 test for deviation from Hardy-Weinberg equilibrium was used to calculate the observed versus expected distribution of genotypes, stratified by self-reported race/ethnicity. In order to better understand genetic linkage, Lewontin's D' and the r^2 statistics were used to estimate linkage disequilibrium between polymorphic variants among African-American participants using SAS Proc Allele (version 9.4; SAS Institute, Inc., Cary, North Carolina). Statistical tests of bivariate differences in continuous and categorical variables according to self-reported race/ethnicity were determined by paired *t* test and McNemar's χ^2 test, respectively. The 2-sided Fisher's exact test was used when cell sizes were equal to 5 or less. Racial/ethnic bivariate differences in non-normally distributed variables were tested using the nonparametric Wilcoxon rank-sum test. The bivariate correlation between SNP genotype and vitamin D metabolite concentrations was determined using the nonparametric Spearman rank-order correlation. Two-sided *P* values of ≤0.05 were defined as statistically significant.

In multiple regression analysis, the association with each genetic variant of interest was tested using pairwise regression using SAS Proc Mixed (SAS Institute, Inc.), using the restricted (or residual) maximum likelihood method. In order to better approximate a normal distribution, plasma 25(OH)D_3 and the proportion 24,25$(OH)_2D_3$ were log-transformed using the natural log. Adjustment factors were entered as continuous variables (unless otherwise indicated) and included age, sex, proportion of West-African genetic ancestry, World Health Organization (2000) body mass index (BMI) (classes I–VI, where class I is underweight and class II is normal weight), oral contraceptive (OC) use (yes/no), tanning bed use (yes/no), total vitamin D intake from diet and supplements combined (IU/day), days after summer solstice at time of blood draw, and time of day of blood draw (0800–1630). Statistically significant genotypes were included in the final models, thereby adjusting for other genetic associations. Adjusted least squares means and 1-way interactions between each variable in the final SAS Proc Mixed model were calculated using SAS Proc GLM. Regression model diagnostics included: goodness of fit (adjusted R^2), collinearity (Durbin-Watson test statistic and variance inflation factor), and missing explanatory variables (plot of residual versus predicted values).

Sorting Intolerant from Tolerant (SIFT) analysis was performed using J. Craig Venter Institute's "SIFT dbSNP rsIDs" version of SIFT based on the dbSNP build 132 database in order to identify polymorphisms most likely to affect protein function (20). The SNPexp tool (version 1.2; Norwegian PSC Research Center, Clinic for Specialized Surgery and Medicine, Rikshospitalet, Oslo University Hospital, Oslo, Norway) was used to visualize differences in gene expression by polymorphism using HapMap samples and the GENEVAR (Gene Expression Variation, https://sourceforge.net/projects/genevar/) database, which employs genome-wide expression arrays from EBV-transformed lymphoblastoid cell lines (using National Center for Biotechnology Information's Reference Genome, Build 36). SIFT predictions were made based on the SIFT scores, with a score of >0.05 indicating a tolerated amino acid substitution and a score of ≤0.05 indicating a damaging substitution (genome range, 0–4.32) (20). A TRANScription FACtor (TRANSFAC) database match analysis was conducted for promoter variants that remained statistically significant in the final model (21).

RESULTS

A total of 404 participants were enrolled. Missing information and failed genotyping calls reduced the sample size to 188 African-American participants matched to 188 European-American participants ($n = 376$). A significantly higher proportion of European Americans reported ever having had an outdoor job, current tanning bed use, and (among women) OC use (Table 1). BMI, systolic blood pressure, melanin index, and proportion of West-African genetic ancestry were significantly higher among African Americans. The timing of blood draw was significantly closer to summer solstice among African Americans (86.16 days), compared with European Americans (96.20 days). Among both men and women, circulating 25(OH)D_3, 24,25$(OH)_2D_3$, and proportion 24,25$(OH)_2D_3$ were significantly lower among African Americans than among European Americans. Among women, 25(OH)D_2 was significantly lower among African Americans. No participants reported statin or hormone replacement use (data not shown).

Tests for Hardy-Weinberg equilibrium indicated that genotype frequencies were within expectation for all polymorphisms except the rs4588 polymorphism among African Americans ($P = 0.019$, Table 2). The absolute value of Lewontin's D' statistic was close or equal to 1.0 for polymorphisms in the *CYP2R1* promoter (rs2060793 and rs10741657), *CYP24A1* promoter (rs2762943 and rs111622401), *CYP24A1* coding region and promoter (rs6022990 and rs2762943, respectively), and the *CYP3A7* coding region and *CYP3A5* promoter (rs2257401 and rs776746, respectively), although r^2 values were not above 0.80 for *CYP24A1* SNPs (Web Tables 1 and 2, available at https://academic.oup.com/aje).

