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Association of vitamin D binding protein (VDBP) polymorphisms and serum 25(OH)D concentrations in a sample of young Canadian adults of different ancestry☆,☆☆

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ABSTRACT

Variants of the vitamin D binding protein (*VDBP*) gene appear to be associated with levels of the main circulating vitamin D metabolite, 25-hydroxyvitamin D [(25(OH)D]. We examined the associations between the common variants of the *VDBP* (*GC*) gene and concentrations of 25(OH)D in a sample of young Canadian adults of East Asian, European and South Asian ancestry, taking into account the effect of vitamin D intake, skin pigmentation, sex, BMI, sun exposure and season. Three hundred and fifty-one (351) healthy young adults were genotyped for two non-synonymous single nucleotide polymorphisms (SNPs), T436K (rs4588) and D432E (rs7041), using a method that ascertains the GC diplotypes of each individual. After controlling for relevant predictor variables in multiple regression models, the number of GC-2 (436 K) alleles was found to be associated with lower 25(OH)D concentrations in the East Asian sample at fall and winter visits. The number of GC-2 alleles also showed a significant negative association with fall 25(OH)D concentration in the European sample. No associations were noted between the number of GC-2 alleles and 25(OH)D in the South Asian sample at either season. Vitamin D intake was also significantly predictive of serum 25(OHD) concentrations, and similarly to what was observed for the GC polymorphisms, the relative strength of the association was influenced by ancestry and season.

1. Introduction

Low levels of vitamin D have been linked to bone mineralization defects, as well as common chronic, autoimmune and infectious diseases, including cancer, cardiovascular disease, diabetes, multiple sclerosis, rheumatoid arthritis and tuberculosis [1–9]. Serum 25-hydroxyvitamin D [25(OH)D] is considered the primary biomarker of vitamin D status. The production of this metabolite in the liver is not tightly regulated, and 25(OH)D has a long half-life (2 or 3 weeks). Thus, serum levels of 25(OH)D serve as a measure of vitamin D coming from all available sources, including diet, sun exposure and supplements [8]. Once in the circulation, vitamin D and its metabolites are bound to vitamin D binding protein (VDBP), also known as group-specific component (or GC), which is the main protein involved in vitamin D transport [10]. VDBP binds with particularly high affinity to 25-hydroxyvitamin D_3 [25(OH)D], leaving less than 1% of circulating 25(OH)D free [11,12]. In contrast to 25(OH)D, which has a half-life of several weeks, VDBP has a short half-life (2.5–3 days) [13,14], suggesting that the protein and its ligand are independently regulated.

The *GC* gene (*VDBP*, NCBI Gene ID#2638) is located on chromosome 4 (4q11-13) and encodes a single chain polypeptide of 474 amino acid residues that consists of 3 domains and is part of the albumin family [15]. Cleavage of a 16-residue signal peptide yields a protein of 458 amino acids that is secreted by the liver in much larger molar amounts than required for release of 25(OH)D. The size difference between the nascent peptide and the secreted protein accounts for the discordance between current standardized molecular numbering of genetic variants and historical amino acid numbering of peptide polymorphisms. There are two well-studied non-synonymous single nucleotide polymorphisms (SNPs) in the *GC* gene, p.T436K (formerly T420K, NCBI rs# rs4588), which results in a C-to-A transversion [threonine (ACG) to a lysine (AAG)], and p.D432E (formerly D416E, NCBI rs# rs7041), which results in a

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T-to-G transversion [an aspartic acid (GAT) to a glutamic acid (GAG)] [16,17]. The combination of these two polymorphisms gives rise to three major electrophoretic serum variants (or isoforms) that are distinguished by their amino acid composition and glycosylation patterns: GC-1f, GC-1s and GC-2 [18]. GC-1f is the wild-type allele and is a combination of aspartic acid at position 432 and threonine at 436; GC-1s has a combination of glutamic acid at position 432 and threonine at 436, and GC-2 has a combination of aspartic acid at position 432 and lysine at 436. The two variants at positions 432 and 436 are in strong linkage disequilibrium (LD), and the combination of glutamic acid at position 432 and lysine at 436 is very rarely observed in human populations. Thus, the three common electrophoretic variants (GC-1f, GC-1s and GC-2) combine to form six diplotypes: 1f-1f, 1f-1s, 1s-1s, 1s-2, 1f-2, and 2-2 [18]. It is important to mention that while some of the previous studies of the *GC* gene focused on the three major GC isoforms resulting from the combination of the haplotypes of the rs7041 and rs4588 SNPs (GC-1f, GC-1s and GC-2), other studies only focused on the comparison between GC-1 (which includes both the GC-1f and GC-1s isoforms) and GC-2. This is equivalent to studying only the rs4588 SNP: GC-1 is characterized by the presence of threonine at position 436 of the protein and GC-2 by the presence of lysine in this site.

