**Research Article**

**Associations between Vitamin D–Binding Protein Isotypes, Circulating 25(OH)D Levels, and Vitamin D Metabolite Uptake in Colon Cancer Cells**

Elizabeth A. Hibler1,2, Elizabeth T. Jacobs1,2, Angelika Dampf Stone3, Christine L. Sardo2, Michael A. Galligan3, and Peter W. Jurutka3

*Abstract*

Vitamin D metabolites have been extensively studied as cancer chemopreventive agents. Gc-globulin (GC) isotypes, based on rs7041 and rs4588 diplootypes, have varying affinities for 1α,25-dihydroxyvitamin D (1,25(OH)2D) and 25-hydroxyvitamin D (25(OH)D), which may affect circulating metabolite concentration as well as delivery at the cellular level. We evaluated associations between GC isotype and circulating vitamin D metabolite concentrations in 403 ursodeoxycholic acid (UDCA) clinical trial participants. Metabolite uptake was evaluated in human colon cancer (HCT-116) cells treated with ethanol vehicle, 1,25(OH)2D, or 25(OH)D, and with plasma from individuals with known GC isotype. Mammalian-2-hybrid and vitamin D–responsive element-based luciferase assays were used to measure the vitamin D receptor pathway activation as a marker for metabolite uptake. Regression analysis demonstrated significantly lower serum 25(OH)D concentration for clinical trial participants with 1F_2, 1S_2, or 2_2 isotypes (P < 0.01) compared with 1S_1S. Consistent with these in vivo observations, cellular data revealed that 25(OH)D uptake varied less by GC isotype only at the higher concentration tested (P = 0.05), while 1,25(OH)2D uptake differed markedly by GC isotype across concentration and assay (P < 0.01). The 1F_1S and 1F_2 isotypes produced the greatest reporter gene induction with 1,25(OH)2D treatment and, while activation varied less with 25(OH)D, the 2_2 isotype demonstrated increased induction at the lower concentration. These results suggest that vitamin D metabolite concentration and delivery to colon cells may vary not only by GC isotype, but also that certain isotypes may more effectively deliver 1,25(OH)2D versus 25(OH)D. Overall, these results may help identify populations at risk for cancer and potential recipients of targeted chemoprevention.

*Research Article*

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**Introduction**

Studies demonstrate that individuals with lower circulating vitamin D metabolite concentrations are at increased risk of several diseases, including cancer (1–4). The prohormone is endogenously synthesized in skin exposed to UVB or ingested through the diet and/or supplement intake (5–8). Gc-globulin (GC), also known as the vitamin D–binding protein, is the primary transport protein for vitamin D metabolites in circulation (9, 10). GC, a serum α2-globulin made of 458 amino acids (51.2 kDa) and part of the albumin superfamily of binding proteins, is primarily synthesized by parenchymal cells in the liver (8, 11). The protein transports 80 to 90% of vitamin D metabolites and has the greatest affinity for 25-hydroxyvitamin D (25(OH)D; ref. 10). However, it is unknown how genetic variation in GC affecting the 1α,25-dihydroxyvitamin D (1,25(OH)2D) or 25(OH)D concentration available in circulation relates to uptake at the cellular level.

There are two commonly studied GC polymorphisms (rs4588 and rs7041) and, as shown in Table 1, the amino acid changes at positions 416 and 420 give rise to the three common phenotypic alleles of 1F, 1S, and 2 (12, 13). There are six combinations of these phenotypic alleles, defined by diplotype of rs4588 and rs7041, which represent the GC protein isotypes, including 1F_1F, 1F_1S, 1F_2, 1S_1S, 1S_2, and 2_2. These GC isotypes demonstrate differences in affinity for vitamin D and vary dramatically in frequency by race-ethnicity (10, 14). White individuals have a lower frequency of the 1F compared with 1S (10, 11, 15), whereas the 2 allele is the least common in all populations, but has a higher frequency in White populations (10). There is also evidence that both GC genotype and GC isotype, as defined by diplotype, are associated with variation in circulating vitamin D metabolite concentrations.
Circulating GC concentration is approximately 1,000-fold higher than that of vitamin D metabolites with only an estimated 0.2% to 0.6% of vitamin D metabolites unbound in the serum (10, 11). GC binding is believed to protect metabolites from catabolism and excretion, which increases the half-life of molecules (10, 16). Our previous work demonstrated strong associations between circulating 25(OH)D and the GC gene overall as well as seven individual polymorphisms, including rs7041, rs222035, rs842999, rs1155563, rs12512631, rs16846876, and rs1746825 (17). Additional epidemiologic studies demonstrate that 25(OH)D levels vary by GC and that higher concentrations are observed for those with 1F_1F or 1S_1S versus 2_2 isotypes (12, 18). Furthermore, studies have identified consistent associations between circulating 25(OH)D levels and colorectal neoplasia risk (2, 19–21). Therefore, variation in the affinity of GC for vitamin D metabolites alters circulating concentrations as well as potential concentrations that reach the cellular level, independent of circulating concentrations of the binding protein.