In multiple regression models, plasma 25(OH)D_3 concentrations were significantly associated with polymorphisms in *CYP3A43* rs680055, *CYP2R1* rs2060793, and Gc-isoform, as well as tanning bed use, sex, age, proportion of West-African genetic ancestry, OC use, vitamin D diet and supplement intake, days from summer solstice at blood draw, and BMI class (Table 3). As expected due to the high pairwise D' value, results for *CYP2R1* rs10741657 with respect to plasma 25(OH)D_3 concentrations were similar when substituted for *CYP2R1* rs2060793 (GA vs. GG genotype ($P = 0.041$)), data not shown.

Proportion 24,25$(OH)_2D_3$ was significantly associated with *CYP3A43* rs680055 and vitamin D receptor (*VDR*) rs2228570, age, OC use, vitamin D diet and supplement intake,

Table 1. Study Sample Characteristics According to Self-Reported Race/Ethnicity Among Healthy African-American and European-American Adults, Pennsylvania, 2006–2009

Characteristic	African-American ($n = 188$)		European-American ($n = 188$)		*P* Value
	%	Mean (SD)	%	Mean (SD)	
Female sex	60.11		61.17		0.833
Mean age, years		21.93 (4.41)		22.30 (4.50)	0.419
Self-reported race/ethnicity					
African-American/African	96.81		9.57		<0.001
American Indian/Alaska Native	16.49		13.30		0.385
Asian	5.32		6.91		0.519
European	22.34		97.34		<0.001
Vitamin D and calcium sources					
Vitamin D/fish oil supplements	13.30		11.70		0.641
Dietary vitamin D intake, IU/day		208.92 (195.42)		269.98 (190.42)	0.003
Total diet + supplement, IU/day[a]		306.39 (247.13)		380.68 (273.78)	0.007
Calcium supplements	11.43		13.89		0.488
Time spent outdoors, hours/week		10.45 (10.28)		11.16 (8.76)	0.467
Outdoor job	28.19		50.27		<0.001
Tanning bed use	3.72		23.40		<0.001
Tanning bed use, hours/week		0.67 (0.29)		1.13 (1.09)	0.084
Oral contraceptive use (women only)	14.16		48.70		<0.001
Other hormonal contraceptive use (women only)	5.31		2.61		0.299
Corticosteroid use	3.19		1.60		0.313
Body mass index[b]		26.59 (6.87)		24.40 (4.23)	0.001
Current smoker	10.11		10.64		0.866
Pack-years of smoking		1.63 (1.74)		2.40 (2.28)	0.246
Systolic blood pressure, mm Hg		115.54 (11.45)		113.21 (11.60)	0.051
Diastolic blood pressure, mm Hg		75.08 (9.03)		73.89 (7.87)	0.176
Melanin index units		55.87 (15.47)		29.51 (5.78)	<0.001
West-African genetic ancestry proportion	71.79		9.42		<0.001
Blood draw past summer solstice, days		86.16 (35.82)		96.20 (24.06)	0.002
25(OH)D_3, nmol/L		48.27 (29.91)		92.66 (52.72)	<0.001
Men		45.63 (28.18)		66.00 (25.21)	<0.001
Women		50.01 (31.00)		109.58 (58.43)	<0.001
25(OH)D_2, nmol/L		3.07 (4.43)		3.82 (4.87)	0.131
Men		3.87 (4.97)		3.54 (4.60)	0.687
Women		2.55 (3.98)		3.99 (5.05)	0.021
24,25$(OH)_2D_3$, nmol/L		5.17 (5.45)		12.60 (9.83)	<0.001
Men		4.80 (4.84)		7.53 (3.51)	0.001
Women		5.40 (5.83)		15.81 (11.13)	<0.001
Proportion 24,25$(OH)_2D_3$		8.82 (3.50)		11.39 (3.72)	<0.001
Men		8.77 (3.38)		10.15 (2.74)	0.008
Women		8.85 (3.58)		12.18 (4.04)	<0.001
Serum total calcium, mg/dL		10.21 (0.81)		10.16 (1.26)	0.640
Men		10.40 (0.77)		10.39 (1.19)	0.973
Women		10.09 (0.81)		10.02 (1.29)	0.602

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; IU, international units; SD, standard deviation.
[a] Includes intake from vitamin D supplements and multivitamins
[b] Body mass index calculated as weight (kg)/height $(m)^2$.