Previous research indicates that there are differences in binding affinity to 25(OH)D among the GC variants, with GC-1f having the highest affinity for 25(OH)D, GC-2 having the lowest affinity and GC-1s having intermediate affinity [16,19–21]. However, the biologic importance of the binding affinity is uncertain, since there is a huge excess of binding sites in relation to physiologic 25(OH)D concentrations. There is also evidence indicating that there are differences in glycosylation between the GC isoforms. In particular, the isoforms GC-1f and GC-1s show trissacharidic glycosylation at position 436, but the trissacharide is absent on the GC-2 isoform [16,22,23]. From the glycosylation differences between the alleles, it has been suggested that the GC-2 allele would be metabolized faster. This was supported by Kawakami et al. who injected radioactively labelled VDBP into healthy young men and observed that the metabolic rate was higher in GC2-2 individuals than in GC1-1 individuals [16,24]. The more rapid metabolism of the GC-2 variant was further supported by studies showing that, while the concentration of VDBP in plasma ranges between 200 and 500 mg/L, GC1-1 individuals have higher concentrations of VDBP than GC1-2 individuals, and the lowest concentrations are found in GC2-2 individuals [16].

GC polymorphisms show substantial allele frequency variation in groups of different ancestry. Kamboh and Ferrell reported a geographical cline in GC-1f and GC-1s allele frequency [25], with the wild-type GC-1f allele found at higher frequencies in African and East Asian populations compared to their European, Middle Eastern and South Asian counterparts [20,25]. Conversely, the GC-1s variant is found in the highest frequencies among the European, Middle Eastern and South Asian populations and has the lowest frequencies in African populations, particularly those of equatorial, west and south Africa [25]. The GC-2 variant is found at relatively lower allele frequencies throughout the studied populations in comparison to the GC-1f and GC-1s alleles [10].

Several studies have reported association between *GC* genotypes and serum 25(OH)D concentrations [13,20,26]. The goal of this study was to examine the association between the common genetic variation of the VDBP protein and serum 25(OH)D concentrations in a sample of young Canadian adults of diverse ancestry and to assess the relative contributions of these markers and other factors relevant to vitamin D status, such as vitamin D intake, skin pigmentation, and UV exposure. We have focused our analysis on the p.T436K (rs4588) polymorphism, which is responsible for the differences in amino acid sequence and glycosylation patterns between GC-1 and GC-2 and has been the focus of many of the previous studies, and also on the GC diplotypes resulting from the combination of the p.D432E (rs7041) and p.T436K (rs4588) haplotypes.

2. Materials and methods

2.1. Participants

Participants for this study were recruited from the Greater Toronto Area. Recruitment took place at the University of Toronto Mississauga (Ontario, Canada) campus (43°N). Detailed description of the study population and data collection are described elsewhere [27]. Briefly, each participant met with the researchers twice during the study, once in the early fall (September to middle of October) and again in the winter (January until end of February). The study was conducted over two years (year 1: fall 2007/winter 2008, year 2: fall 2008/winter 2009). At the first visit, participants provided informed consent and completed a questionnaire that assessed their ancestry. During each visit, the participant was measured for weight and height and skin pigmentation, completed a food frequency questionnaire (FFQ) to assess dietary intake of vitamin D and calcium, completed a UVR exposure questionnaire and provided a blood sample. This study was approved by the University of Toronto Health Sciences Research Ethics Board, and all participants provided written informed consent.

The exclusion criteria for the present study were: age (<18 and >35 years old); diagnosis of kidney/liver disease or other active or chronic diseases potentially affecting vitamin D metabolism or absorption (e.g., inflammatory bowel disease, cystic fibrosis etc.) [28]; use of medications that affect vitamin D metabolism (including corticosteroids and anticonvulsants, etc.) [8,29]. Use of vitamin D supplements was not a criterion for exclusion, since we were interested in evaluating the use of vitamin D supplements and the effect of supplementation on 25(OH)D levels.

2.2. 25(OH)D measurement

An aliquot of whole blood was centrifuged and the serum fraction decanted and stored at −80 °C. Serum 25-hydroxyvitamin D [25(OH)D] was measured by liquid-chromatography tandem mass spectrometry (LC–MS/MS). A detailed summary of the method used is found elsewhere [27].

2.3. Anthropometric measurements

Study staff measured weight and height for each participant, and body mass index (BMI = kg/m^2) was calculated.

2.4. Ancestry

The participants completed a personal questionnaire that assessed ancestry. The questionnaire asked detailed questions regarding the places of birth, ethnicity, native language, migration history, and present residence of each participant, and we also included questions about parental and grandparental places of birth, ethnicity and native language. For data analysis, participants were grouped into broad geographic regions: East Asia, Europe, Middle East, Africa and South Asia. For example, individuals who stated that their ancestors were from China, Japan, and Korea, were grouped as East Asian, while those who reported ancestors from India and Pakistan were grouped as South Asian. Individuals who reported being of multiple ancestries were placed into a subgroup designated as “Other”.