We hypothesize that GC isotypes may affect not only circulating vitamin D metabolite concentrations, but also delivery at the cellular level. Chun and colleagues demonstrated that availability of 25(OH)D in cells differed by GC isotype, as measured by 24-hydroxylase expression in monocytes (22). However, this relationship had not previously been tested with 1,25(OH)2D treatment or in colon cells. The current study expanded upon previous work to evaluate associations between circulating vitamin D metabolite concentration and GC isotypes at the population level, as well as to establish a novel experimental screening system to determine whether GC isotype influenced vitamin D metabolite uptake in colorectal carcinoma cells, with measurable biologic endpoints relevant to tumorigenesis. The overall goal of this translational research is to identify factors that may influence colorectal neoplasia risk to identify individuals at risk for cancer or potential recipients for targeted chemoprevention.

Materials and Methods

Epidemiologic analysis

Study population. The epidemiologic analysis included participants from the ursodeoxycholic acid (UDCA) clinical trial conducted at the Arizona Cancer Center (Tucson, AZ), which has been previously described (23–25). Briefly, the UDCA trial was a phase III randomized, double-blind, placebo-controlled trial conducted to test the effect of UDCA on recurrence of colorectal neoplasia (23). The study recruited Arizona residents between 40 to 80 years of age with a history of removal of one or more colorectal adenomas (>3 mm in diameter) during a colonoscopy before study enrollment (23). There were 1,192 participants in the overall sample with complete genotype data; however, the sample was further restricted (N = 403) to individuals who reported White race with complete vitamin D metabolite and genotype measurements (23, 26). Restriction was necessary because there were not enough individuals of varied race/ethnicity to account for population stratification. The University of Arizona Human Subjects Protection Program approved the UDCA trial and informed consent was obtained for all the subjects before enrollment.

Genotyping and vitamin D metabolite measurement.

Genotyping of participants has been described previously and two GC polymorphisms (rs7041 and rs4588) were chosen a priori and included as part of the original Illumina Golden Gate platform (Illumina; refs. 27, 28). Circulating vitamin D metabolite concentrations were measured at the Bruce Hollis Lab (University of South Carolina, Columbia, SC; refs. 29, 30). This laboratory utilized multiple quality assurance measures, as described previously, with demonstrated coefficient of variations less than 7.0% for 25(OH)D and 11.0% for 1,25(OH)2D (31, 32).

Statistical analysis. Linear regression models were utilized to evaluate associations between GC isotype and circulating 25(OH)D concentration. Common factors related to circulating 25(OH)D concentration, including age, body mass index (BMI), and gender, were assessed for confounding and none were statistically significant. Each GC group was compared with the 1S_1S isotype as a standard. Because of a low count of individuals with the 1F_1F isotype (N = 9), as expected in a White population, this group was combined with the 1S_1S group. Biologically, these groups differ only by the rs7041 polymorphism, and combining these groups increased power without significantly altering the results.

Cell-based assays

Cell culture, transfection, and dosing. HCT-116 colorectal carcinoma cells were purchased from the American Tissue Culture Collection and grown at 37°C in a humidified 5% CO2 incubator. Cells were seeded in 24-well plates at a density of 100,000 cells/well and grown overnight in Dulbecco’s Modified Eagle Medium supplemented with 5% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The cells were transfected using Express-In Transfection Reagent supplied by Thermo Scientific and plasmids appropriate to the vitamin D receptor-retinoid X receptor (VDR-RXR) Mammalian-two-hybrid (M2H) bait.