Table 2. Nonsummer Unadjusted Mean Plasma 25-hydroxyvitamin D_3 (nmol/L) and Percent 24,25-dihydroxyvitamin D_3 According to Genotype and Self-Reported Race/Ethnicity Among Healthy African-American and European-American Adults, Pennsylvania, 2006–2009

Variable	25(OH)D_3					Proportion 24,25(OH)$_2D_3$				
	African-American		European-American		P Value	African-American		European-American		P Value
	Mean (SD)	No. of Participants	Mean (SD)	No. of Participants		Mean (SD)	No. of Participants	Mean (SD)	No. of Participants	
CYP3A43 (dbSNP ID rs680055)										
CC (Ala340Ala)	31.63 (17.09)	16	–[a]	–[a]	0.160	7.37 (3.35)	16	–[a]	–[a]	0.952
CG (Ala340Pro)	49.31 (29.68)	87	85.82 (44.32)	19	0.002	8.84 (3.60)	87	10.57 (2.94)	19	0.053
GG (Pro340Pro)	50.63 (31.37)	84	93.77 (53.86)	167	<0.001	9.07 (3.41)	84	11.50 (3.80)	167	<0.001
Spearman's correlation (*P* value)	0.16 (0.028)		0.06 (0.441)			0.10 (0.178)		0.09 (0.227)		
Hardy-Weinberg *P* value	0.403		0.450			0.403		0.450		
CYP24A1 (dbSNP ID rs2762943)										
GG	48.62 (30.33)	178	92.94 (54.05)	164	<0.001	8.95 (3.52)	178	11.34 (3.87)	164	<0.001
GT	42.00 (21.32)	10	91.10 (45.17)	22	<0.001	6.50 (2.02)	10	11.65 (2.57)	22	<0.001
TT	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]	–[a]
Spearman's correlation (*P* value)	–0.05 (0.507)		0.01 (0.844)			–0.17 (0.015)		0.05 (0.456)		
Hardy-Weinberg *P* value	1.000		0.543			1.000		0.543		
CYP2R1 (dbSNP ID rs2060793)										
AA	36.27 (19.43)	24	83.31 (35.15)	21	<0.001	8.69 (3.51)	24	11.29 (3.36)	21	0.016
AG	51.27 (27.83)	90	94.22 (58.14)	81	<0.001	8.90 (3.00)	90	11.48 (3.44)	81	<0.001
GG	48.74 (34.58)	72	93.74 (51.41)	85	<0.001	8.73 (4.10)	72	11.32 (4.10)	85	0.001
Spearman's correlation (*P* value)	0.01 (0.857)		0.03 (0.690)			–0.04 (0.621)		–0.02 (0.800)		
Hardy-Weinberg *P* value	0.745		0.865			0.745		0.865		
VDR (dbSNP ID rs2228570)										
GG (short/short)	45.60 (27.05)	102	95.82 (55.61)	79	<0.001	8.66 (3.26)	102	11.32 (4.29)	79	<0.0001
GA (short/long)	50.90 (33.61)	71	91.06 (53.11)	83	<0.001	8.60 (2.92)	71	11.45 (3.46)	83	<0.0001
AA (long/long)	53.92 (30.00)	15	88.81 (43.51)	25	0.001	11.02 (6.21)	15	11.36 (2.67)	25	0.843
Spearman's correlation (*P* value)	0.07 (0.312)		–0.06 (0.445)			0.05 (0.482)		0.06 (0.389)		
Hardy-Weinberg *P* value	0.571		0.636			0.571		0.636		

Table continues

Table 2. Continued

Variable	$25(OH)D_3$					Proportion $24,25(OH)_2D_3$				
	African-American		European-American		*P* Value	African-American		European-American		*P* Value
	Mean (SD)	No. of Participants	Mean (SD)	No. of Participants		Mean (SD)	No. of Participants	Mean (SD)	No. of Participants	
Gc-isoform[b]										
Gc-1f/Gc-1f	42.54 (25.74)	83	92.70 (28.64)	8	0.001	8.63 (3.17)	83	12.37 (3.86)	8	0.029
Gc-1f/Gc-2	39.68 (19.68)	29	94.78 (27.11)	11	<0.001	8.66 (2.76)	29	10.99 (2.99)	11	0.038
Gc-2/Gc-2	54.67 (22.85)	6	75.35 (38.96)	20	0.232	9.85 (4.16)	6	12.23 (4.32)	20	0.254
Gc-1f/Gc-1s	54.56 (35.42)	55	93.70 (49.10)	32	<0.001	9.03 (3.23)	55	11.10 (3.64)	32	0.010
Gc-1s/Gc-2[c]	79.87 (37.82)	5	81.35 (37.16)	54	0.937	10.08 (2.61)	5	11.32 (3.45)	54	0.436
Gc-1s/Gc-1s	75.50 (32.02)	8	107.91 (71.51)	60	0.213	9.70 (8.44)	8	11.25 (4.01)	60	0.624
Spearman's correlation (*P* value)	0.24 (<0.001)		0.08 (0.277)			0.02 (0.827)		−0.07 (0.344)		
Hardy-Weinberg *P* values	0.019/0.827		0.068/0.457			0.019/0.827		0.068/0.457		

Abbreviations: $25(OH)D_3$, 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; Ala, alanine; Asp, aspartic acid; CYP, cytochrome P450; dbSNP, database of single nucleotide polymorphisms; Gc, vitamin D binding protein; Glu, glutamic acid ; IU, international units; Lys, lysine; Pro, proline; Thr, threonine; SD, standard deviation; VDR, vitamin D receptor.