2.5. Dietary assessment

Daily intake of vitamin D from dietary and supplemental sources was estimated using a food frequency questionnaire (FFQ). We have previously validated this FFQ for assessment of vitamin D and calcium intake in young adults of diverse ancestry [30]. Subjects were provided with portion size aids and recorded their food, beverage and supplement intake. The FFQs were analyzed with the computer program Food Processor (version 8.0 and its revisions, ESHA Research Inc., Salem OR, which included the 2007 Canadian Nutrient File from Health Canada); Canadian foods were always chosen where Canadian fortification was different from elsewhere (e.g., margarine and breakfast cereals).

2.6. DNA collection and genetic analysis

A sample of each participant's blood was collected in a 4-mL EDTA tube. Collected blood was stored at −20 °C until DNA extraction took place. Genomic DNA extraction was carried out using the E.Z.N.A. Blood DNA Midi Kit (Omega Bio-Tek, Georgia, United States).

The p.D432E and p.T436K SNPs were genotyped with phase assignment based on allele-specific amplification of the p.T436K site followed by restriction endonuclease digestion of the p.D432E site. Allele-specific amplification was carried out in a 20 μL reaction mixture containing 1X PCR buffer (Qiagen), 0.2 mM each of dNTPs, 50 ng genomic DNA, 0.5 U HotStarTaq™ (Qiagen) and 0.3-1 μM of the following primers: 5′-GGCATGTTTCACTTTCTGATCTC-3′ (forward), 5′-ACCAGCTTTGCCAGTACCG-3′ (wild-type reverse) and 5′-GCAAAGTCTGAGTGCTTGTTATGCAGCTTTGCCAGTTGCT-3′ (mutant reverse). The underlined bases in the primer sequences are mismatched nucleotides introduced to avoid cross priming. After the initial DNA denaturation and HotStarTaq™ activation at 95 °C for 15 min, the amplification went through 35 cycles of denaturation at 94 °C for 20 s, annealing at 58 °C for 20 s and extension at 72 °C for 20 s with an increment of 1 s after each subsequent cycle and a final extension at 72 °C for 5 min. Eight microliters of the amplified products were run in a 2% NuSieve gel containing ethidium bromide and then visualized using UV illumination. The p.T436K wild-type allele produced a 246 bp band and the mutant allele a 270 bp band. Another 8 μL of amplicon was digested with *Hae*III (New England BioLabs) at 37 °C overnight and the digested products were analyzed by repeat electrophoresis in 2% NuSieve gel. The DNA bands containing the wild-type p.D432E allele remained unchanged while those with p.D432E mutant allele were cut, producing 221 bp fragments.

2.7. Sun/UVR exposure

Each participant completed a UVR exposure questionnaire that assessed sun exposure habits, use of sunscreen and extent of body exposure. For the present study we used the variable of "average time spent outside between 9 a.m. and 5 p.m. during the summer months" as an estimate of summertime sun exposure, dichotomized to be either less than or greater than 60 min per day.

2.8. Skin pigmentation

Constitutive skin pigmentation (pigmentation in unexposed areas of the skin) was measured in the inner upper arm using a narrow-band reflectometer (Dermaspectrometer, Cortex Technology, Hadsund, Denmark) [31]. Melanin Index values calculated using the Dermaspectrometer typically range from the low 20 s to almost 100, with individuals with the lightest skin pigmentation having the lowest values and those with the darkest pigmentation having the highest [31].

2.9. Statistical analyses

Deviations from Hardy–Weinberg proportions were evaluated using a chi-square test for each SNP, by ancestry group, with a cutoff of $p < 0.05$ [32]. Multiple regression was used to test the association of the GC variants on with serum 25(OH)D concentrations in fall and the winter in two separate analyses per ancestry group. In the first analysis, we assessed the association between 25(OH)D and the number of GC-2 (436 K) alleles. In the second analysis, we incorporated the six diplotypes resulting from the combination of the T436K and D432E haplotypes into the regression analysis. In addition to the relevant genetic markers, other variables were also added to the multiple regression model because of their known effect on 25(OH)D concentrations (sex, BMI, total vitamin D intake, UV exposure and skin pigmentation).

Because we had information for the same participants for both the fall and winter, we conducted a separate regression analysis for each season to test if associations differed by season. In the Multiple Regression, the dependent variable 25(OH)D had a significantly skewed distribution and was log transformed before the analysis. In the fall regression models, log serum 25(OH)D was the dependent variable while BMI, sex, skin pigmentation, reported sun exposure, vitamin D intake, study year and number of GC-2 alleles/diplotype were independent variables. For the winter visit, the dependent variable was log winter serum 25(OH)D concentration, while the independent variables were BMI, sex, skin pigmentation, reported vitamin D intake, study year and number of GC-2 alleles/diplotype. The goal of the regression was the calculation of the partial r^2 statistic for each predictor variable, where the statistic reflects the amount of variation captured by each predictor in the model, adjusted for the effect of the other predictors.