Table 1. Changes in nucleotides and amino acids by GC isotype

<table>
<thead>
<tr>
<th>Phenotypic allele</th>
<th>Diplootype from rs7041/rs4588</th>
<th>Amino acid (position 416/420)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F_1F</td>
<td>TT/CC</td>
<td>Asp/Thr</td>
</tr>
<tr>
<td>1S_1S</td>
<td>GG/CC</td>
<td>Glu/Thr</td>
</tr>
<tr>
<td>2_2</td>
<td>TT/AA</td>
<td>Asp/Lys</td>
</tr>
</tbody>
</table>

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The VDRE-based assay included transfection of cells with 20 ng human CYP3A4 gene promoter region linked to luciferase. The Renilla gene is included for measurement and control of transfection efficiency. Each well received 2 μL Express-In, along with 25 ng pCMV-BD-hRXRα (bait) and 25 ng pCMV-AD-hVDR (prey), plus 200 ng pFR-Luc (reporter gene) and 20 ng pRL-NUL (Renilla reniformis) for the M2H system. The VDRE-based assay included transfection of cells with 20 ng pRL-NUL (Renilla reniformis), 50 ng pSG5-hVDR (vitamin D receptor), and 250 ng PER6-Luc (VDRE from the human CYP3A4 gene promoter region linked to luciferase).

Cells were incubated for 24 hours at 37°C and 5% CO₂ then dosed with ethanol vehicle or different concentrations of 1,25(OH)₂D or 25(OH)D plus 2.5% plasma from individuals with known GC isotype. Plasma samples from six UDCA trial participants, each representing one GC isotype group, were utilized to provide GC for uniform addition to cell culture experiments. We tested the ethanol vehicle plus participant plasma alone with no vitamin D metabolites added, and observed minimal activation of the luciferase systems compared with cells treated with 25(OH)D or 1,25(OH)₂D (Fig. 1A and B). From this, we concluded that differences in the circulating vitamin D metabolite concentrations or GC concentration in the plasma did not significantly affect the activation of the luciferase systems. Furthermore, utilization of 2.5% plasma in each well was also tested compared with 5% plasma, which is traditionally used in cell culture experiments, and no appreciable difference in cell growth or uptake of vitamin D metabolites was detected (data not shown). The lower percent plasma was chosen to conserve the limited plasma available from selected participants. Finally, we took into consideration that circulating GC concentration is approximately 1,000-fold higher than vitamin D metabolites in vivo. Therefore, using a lower concentration of plasma was still expected to provide sufficient GC concentration in the experimental model for the majority of vitamin D metabolites to be bound to GC, similarly to in vivo, and was not expected to affect delivery at the cellular level.

Experiments were conducted to determine the best concentrations of 1,25(OH)₂D and 25(OH)D to be used for the current work. We began by testing concentrations utilized in previous experiments (data not shown; ref. 34), and chose final concentrations that demonstrated a consistent dose response in the M2H system (Fig. 1A), as well as activation of the VDRE system (Fig. 1B), with 2.5% serum. For both M2H and VDRE assays with 1,25(OH)₂D, 1 × 10⁻⁸ and 5 × 10⁻¹⁰ mol/L were included (final concentration). To account for variation in the uptake of 25(OH)D, higher concentrations of 5 × 10⁻⁷ and 5 × 10⁻⁸ mol/L were tested. HCT-116 cells have demonstrated activity of CYP27B1, which is required for intracellular conversion of 25(OH)D to 1,25(OH)₂D (34). Plates were then again incubated for 24 hours, cells lysed, and luciferase assays (Promega) were performed to measure luciferase activity as an index of uptake of vitamin D metabolites.