[a] Data not presented for cells with 5 or fewer subjects.

[b] Gc-isoform predicted from rs7041 and rs4588 genotype combinations as follows: Gc-1f/Gc-1f = $432^{Asp/Asp}$ (TT)/$436^{Thr/Thr}$ (CC); Gc-1f/Gc-2 = $432^{Asp/Asp}$ (TT)/$436^{Thr/Lys}$ (CA); Gc-2/Gc-2 = $432^{Asp/Asp}$ (TT)/$436^{Lys/Lys}$ (AA); Gc-1f/Gc-1s = $432^{Asp/Glu}$ (TG)/$436^{Thr/Thr}$ (CC); Gc-1s/Gc-2 = $432^{Asp/Glu}$ (TG)/$436^{Thr/Lys}$ (CA); Gc-1s/Gc-1s = $432^{Glu/Glu}$ (GG)/$436^{Thr/Thr}$ (CC). Hardy-Weinberg *P* values are presented in the table for rs4588/rs7041, respectively.

[c] Hypothetical Gc-isoforms for double heterozygote genotype combination (rs4588 = "CA" and rs7041 = "TG") group. The other alternative Gc-isoform possibility for this genotype combination is Gc-1f/undefined.

Table 3. Final Multiple Regression Models of Log Plasma 25-Hydroxyvitamin D_3 and Log Proportion Plasma 24,25-Dihydroxyvitamin D_3 Among Healthy African-American and European-American Adults, Pennsylvania, 2006–2009

Variable	Log 25(OH)D_3			Log Proportion 24,25(OH)$_2$$D_3$		
	Beta Coefficient[a]	*P* Value, Recessive	*P* Value, Additive	Beta Coefficient[a]	*P* Value, Recessive	*P* Value, Additive
CYP3A43 (dbSNP ID rs680055)						
CC (Ala340Ala)	Referent			Referent		
CG (Ala340Pro)	0.21	0.026		0.17	0.043	
GG (Pro340Pro)	0.25	0.011		0.22	0.009	
P value		0.015	0.047		0.018	0.016
CYP2R1 (p-1559) (dbSNP ID rs2060793)						
AA	Referent			–[b]		
AG	0.15	0.038		–[b]		
GG	0.06	0.392		–[b]		
P value		0.109	0.945			
VDR (dbSNP ID rs2228570)						
GG (short/short)	–[b]			Referent		
GA (short/long)	–[b]			0.05	0.135	
AA (long/long)	–[b]			0.17	0.003	
P value					0.025	0.003
Gc-isoform[c]						
Gc-1f/Gc-1f	Referent			Referent		
Gc-1f/Gc-2	0.02	0.975		0.04	0.521	
Gc-2/Gc-2	–0.12	0.261		0.07	0.350	
Gc-1f/Gc-1s	0.20	0.005		0.03	0.592	
Gc-1s/Gc-2[d]	0.08	0.361		–0.01	0.869	
Gc-1s/Gc-1s	0.29	0.001		–0.01	0.841	
Tanning bed use	0.33	<0.001		0.09	0.072	
Female sex	0.13	0.015		0.03	0.412	
Age, years	–0.01	0.007		0.01	0.013	
West-African ancestry proportion	–0.70	<0.001		–0.13	0.078	
Oral contraceptive use (women only)	0.26	<0.001		0.23	<0.001	
Vitamin D diet and supplement intake, per 100 IU/day	0.03	0.002		0.01	0.032	
Days from summer solstice, per 10 days	–0.02	0.001		0.04	<0.001	
Time of day of blood draw, per hour[e]	–[b]			–0.01	0.023	
Body mass index class						
I (underweight)	–0.05	0.703		–0.08	0.464	
II (normal weight)	Referent			Referent		
III	–0.09	0.096		–0.05	0.176	
IV	–0.24	0.005		–0.23	<0.001	
V	–0.15	0.172		–0.29	<0.001	
VI	–0.09	0.506		+0.05	0.582	
P for trend		0.015			0.003	

Table continues

days from summer solstice at blood draw, time of day of blood draw, and BMI class (Table 3). Unlike with plasma 25(OH)D_3, tanning bed use, sex, and West-African genetic ancestry proportion were not significantly associated with proportion 24,25(OH)$_2$$D_3$.