Outliers and influential points were assessed using normality tests (Shapiro–Wilk and Anderson–Darling) and Cook's distance, respectively. When outliers and/or influential points were identified, the test was run with and without outliers, and the results compared. Subsequently, all results are presented with outliers and influential points removed. All statistical tests were performed with SPSS (Version 15.0, SPSS Inc., 2006) and Minitab (Version 15.1.1, Minitab Inc., 2007).

Power analysis was conducted using the software Quanto (Version 1.2.4). Using a sample size of 100 individuals per group and an additive model of inheritance, we estimated that there is more than 80% power to detect a marginal proportion of the variance in 25(OH)D levels explained by genetic effects of 0.08 or higher in each of the three population groups (East Asian, European and South Asian).

3. Results

3.1. Sample characteristics, 25(OH)D concentrations and associated variables

Genetic data and corresponding serum 25(OH)D measurements were available for 351 subjects (225 females, 126 males) in the fall and 322 (202 females, 120 males) in the winter. The difference between fall and winter participant numbers was due to study drop out and/or failure to attend the second visit. Participants were grouped into broadly defined ancestral groups based on the geographic origin of the subjects, their parents and grandparents. The majority of the participants were of African ($N = 12$), East Asian ($N = 104$), European ($N = 110$), Middle Eastern ($N = 16$) or South Asian ($N = 95$) ancestry. However, because of the small sample size, the African and Middle Eastern ancestry groups were not included in further analyses.

Table 1 summarizes the relevant characteristics for the three population groups (East Asian, European and South Asian) and also

Table 1
Description of the raw variables collected in the total sample, and stratified by ancestral subgroup.[a,b]

Fall	East Asian	European	South Asian	Total Sample
	(*N* = 104)	(*N* = 111)	(*N* = 95)	(*N* = 351)
Sex (female, male)	74, 30	73, 38	61, 34	225, 126
Age (yrs)	20.4 ± 2.31	22.1 ± 3.97	20.7 ± 1.89	21.1 ± 2.99
BMI (kg/m^2)	22.3 ± 3.69	23.6 ± 4.97	23.3 ± 3.74	23.2 ± 4.22
Skin pigmentation (melanin index)	32.4 ± 2.75[b]	30.4 ± 2.91[c]	39.2 ± 6.00[a]	34.6 ± 7.30
25(OH)D (nmol/L)	48.2 ± 15.3[b]	76.9 ± 24.5[a]	37.5 ± 15.1[c]	54.4 ± 24.7
Total vitamin D intake (μg/day)	6.33 ± 5.55	8.45 ± 7.78	7.78 ± 6.35	7.60 ± 7.45
Winter	(*N* = 92)	(*N* = 97)	(*N* = 94)	(*N* = 322)
Sex (female, male)	65, 27	61, 36	60, 34	202, 120
Age (yrs)	20.7 ± 2.28	22.6 ± 4.08	21.0 ± 1.89	21.4 ± 3.00
BMI (kg/m^2)	22.6 ± 3.88	23.8 ± 5.09	23.6 ± 3.93	23.5 ± 4.38
Skin pigmentation (melanin index)	30.9 ± 2.94[*,b]	28.1 ± 2.63[*,c]	37.8 ± 5.50[a]	33.1 ± 7.35
25(OH)D (nmol/L)	33.3 ± 15.4[*,b]	52.4 ± 19.7[*,a]	29.4 ± 14.7[*,c]	38.4 ± 19.4
Total vitamin D intake (μg/day)	5.63 ± 4.88	7.48 ± 7.53	7.60 ± 7.33[*]	7.25 ± 7.95

[a] Means ± SD in a row with different superscripts differ, $p < 0.005$ (ANOVA controlling for study year and sex).
[b] Total sample includes participants of East Asian, European, and South Asian ancestry, as well as individuals of other ancestry (see text).
[*] Differs from subgroup mean for fall, $p < 0.05$ (repeated measures).

for the total sample. There were differences between the three groups in skin pigmentation and 25(OH)D concentrations in both seasons (ANOVA controlling for study year and sex, see Table 1). There were also significant within-group seasonal differences in serum 25(OH)D concentrations, with higher levels observed in fall than in winter (Repeated Measures ANOVA controlling for study year and sex, see Table 1).

Significant sex differences were found for: BMI, 25(OH)D and vitamin D intake. Significant study year differences were found for: 25(OH)D, skin pigmentation and vitamin D intake (one way ANOVAs, data not shown). To control for these effects, study year and sex were subsequently included as predictors in the multiple regression analyses.