**Statistical analysis.** For cellular experiments, luciferase expression was normalized to Renilla levels by dividing luciferase by Renilla values and then multiplying by 10,000. The mean and SD of at least replicate experiments were calculated (N = 7 for M2H and N = 4 for VDRE), with triplicate samples in each treatment group within each independent experiment. As described above, the 1S_1S isotype was used as the standard and percent 1S_1S expression was calculated. ANOVA regression was performed to test for an overall pattern of statistically significant differences between the GC isotypes then, if the overall P value was...
Our analysis demonstrated statistically significantly lower concentration and GC isotype compared with 1S_1S (P<0.001). At 1×10⁻⁶ mol/L of 1,25(OH)₂D (Fig. 2A), the 1F_1S, 1F_2, and 1S_2 isotypes demonstrated increased activation relative to 1S_1S. However, at 5×10⁻¹⁰ mol/L 1,25(OH)₂D (Fig. 2B), overall induction of luciferase decreased compared with 1S_1S, but the majority of isotypes followed a similar pattern as the higher concentration. At both concentrations, the reporter gene induction with the 1F_2 isotype was statistically significantly greater than the 1S_1S isotype (P<0.05) using the Tukey method for pairwise comparisons. We were unable to detect a statistically significant difference in activation of the M2H luciferase system with 25(OH)D treatment. However, all isotypes demonstrated higher activation compared with 1S_1S at 5×10⁻⁷ mol/L 25(OH)D (Fig. 2C), whereas only the 1F_1F and 2_2 isotypes activated luciferase to a greater extent than 1S_1S at the lower concentration of 5×10⁻⁸ mol/L 25(OH)D (Fig. 2D).

Results

Baseline characteristics: UDCA study participants

The baseline characteristics of the UDCA clinical trial participants (N = 1192), as well as the subset used for the current analysis (N = 403), are presented in Table 2 and have been previously described in detail (17, 26). The subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%). This subset of UDCA trial participants had a mean age of 66.1 ± 8.5 years and 65.5% were male. Mean BMI was 28.4 ± 4.8 kg/m², whereas total calcium intake was within the range of the recommended daily allowance (1,034.5 ± 116.8 g/day), and reported supplement use was high (77.9%).

Epidemiologic analysis: associations between GC isotype and circulating vitamin D metabolites

The results for associations between circulating 25(OH)D concentration and GC isotype are presented in Table 3. Our analysis demonstrated statistically significantly lower circulating 25(OH)D with the isotypes, including the 2 phenotypic allele compared with 1S_1S (P<0.001). Circulating 25(OH)D concentrations decreased with additional copies of the 2 allele, with the 2_2 isotype demonstrating a mean concentration 6.8 ng/mL [95% confidence interval (CI), −10.23−3.39] less than the 1S_1S isotype reference group, which had an observed mean of 28.4 ng/mL (95% CI, 26.8–30.0). The 1S_2 and 1F_2 isotypes also differed with respect to any of the selected characteristics from the UDCA study as a whole.

Cellular-based assays: GC isotype and uptake of vitamin D metabolites

The in vitro experiments using HCT-116 cells demonstrated statistically significant differences in 1,25(OH)₂D and 25(OH)D uptake by GC isotype, as illustrated in Figs. 2 and 3. The percent transactivation of the luciferase reporter gene in HCT-116 cells by GC isotype, using 1S_1S set to 100 as a reference, was significantly different overall using ANOVA analysis in the M2H system (Fig. 2), at both concentrations of 1,25(OH)₂D tested (P<0.01). At 1×10⁻⁶ mol/L of 1,25(OH)₂D (Fig. 2A), the 1F_1S, 1F_2, and 1S_2 isotypes demonstrated increased activation relative to 1S_1S. However, at 5×10⁻¹⁰ mol/L 1,25(OH)₂D (Fig. 2B), overall induction of luciferase decreased compared with 1S_1S, but the majority of isotypes followed a similar pattern as the higher concentration. At both concentrations, the reporter gene induction with the 1F_2 isotype was statistically significantly greater than the 1S_1S isotype (P<0.05) using the Tukey method for pairwise comparisons. We were unable to detect a statistically significant difference in activation of the M2H luciferase system with 25(OH)D treatment. However, all isotypes demonstrated higher activation compared with 1S_1S at 5×10⁻⁷ mol/L 25(OH)D (Fig. 2C), whereas only the 1F_1F and 2_2 isotypes activated luciferase to a greater extent than 1S_1S at the lower concentration of 5×10⁻⁸ mol/L 25(OH)D (Fig. 2D).