After excluding users of OCs, *CYP2R1* rs10741657 remained associated (Web Table 3) but *CYP2R1* rs2060793 was no longer significantly associated with 25(OH)D_3 (data not shown). Residual versus predicted plots did not suggest

Table 3. Continued

Variable	Log 25(OH)D_3			Log Proportion 24,25$(OH)_2D_3$		
	Beta Coefficient[a]	*P* Value, Recessive	*P* Value, Additive	Beta Coefficient[a]	*P* Value, Recessive	*P* Value, Additive
Adjusted R^2	0.50			0.37		
Durbin-Watson	1.76			1.53		

Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D_3; 24,25$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; Ala, alanine; Asp, aspartic acid; CYP, cytochrome P450; dbSNP, database of single nucleotide polymorphisms; Gc, vitamin D binding protein; Glu, glutamic acid; IU, international units; Lys, lysine; Pro, proline; Thr, threonine; SD, standard deviation; VDR, vitamin D receptor.

[a] Adjusted for age, sex, West-African genetic ancestry proportion, body mass index class, oral contraceptive use, tanning bed use, vitamin D diet and supplement intake, days from summer solstice at time of blood draw, and other variables in table. Proportion 24,25$(OH)_2D_3$ also adjusted for time of day of blood draw.

[b] Not in model.

[c] Gc-isoform predicted from rs7041/rs4588 genotype combinations as follows: Gc-1f/Gc-1f = $432^{Asp/Asp}$ (TT)/$436^{Thr/Thr}$ (CC); Gc-1f/Gc-2 = $432^{Asp/Asp}$ (TT)/$436^{Thr/Lys}$ (CA); Gc-2/Gc-2 = $432^{Asp/Asp}$ (TT)/$436^{Lys/Lys}$ (AA); Gc-1f/Gc-1s = $432^{Asp/Glu}$ (TG)/$436^{Thr/Thr}$ (CC); Gc-1s/Gc-2 = $432^{Asp/Glu}$ (TG)/$436^{Thr/Lys}$ (CA); Gc-1s/Gc-1s = $432^{Glu/Glu}$ (GG)/$436^{Thr/Thr}$ (CC).

[d] Hypothetical Gc-isoforms for double heterozygote genotype combination (rs4588 = "CA" and rs7041 = "TG") group. The alternative combination is Gc-1f/undefined.

[e] Hours 0800–1630.

missing variables, and variance inflation factor and Durbin-Watson test statistics did not indicate high collinearity for any the statistical models.

In models of plasma 25(OH)D_3 stratified on skin melanin index, *CYP3A43* rs680055 and *CYP2R1* rs2060793 remained statistically significant among individuals with a melanin index >40 (*P* value, recessive = 0.030 and 0.015, respectively—Web Table 4). Vitamin D binding protein Gc-1s/Gc-1s remained significantly associated with plasma 25(OH)D_3 among individuals with a melanin index of ≤40 and individuals with melanin index of >40 (*P* = 0.006 and 0.004, respectively).

In SIFT analysis, the *CYP24A1* rs6022990 Met374Thr amino acid change (score = 0, median conservation value = 2.51) and the *CYP3A43* rs680055 Pro340Ala amino acid change (score = 0, median conservation value = 4.15) were classified as damaging. There was low confidence in the prediction for rs680055 due to low sequence diversity (20).

TRANSFAC analysis identified transcription factors Helios A and NF-AT1 to bind on the negative strand ~30 nucleotides upstream of the *CYP2R1* rs2060793 polymorphism. This polymorphism lies within a polycomb-repressed element based on the ENCODE Broad Institute's ChromHMM data set using the GM12878 (chr11:14914224-14915424), NHLF (chr11:14914224-14921224), and HUVEC cell types (chr11:14914424-14917824) analyses (22). In addition, it lies within a transcriptional transition element in the K562 cell type (chr11:14914424-14915824).

Interaction analysis of the final models identified a statistically significant interaction between *CYP2R1* rs2060793 and OC use (*P* = 0.030, Figure 1) but not between *CYP2R1* rs10741657 and OC use (*P* = 0.140, data not shown). There was also a significant interaction between *VDR* rs2228570 and *CYP3A43* rs680055 in relation to proportion 24,25$(OH)_2D_3$ (Figure 2, *P* = 0.013). Web Tables 5 and 6 present adjusted least squares means and 95% confidence intervals for Figures 1 and 2, respectively.