3.2. Vitamin D binding protein (VDBP) polymorphisms, ancestry and 25(OH)D levels

Table 2 shows the allele and genotype frequencies for the polymorphism T436K (rs4588), which is responsible for the differences between the GC 1 (threonine) and 2 (lysine) proteins. The table also reports the frequencies of the three haplotypes defined by the combination of the D432E and T436K alleles: GC-1f (aspartic acid and threonine), GC-1s (glutamic acid and threonine) and GC-2 (aspartic acid and lysine), as well as the resulting diplotypes. No significant deviations from Hardy Weinberg proportions ($p < 0.05$) were observed in either of the two polymorphisms (results not shown). Table 3 shows the mean 25(OH)D concentrations, stratified according to T436K genotype and GC diplotype, for each population during the fall and winter visits.

Tables 4 and 5 report the contribution of each of the independent variables to 25(OH)D levels during the fall and the winter, measured as the partial correlation coefficient (and *p*-value). The square of this statistic (the partial r^2) is important, in that it reflects the amount of variation captured by each variable in the model, while controlling for the effect of all other variables. For each ancestry group, two regression analyses were conducted per season to test for the association with the GC polymorphisms: one with the T436K polymorphism (e.g. number of GC-2 alleles, Table 4) and another with the actual GC diplotypes (Table 5).

Table 2
Proportion of individuals with specific genotypes for the two vitamin D binding protein SNPs and the GC diplotypes for the full sample, and stratified by ancestry.

Genetic polymorphism		East Asian		European		South Asian	
		N	%	*N*	%	*N*	%
D432E (rs7041)							
Alleles	G		32		59		58
	T		68		41		42
Genotypes	GG	12	12	39	35	31	33
	TG	43	41	52	47	48	50
	TT	49	47	20	18	16	17
T436K (rs4588)							
Alleles	C (GC-1)		73		72		74
	A (GC-2)		27		28		26
Genotypes	CC (GC1-1)	54	52	57	52	52	55
	CA (GC1-2)	43	41	46	41	36	38
	AA (GC2-2)	7	7	8	7	7	7
GC Diplotypes (rs7041 + rs4588)							
Alleles	1f (T + C)		44		19		24
	1s (G + C)		30		55		52
	2 (T + A)		26		26		24
Diplotypes	1f-1f	20	19	9	8	10	11
	1f-1s	25	24	15	4	19	20
	1f-2	26	25	9	8	7	7
	1s-1s	11	11	37	33	27	28
	1s-2	14	14	34	31	25	26
	2-2	7	7	7	6	7	7

Table 3
Serum 25(OH)D concentrations ± SD, stratified by T436K genotype (e.g. number of copies of allele 2) and GC diplotypes.

Genetic polymorphism	East Asian		European		South Asian	
	Fall	Winter	Fall	Winter	Fall	Winter
T436K						
rs4588						
CC (GC 1-1)	54.4 ± 15.7	31.3 ± 17.1	81.4 ± 26.1	52.5 ± 20.4	36.8 ± 16.5	30.4 ± 16.0
AC (GC 1-2)	42.5 ± 12.0	29.5 ± 12.5	73.4 ± 22.6	52.2 ± 19.7	37.8 ± 12.8	29.1 ± 13.8
AA (GC 2-2)	34.2 ± 6.50	24.4 ± 7.39	59.6 ± 20.6	53.5 ± 16.6	41.5 ± 15.8	23.4 ± 7.67
GC diplotypes						
rs7041 + rs4588						
GC 1f-1f	55.5 ± 18.2	40.8 ± 18.9	81.4 ± 40.5	47.6 ± 19.9	33.2 ± 17.3	30.1 ± 22.6
GC 1f-1s	49.6 ± 12.7	32.2 ± 11.0	87.3 ± 32.1	59.2 ± 31.3	36.0 ± 16.6	32.0 ± 18.2
GC 1f-2	43.4 ± 12.7	26.6 ± 12.0	74.5 ± 22.7	48.6 ± 11.1	37.0 ± 15.0	28.6 ± 10.5
GC 1s-1s	63.8 ± 14.7	44.8 ± 23.9	79.1 ± 22.8	50.6 ± 15.5	37.8 ± 16.9	29.5 ± 14.7
GC 1s-2	41.2 ± 11.2	33.4 ± 12.4	73.2 ± 22.9	53.3 ± 21.6	38.0 ± 12.5	29.2 ± 14.7
GC 2-2	34.2 ± 6.53	24.4 ± 7.39	59.6 ± 10.6	53.5 ± 16.6	41.5 ± 15.8	23.4 ± 7.67

Table 4
Relative contribution (partial correlations, *p* values) for predictors in regression models for 25(OH)D concentrations, including the T436K polymorphism.