Table 2. Baseline characteristics of study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>UDCA N = 1,192</th>
<th>Subset N = 403*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y ± SD</td>
<td>66.2 ± 8.5</td>
<td>66.1 ± 8.5</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
<td>804 (67.5)</td>
<td>294 (65.5)</td>
</tr>
<tr>
<td>Race, White, n (%)</td>
<td>1108 (94.5)</td>
<td>403 (100.0)</td>
</tr>
<tr>
<td>Mean BMI, kg/m² ± SD</td>
<td>28.2 ± 4.8</td>
<td>28.4 ± 4.8</td>
</tr>
<tr>
<td>Aspirin use, n (%)</td>
<td>331 (27.8)</td>
<td>120 (29.8)</td>
</tr>
<tr>
<td>Ever smoker, n (%)</td>
<td>802 (69.3)</td>
<td>271 (68.3)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>140 (11.7)</td>
<td>52 (12.9)</td>
</tr>
<tr>
<td>Total fat, g/d ± SD</td>
<td>62.2 ± 31.7</td>
<td>61.1 ± 30.3</td>
</tr>
<tr>
<td>Energy, kcal/d ± SD</td>
<td>1,987.9 ± 815.6</td>
<td>1,998.1 ± 812.6</td>
</tr>
<tr>
<td>Calcium intake, g/d ± SD</td>
<td>1,008.5 ± 494.4</td>
<td>1,034.5 ± 516.8</td>
</tr>
<tr>
<td>25(OH)D concentration, ng/mL ± SD</td>
<td>N/A</td>
<td>26.6 ± 9.2</td>
</tr>
<tr>
<td>1,25(OH)₂D concentration, pg/mL ± SD</td>
<td>N/A</td>
<td>35.3 ± 9.2</td>
</tr>
<tr>
<td>Supplemental vitamin D use, yes, n (%)</td>
<td>856 (71.8)</td>
<td>314 (77.9)</td>
</tr>
</tbody>
</table>

We also tested uptake of vitamin D metabolites using a VDRE-based luciferase system as shown in Fig. 3. In this VDRE-based system, the percent transactivation of the luciferase reporter gene was significantly different (overall ANOVA \( P < 0.001 \) and \( P < 0.01 \), respectively) with the two concentrations of 1,25(OH)\(_2\)D tested (Fig. 3A and B). Again,

Table 3. Associations between GC isotype and circulating 25(OH)D concentrations

<table>
<thead>
<tr>
<th>Gc isotype</th>
<th>Frequency</th>
<th>Difference in 25(OH)D (ng/mL) from 1S_1S isotype</th>
<th>( p^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1S_1S</td>
<td>120 (29.8)</td>
<td>Ref. ( c )</td>
<td>Ref.</td>
</tr>
<tr>
<td>1F_1F/1F_1S</td>
<td>82 (20.4)</td>
<td>0.30 (−2.23 to −2.82)</td>
<td>0.82</td>
</tr>
<tr>
<td>1S_2</td>
<td>125 (31.0)</td>
<td>−3.03 (−5.29 to −0.78)</td>
<td>0.01</td>
</tr>
<tr>
<td>1F_2</td>
<td>42 (10.4)</td>
<td>−3.76 (−6.92 to −0.60)</td>
<td>0.02</td>
</tr>
<tr>
<td>2_2</td>
<td>34 (8.4)</td>
<td>−6.81 (−10.23 to −3.39)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

\( p_{\text{trend}} = 0.001 \)

\( a N = 403. \) One participant was excluded because the diplotype was not represented in the common categories, as previously described by Abbas et al. (12).

\( b \) Compared with 1S_1S using linear regression among White participants.

\( c \) The reference group mean is 28.42, 95% CI (26.8–30.0).

Figure 2. Assessment of 1,25(OH)\(_2\)D and 25(OH)D uptake in colon cancer cells using a M2H luciferase reporter system. Each panel depicts the percent transactivation (luciferase/Renilla RLU) of the luciferase system compared with 1S_1S isotype as a control (set to 100%) in HCT-116 cells using the M2H system. Seven replicate experiments with 3 wells per group were conducted for the M2H system. Cells dosed with either 1 \( \times 10^{-8} \) mol/L 1,25(OH)\(_2\)D (A) or 5 \( \times 10^{-10} \) mol/L 25(OH)D (B) demonstrate significantly different activation of the M2H system (overall ANOVA \( P < 0.01 \)). The 1F_1S and 1F_2 isotypes led to a statistically significant increased activation compared with 1S_1S in this system (Tukey pairwise *, \( P < 0.05 \)). Cells treated with either (C) 5 \( \times 10^{-7} \) or (D) 5 \( \times 10^{-8} \) mol/L 25(OH)D did not reveal statistically significant variation in activation of the luciferase system (overall ANOVA \( P = 0.24 \) and 0.28, respectively), though the 2_2 isotype demonstrates higher activation at the lower concentration of 25(OH)D (D).
GC isotype, 25(OH)D Levels, and Uptake in Colon Cancer Cells