DISCUSSION

CYP2R1

Genetic variability in *CYP2R1* has been associated with serum 25(OH)D concentrations in Northern European and European-American populations (3, 23). The rs2060793 promoter (p-1559)

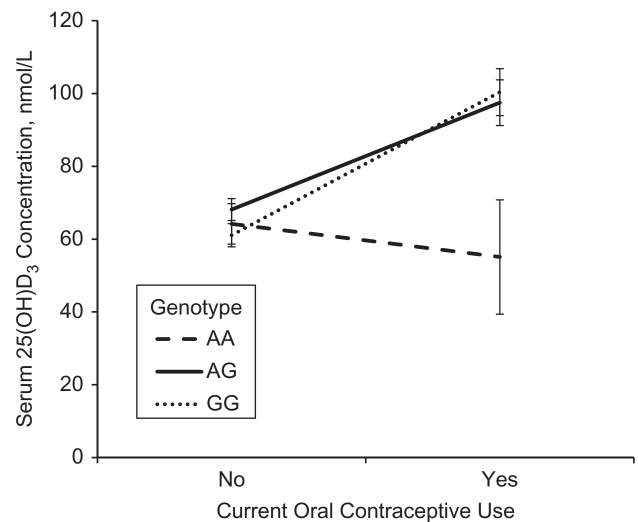

Figure 1. Adjusted least-squares mean plasma 25-hydroxyvitamin D (25(OH)D_3) according to *CYP2R1* (rs2060793) genotype and oral contraceptive use among healthy, age-matched African-American (*n* = 188) and European-American (*n* = 188) adults, Pennsylvania, 2006–2009. Mean plasma 25(OH)D_3 values adjusted for age, sex, proportion of West-African genetic ancestry, body mass index class, tanning bed use, vitamin D diet and supplement intake, days from summer solstice at time of blood draw, and vitamin D binding protein isoform. *P* for interaction from type III (partial) sum of squares = 0.030.

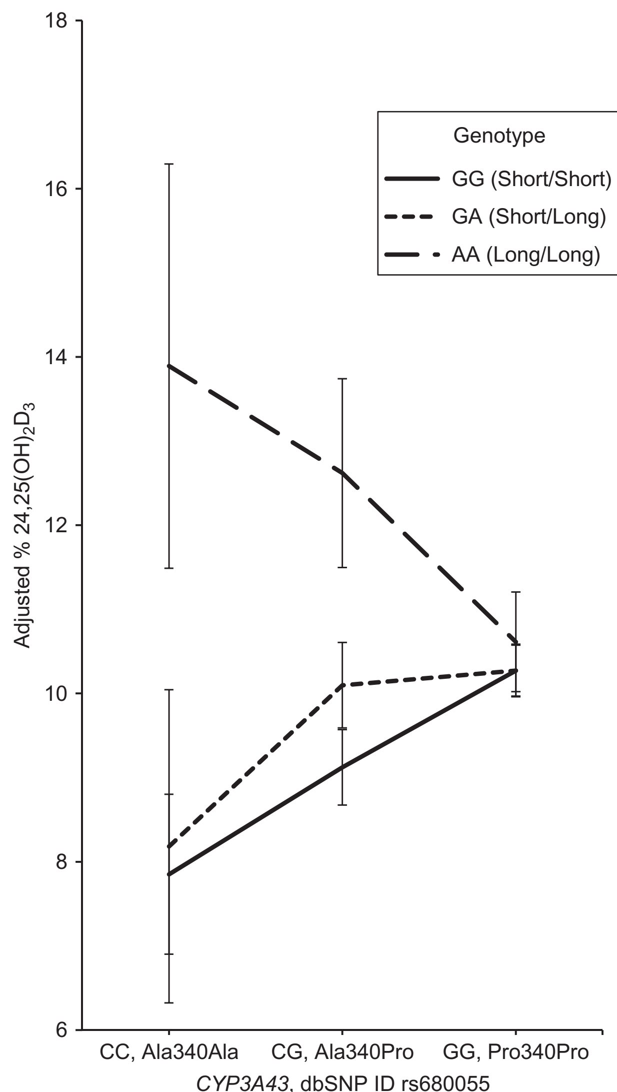

Figure 2. Adjusted least-squares mean proportion 24,25-dihydroxyvitamin D3 (24,25$(OH)_2D_3$) according to *CYP3A43* (rs680055) and vitamin D receptor (*VDR*) (rs2228570) genotypes among healthy, age-matched African-American (n = 188) and European-American (n = 188) adults, Pennsylvania, 2006–2009. Mean proportion 24,25$(OH)_2D_3$ values adjusted for age, sex, proportion of West-African genetic ancestry, body mass index class, oral contraceptive use, tanning bed use, vitamin D diet and supplement intake, days from summer solstice at time of blood draw, time of day of blood draw, and Gc-isoform. P for interaction from type III (partial) sum of squares = 0.013. Ala, alanine; dbSNP, database of single nucleotide polymorphisms; Gc, vitamin D binding protein; Pro, proline.

polymorphism has been associated with circulating 25(OH)D in 2 genome-wide association studies of non-Hispanic white individuals living in the United States and reproduced among healthy Hispanic breast-cancer controls (3, 4, 24), but not all reported a significant association (25). One meta-analysis found significant heterogeneity among white cohorts (3).