Variables	Ancestry											
	East Asians				Europeans				South Asians			
	Fall		Winter		Fall		Winter		Fall		Winter	
	Partial	*p*	Partial	*p*	Partial	*p*	Partial	*p*	Partial	*p*	Partial	*p*
Study year (1 or 2)	−0.115	0.263	0.045	0.683	−0.167	0.094	−0.007	0.950	−0.186	0.082	−0.018	0.871
Sex (female is reference category)	0.196	0.056	0.094	0.397	−0.022	0.829	−0.170	0.108	0.248	0.020	0.161	0.134
BMI (kg/m^2)	0.106	0.305	−0.055	0.619	−0.212	0.032	−0.076	0.476	−0.125	0.246	−0.281	0.008
Skin pigmentation (melanin index)	0.204	0.046	0.194	0.078	0.061	0.542	−0.165	0.117	0.009	0.932	0.085	0.429
Total vitamin D intake (IU/day)	0.382	<0.001	0.585	<0.001	0.179	0.072	0.268	0.010	0.398	<0.001	0.690	<0.001
UVR exposure (⟨60⟩ min/day)	0.091	0.378	n/a	n/a	0.096	0.335	n/a	n/a	0.070	0.518	n/a	n/a
T436K (Allele 2)	−0.463	<0.001	−0.384	<0.001	−0.256	0.009	−0.066	0.535	0.126	0.240	−0.081	0.450

3.2.1. Effect of the GC polymorphisms on 25(OH)D levels

3.2.1.1. East Asians. The GC-2 allele (436 K allele) was significantly associated with lower serum 25(OH)D concentrations in the East Asian subgroup in both the fall ($p < 0.001$) and in the winter (<0.001) (Table 4). When controlling for other variables in the regression, the T436K polymorphism was able to account for approximately 21% and 15% of the variation in 25(OH)D in the fall and winter, respectively.

When the diplotypes resulting from the combination of the D432E and T436K haplotypes were inserted into a separate regression analysis (Table 5), there were significant negative associations (in agreement with the previous analysis) between 25(OH)D levels and GC1f-2 ($p = 0.014$), GC1s-2 ($p = 0.021$) and GC2-2 ($p = 0.003$) for the fall and with GC1f-2 ($p = 0.005$) and GC2-2 ($p = 0.009$) for the winter (Table 5). When controlling for the effect of the other variables, the individual diplotypes explained 7 to 10% of the overall variation in 25(OH)D.

3.2.1.2. Europeans. The GC-2 allele (436 K allele) was significantly associated with lower serum 25(OH)D concentrations in the European sample in the fall only ($p = 0.009$) (Table 4). The T436K polymorphism accounted for approximately 7% of the variation in fall 25(OH)D concentrations in Europeans, after controlling for the effects of the remaining variables. No significant associations were observed between T436K and 25(OH)D concentrations in the winter.

None of the six individual diplotypes showed a significant association with 25(OH)D concentrations in either the fall or winter in the European sample (see Table 5), although there was a trend

Table 5
Relative contribution (partial correlations, *p* values) for predictors in regression models for 25(OH)D concentrations, including individual *GC* diplotypes.

VARIABLES	Ancestry											
	East Asians				Europeans				South Asians			
	Fall		Winter		Fall		Winter		Fall		Winter	
	Partial	*p*	Partial	*p*	Partial	*p*	Partial	*p*	Partial	*p*	Partial	*p*
Study year (1 or 2)	−0.147	0.162	0.045	0.692	−0.164	0.107	−0.004	0.973	−0.192	0.080	−0.017	0.876
Sex (female is reference category)	0.187	0.075	0.074	0.517	−0.031	0.762	−0.171	0.113	0.231	0.035	0.153	0.165
BMI (kg/m^2)	0.069	0.514	−0.058	0.612	−0.207	0.041	−0.080	0.459	−0.124	0.259	−0.269	0.013
Skin pigmentation (melanin index)	0.209	0.045	0.188	0.096	0.068	0.508	−0.163	0.133	0.002	0.987	0.083	0.453
Total vitamin D intake (IU/day)	0.367	<0.001	0.558	<0.001	0.183	0.071	0.267	0.012	0.396	<0.001	0.682	<0.001
UVR exposure (⟨60⟩ min/day)	0.063	0.549	n/a	n/a	0.108	0.289	n/a	n/a	0.069	0.532	n/a	n/a
GC diplotypes (1F-1F is reference category)												
1F-1S	−0.035	0.744	−0.102	0.373	0.039	0.700	0.021	0.847	0.042	0.707	0.038	0.733
1F-2	−0.256	0.014	−0.311	0.005	0.007	0.942	0.008	0.940	0.070	0.526	0.016	0.882
1S-1S	0.156	0.139	0.018	0.873	0.051	0.619	0.037	0.733	0.054	0.624	0.041	0.709
1S-2	−0.240	0.021	−0.191	0.092	−0.018	0.858	0.016	0.880	0.069	0.530	0.030	0.786
2-2	−0.303	0.003	−0.291	0.009	−0.100	0.328	−0.010	0.925	0.103	0.349	−0.008	0.939

toward lower 25(OH)D concentrations for participants with the GC 2-2 diplotype.

3.2.1.3. South Asians. Neither the T436K polymorphism (Table 4), nor any of the individual diplotypes (Table 5) had any significant effect on 25(OH)D concentrations in the South Asian subgroup at either season.

3.2.2. Effect of other variables on 25(OH)D concentrations

Of all the other variables included in the regression models, total vitamin D intake had the strongest effect on serum 25(OH)D concentrations. Higher intakes of vitamin D were associated with higher 25(OH)D levels, both in the fall and the winter. This effect was evident in all the population groups (Tables 4 and 5). When controlling for the other variables tested, total vitamin D intake alone was able to explain 3%, 15% and 16% of the variation in fall 25(OH)D concentrations in the European, East Asian and South Asian samples, respectively. In the winter, total vitamin D intake alone was able to account for 7%, 34% and 48% of the 25(OH)D concentrations in the European, East Asian, and South Asians subgroups, respectively (Table 4).

4. Discussion

We characterized the GC T436K (rs4588) and D432E (rs7041) polymorphisms in a sample of young Canadian adults of East Asian, European and South Asian ancestry. No significant deviations from Hardy–Weinberg proportions were observed in any population group, suggesting that there is no strong genetic stratification in any of the samples, which would be reflected in an excess of homozygotes (Wahlund effect). We found that the *GC* polymorphisms were significant predictors of 25(OH)D concentrations (Tables 4 and 5). However, the strength of the associations was dependent on ancestry and season. We observed that the GC-2 (436 K) allele was significantly associated with lower serum 25(OH)D concentrations in both East Asians and Europeans at the fall visit, and in East Asians at the winter visit (Table 4). We also observed significant associations between some of the GC diplotypes, which are determined by the combination of the D432E and T436K haplotypes, and 25(OH)D levels. In particular, the GC diplotypes that contain the GC-2 allele (IF-2, IS-2, 2-2) showed lower 25(OH)D concentrations in the fall in the East Asian sample and the diplotypes 1F-2 and 2-2 were also significantly associated with low 25(OH)D concentrations in the wintertime (diplotype 1S-2 approached significance, $p = 0.092$) in the East Asian sample (Table 5). It is important to mention that when considering the diplotypes, the increased number of dependent variables may have affected our ability to identify significant associations with 25(OH)D concentrations. In contrast to the results described for the East Asian and European samples, we did not observe any significant associations between the GC-2 allele or any of the GC diplotypes and 25(OH)D concentrations in the South Asian sample (see Tables 4 and 5). Interestingly, the relative contribution of the *GC* genetic markers to 25(OH)D concentrations appears to differ not only by ancestry but also by season. The partial correlation (and resultant partial r^2) values corresponding to the genetic markers are substantially higher in the fall than in the winter (Tables 4 and 5) pointing to the presence of gene–environment interaction. The main difference between the two seasonal measurements is that, during Canadian summer and fall seasons, there is significant endogenous vitamin D synthesis, but not in winter. This is reflected in substantially higher 25(OH)D concentrations in the fall (see Table 1), a well-known finding in high-latitude countries. Thus, our data indicate that the genetic effects of the *GC* polymorphisms are easier to identify during the fall, when there is endogenous synthesis of vitamin D in the skin and higher circulating levels of 25(OH)D. It is important to note that in a recent study of older Canadian women, Sinotte et al. observed that the relationship between the *GC* SNPs and 25(OH)D concentrations was more obvious when the concentrations of 25(OH)D are higher, particularly in the months when cutaneous vitamin D synthesis is likely significant [13]. Given these findings, one explanation for the failure to observe significant effects in our South Asian sample is that 25(OH)D concentrations were very low in this sample in both seasons (Table 1), possibly due to the more limited cutaneous synthesis of vitamin D in South Asians during the fall as a result of their higher melanin levels [27]. In summary, the seasonal and ancestral heterogeneity observed in our dataset may be due to the fact that our ability to identify genetic effects is influenced by circulating serum 25(OH)D concentrations, which are themselves determined by factors such as UV exposure or skin pigmentation. However, we cannot discard other alternative explanations for the differences observed between ancestral groups, such as population heterogeneity in genetic effects. Further studies evaluating seasonal variation of 25(OH)D levels in much larger samples will be necessary to clarify these issues.