In silico analysis supports the possibility of a functional role for the rs2060793 region of *CYP2R1*, where Helios and ATF1 transcription factors are predicted to bind. While no vitamin D response elements are known to occur in the *CYP2R1* promoter, in other genomic regions, noncanonical vitamin D response elements have been shown to overlap with the ATF1-binding site where VDR competes with ATF1 to alter T-cell response (26). Helios expression, restricted to T-lymphocytes, has been described as a key transcription factor stabilizing T-regulatory cells during inflammation (27, 28), particularly in FoxP3+ CD4 T-regulatory cells. Helios expression is influenced by 1,25 $(OH)_2D_3$ and estrogen (29).

To date, there is limited evidence of a positive correlation between *FOXP3* expression in the peripheral regulatory T-cell population and seasonal circulating 25(OH)D concentrations among healthy individuals (30). In patients with moderate to severe asthma, a significant positive correlation between the frequency of circulating FoxP3+ T-regulatory cells and serum 25(OH)D ($r = 0.70$) has been reported (31). Taken together, these results suggest an interplay between T-lymphocyte regulation and circulating vitamin D metabolite concentrations. The consistent association between rs2060793 and circulating 25(OH)D among different racial/ethnic groups, and the possible interaction with estrogen, suggests the need for further molecular characterization of this genetic region.

CYP3A43

The CYP3A family of enzymes catalyzes the hydroxylation of steroids—including testosterone, progesterone, and cortisol—and metabolizes antidepressants, immunosuppressive agents, macrolide antibiotics, calcium channel blockers, and other toxins (32). The human *CYP3A* gene cluster, located on the long arm of chromosome 7, has been evolutionarily implicated in selective environmental pressures in non-African populations (33, 34). Differences in genotype frequency within CYP3A family gene loci between African-American and European-American population groups have been previously described (35), with between-individual differences in gene and protein expression as high as 40-fold, and the relative balance of *CYP3A4/CYP3A5* gene expression substantially different by racial/ethnic group (36).

Clinically, genetic variability in *CYP3A4* and *CYP3A5* has been associated with racial/ethnic differences in response to blood pressure and cholesterol-lowering drugs (37, 38). Discovered in 2000, much less is known regarding the CYP3A43 enzyme, with protein expression in human liver microsomes at levels comparable to CYP3A5 and CYP3A7 (approximately 4 pmol/mg) and approximately 15-fold lower than CYP3A4 (39). Splice variants of CYP3A43 protein add spliced exon sequences of either the *CYP3A4* or *CYP3A5* genes, and have been identified in human hepatocytes with a 250-fold range (0.02%–5.0%) of expression, yet little is known regarding their role in human metabolism (40). While *CYP3A43* tissue expression is greatest in the prostate (11), expression is higher in the human brain relative to liver, kidney, lung, and heart tissue—and reported to be as high as 170-fold greater than *CYP3A4* in various brain tissues, also differing by racial/ethnic group (41).

Although less frequent among both African-American and European-American populations, the *CYP3A43* Ala340Ala genotype occurs among individuals of sub-Saharan African ancestry more frequently than any other population group (42).

The pyrrolidine ring of Pro allows fewer protein conformational possibilities relative to other amino acids, and the Ala-to-Pro amino acid substitution, in particular, may alter confirmation and stability of the CYP3A43 protein (43, 44). In our study, 1 or 2 copies of the Pro340 allele resulted in approximately 21% and 25% increases in circulating 25(OH)D_3 concentration, respectively, compared with Ala340 homozygotes. By comparison, administration of the *CYP3A4*-inducing drug rifampicin resulted in a 3% suppression of 25(OH)D_3 concentration in healthy volunteers (9). Vitamin D response elements have been identified in the promoter region of *CYP3A43* (45). It is not known whether the CYP3A43 enzyme has direct vitamin D metabolic activity.

VDR and CYP3A43 interaction

Both the *VDR* rs2228570 and *CYP3A43* rs680055 polymorphisms have been independently associated with cancer risk. *CYP3A43* Ala340 has been consistently associated with an increased risk of prostate cancer in multiethnic studies (46, 47). Meta-analyses of *VDR* rs2228570 have identified associations with multiple cancer types (48), including breast and ovarian cancer (49), but not consistent associations for prostate cancer (50). To our knowledge, interactions with cancer risk have not been identified between *CYP3A43* and *VDR*. *CYP3A4* rs2740574 was not associated with 25(OH)D_3 or proportion 24,25$(OH)_2D_3$, although this polymorphism appears to be consistently associated with prostate cancer risk (51).

Vitamin D binding protein (Gc-isoform)

A meta-analysis has identified associations between SNPs in the *GC* gene and circulating 25(OH)D concentrations (3). It is possible that variability in circulating concentrations of 25(OH)D (i.e., both 25(OH)D_2 and 25(OH)D_3) is a function of circulating Gc concentrations (i.e., because more 25(OH)D remains bound in the circulating compartment, with less diffusion of the unbound fraction of 25(OH)D into tissues). However, the relationship between circulating vitamin D metabolites and circulating Gc concentrations is not straightforward. Gc circulates in a concentration at roughly 1,000-fold higher than 25(OH)D_3, with less than 5% of circulating Gc bound to vitamin D sterols (52). In addition, differential binding affinities according to Gc-isoform might play a role, although the existing literature is limited and not all binding affinity studies agree (53). Further understanding regarding the distribution of vitamin D metabolites in human blood and adipose tissue are needed to determine whether Gc-isoform may be associated with the bioavailable fraction of vitamin D metabolites.

Oral contraceptives

Estrogen has long been recognized to be a potent regulator of vitamin D metabolism (54). Observational and clinical trials have identified higher circulating 25(OH)D concentrations among OC users, with postadministration increases ranging from 5% to 30% (55–57). Postmenopausal estrogen use is also associated with increased circulating 25(OH)D (58). Estrogen is known to influence the expression of hundreds of genes in a cell-dependent manner, including genes involved in apoptosis, cell adhesion, cell cycle, enzymes, growth factors, protein processing, and signal transduction. With respect to vitamin D metabolism, there are three observed associations following estrogen use: 1) higher circulating Gc protein concentration (59); 2) increased intestinal calcium absorption (60); and 3) decreased urinary calcium excretion (61). In our study, OC use was positively associated both with circulating concentrations of 25(OH)D_3 and with the circulating proportion 24,25$(OH)_2D_3$, suggesting that estrogen not only influences the absolute concentration but also the relative balance of circulating vitamin D metabolites. The clinical significance of these observations is not known. Approximately 28% of women of reproductive age in the United States report OC use (62).

Statistical adjustment and/or design control for exogenous estrogen use is not routinely conducted in epidemiologic studies of vitamin D. We observed a magnitude of association between OC use and plasma 25(OH)D_3 that was similar to the magnitude of association with tanning bed use. Methodologically, control for exogenous estrogens as a possible source of confounding might reduce variability and improve *P* value estimates in genetic studies.

Limitations

Because this study included healthy young participants, these results may not be generalizable to other populations, including pregnant, chronically ill, or older individuals. Due to small cell sizes, interaction results could be the result of chance. While energy-adjusted vitamin D intakes have been more strongly correlated with 25(OH)D concentrations than the nonadjusted values used in this study (58), the *P* values for genetic variables in this study were improved after adjustment for total vitamin D intake (data not shown). Finally, because the results for diet and supplement intake are from a cross-sectional study and not a study of individuals over time, the increase in plasma 25(OH)D_3 per 100 IU/day should not be extrapolated to expected changes for a single individual over time at a given dose.

Strengths

This study was designed to assess differences in vitamin D metabolites and was conducted in nonsummer months in order to reduce seasonal variability. Our genetic ancestry estimation method is highly concordant with other published methods (12). Our abbreviated method of assessment of total mean intake of vitamin D was significantly associated with plasma 25 (OH)D_3 concentrations. Average intakes among participants in this study were above the US average of 232–264 IU/day for women and men, aged 18–30 years in the United States, but below the recommended daily intake of 600 IU/day (63). Our study accounted for approximately half of the variability in circulating 25(OH)D_3, with a higher R^2 value than other published studies (range, 0.13–0.40 (22, 64)). Finally, mass spectrometry was used to separately assess vitamin D_2 and D_3 metabolite concentrations, allowing the study to reduce variability due to differences in side-chain metabolism (65).

Conclusions

Genetic polymorphisms in *CYP3A43*, *VDR*, and *GC* may play a role in circulating concentrations of multiple vitamin D metabolites, independent from genetic ancestry. In spite of several statistically significant findings by our research and other studies, at least half of the variability of circulating vitamin D metabolite concentrations remains unexplained. Methodologically, gene × gene, and gene × environment interactions should be more fully investigated and accounted for in future studies of circulating vitamin D metabolite concentrations.

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