Significant associations between *GC* polymorphisms and serum 25(OH)D concentrations have been observed by others. In a cross-sectional study of postmenopausal women, Lauridsen et al. reported that the highest 25(OH)D concentrations were observed in subjects with the GC1-1 phenotype and the lowest in subjects with the GC2-2 phenotype, while individuals with the GC1-2 phenotype had intermediate concentrations [26]. Kurylowicz et al. observed that a greater proportion of Graves disease patients that were either heterozygous (TK) or homozygous (KK) at T436K (rs4588) (corresponding to 1 or 2 copies of the GC-2 allele, respectively) had 25(OH)D concentrations below 50 nmol/L compared to homozygotes for the wildtype variant (TT) (no copies of GC-2) [33]. Engelman et al. reported that the GC-2 allele was associated with lower levels of 25(OH)D in their study of Hispanics and African Americans, but concentrations were not specified [20]. In a sample of postmenopausal women in Germany, Abbas et al. observed that mean 25(OH)D concentrations were lowest in women with the GC2-2 diplotype and the highest concentrations were observed in women with the GC1f-1s diplotype [17]. In the Rotterdam study ($N = 1317$) a significant association was observed between GC haplotypes and 25(OH)D concentrations: GC-1S was associated with a 7 nmol/L increase in 25(OH)D concentrations and GC-2 was associated with a 16 nmol/L decrease in 25(OH)D [34]. In a small sample of healthy subjects from Toronto, Fu et al. also observed that KK homozygotes (GC1-1) at T436K had lower mean 25(OH)D concentrations (46 nmol/L) than TK heterozygotes (GC1-2, 65 nmol/L) and TT homozygotes (GC1-1, 66 nmol/L) [35]. Finally, two recent genome wide studies in European populations have found significant associations at the genome-wide level between 25(OH)D concentrations and polymorphisms in the *GC* gene [36,37]. Interestingly, in both GWA studies, the lead SNPs include either rs4588 [$p = 4.1 \times 10^{-22}$, 36] or markers in almost complete linkage disequilibrium with rs4588 in European populations (rs2282679, $p = 1.9 \times 10^{-109}$, r^2 with rs4588 = 0.983, 37], strongly suggesting that this non-synonymous polymorphism (or other SNPs nearby) may be the causal variant.

When considering all the potential factors that may affect serum 25(OH)D concentrations, the most important factor appears to be dietary intake. Higher vitamin D intakes were positively associated with higher 25(OH)D levels in all groups (Tables 4 and 5). Interestingly, the effect of vitamin D intake also seems to depend on factors such as season and ancestry. The proportion of the variation in 25(OH)D levels explained by vitamin D intake is higher in winter than fall for all groups. This is not surprising because in Canada there is not enough UV exposure to endogenously synthesize sufficient vitamin D during the winter, and vitamin D intake (through diet and supplements) becomes the only source in winter.

Also, the proportion of the variation in 25(OH)D levels explained by vitamin D intake is substantially higher in South Asians and East Asians than in Europeans. Again, this provides evidence that some factors affecting 25(OH)D concentrations show heterogeneity between population groups. It is of note that vitamin D intake did not show a significant association with 25(OH)D in the European sample in the fall (Tables 4 and 5), suggesting that endogenous synthesis was perhaps the main source of vitamin D in this group during the fall. It is important to indicate that some of the factors included in the analysis, such as BMI or skin pigmentation, show limited variation within each population group, and our study is not adequately powered to detect significant effects for these factors. In the case of skin pigmentation, a previous study of ours showed that melanin levels have a significant negative association with 25(OH)D concentrations in the total sample [27]. This does not contradict our present findings, because the amount of skin pigmentation variation in the total sample is considerably higher than in each of the population groups, thereby enhancing our ability to identify significant effects.

A limitation of this study is the relatively small sample size for each ancestral group, thereby resulting in reduced statistical power to identify small effects. However, our study is adequately powered to detect a marginal proportion of the variance in 25(OH)D levels explained by genetic effects of 0.08 or higher.

Our research has several strengths in comparison with previous studies. Our sample included subjects from three different population groups, East Asian, European and South Asian. We were able to measure many of the most important factors that affect vitamin D levels, including skin pigmentation, vitamin D intake and UV exposure, and this makes it possible to explore the relative effects of the different predictors on 25(OH)D concentrations. Another strength of our study is that we were able to measure 25(OH)D concentrations sequentially from late summer/early fall to winter, thereby capturing the intra-individual seasonal fluctuations that we expect in 25(OH)D concentrations at our latitude.

In conclusion, we report that *GC* polymorphisms are significantly associated with 25(OH)D concentrations in a sample of young Canadian adults of diverse ancestry. In particular, individuals carrying more 1 or 2 copies of the 436 K (GC-2) allele appear on average to have lower 25(OH)D concentrations. Our study, along with others, including several recent genome-wide association studies, indicate that genetic markers within the *GC* gene influence vitamin D status, and are therefore relevant to the study of vitamin D associated diseases. However, our study also indicates that vitamin D status is influenced by a complex set of factors that appear to show heterogeneity between population groups. This is reflected by the different strength of associations of vitamin D intake and GC polymorphisms with 25(OH)D depending on season or ancestral group.

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