The results of the present analyses support the hypothesis that GC isotype, as defined by diplotypes of two commonly studied polymorphisms, are associated with circulating 25(OH)D concentration as well as delivery of vitamin D metabolites at the cellular level to colon cells. The epidemiologic analysis identified associations between GC isotypes and clinically relevant differences in circulating 25(OH)D concentration. Furthermore, at the cellular level in human colonocytes, the experiments demonstrated statistically significant differences in activation of two VDR-based luciferase systems, across multiple concentrations of 1,25(OH)2D and a single concentration of 25(OH)D, by GC isotype. Overall, this evidence provides a translational basis for further studies of the vitamin D endocrine system and cancer prevention in diverse and at-risk populations.

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The results of the present analyses support the hypothesis that GC isotype, as defined by diplotypes of two commonly studied polymorphisms, are associated with circulating 25(OH)D concentration as well as delivery of vitamin D metabolites at the cellular level to colon cells. The epidemiologic analysis identified associations between GC isotypes and clinically relevant differences in circulating 25(OH)D concentration. Furthermore, at the cellular level in human colonocytes, the experiments demonstrated statistically significant differences in activation of two VDR-based luciferase systems, across multiple concentrations of 1,25(OH)2D and a single concentration of 25(OH)D, by GC isotype. Overall, this evidence provides a translational basis for further studies of the vitamin D endocrine system and cancer prevention in diverse and at-risk populations.
studies primarily evaluated associations by genotype or allele. Our previous work (17) as well as analyses by Ahn and colleagues (35) identified GC polymorphisms significantly associated with serum 25(OH)D concentrations, including rs7041 and rs4588. Sinotte and colleagues also found that, in premenopausal women, 25(OH)D levels significantly declined with additional copies of the rare genotypes defining 1F/1S and 2 (36). However, more recent studies have demonstrated that the 2 isotype is associated with decreased circulating concentrations of vitamin D metabolites (12, 37). It has been proposed that the 1F isotype, which has a greater affinity for vitamin D metabolites, may lead to more efficient transport and protection from catabolism (10, 14). However, there may be factors not accounted for in these population level studies as there is also evidence, as described below, that the reported differences in circulating vitamin D metabolites by GC isotype may translate to tissue-specific and/or cellular level effects.

There are two models related to vitamin D metabolite delivery at the cellular level. The "free-hormone hypothesis" states that unbound metabolites act upon target tissues, whereas the GC-bound metabolites act as stores in the body (8, 38). A second, complementary theory identifies the additional proteins of megalin and cubilin that act to take up the GC-bound metabolites into cells (39, 40). With either mechanism, variations in affinity of GC isotypes for vitamin D metabolites could alter the intracellular concentration and ability to activate the transcriptional activity of VDR. At the cellular level, 1,25(OH)2D has been demonstrated to reduce cancer-promoting phenotypes in a variety of cell types (7, 8, 18, 41, 42). However, only one previous study to date has attempted to quantify the effect of factors that influence intracellular metabolite concentration and hence the potential anticancer activity of vitamin D. Using induction of 24-hydroxylase (CYP24A1) as a surrogate for intracellular 1,25(OH)2D concentration, Chun and colleagues demonstrated that the 1S and 2 isotypes led to the greatest induction of the enzyme when monocytes were treated with 25(OH)D (22). We only detected a marginally significant difference by GC genotype with $5 \times 10^{-7}$ mol/L 25(OH)D in the VDRE-based assay; however, the 2 isotype did demonstrate increased activity in the M2H assay at the lower concentration tested. Furthermore, with 1,25(OH)D treatment, similar patterns of activation across GC isotypes and assay were observed. In contrast, only 25(OH)D treatment was tested by Chun and colleagues using real-time PCR in monocytes, cells which they also demonstrated do not utilize megalin-mediated endocytosis (22). Our previous work has demonstrated that HCT-116 cells do utilize megalin for uptake of vitamin D metabolites (submitted for publication), which could explain the differences between the results of these studies and requires further exploration. The results of the present study may indicate that additional factors play a role in vitamin D metabolite uptake in colon cells, including the potential role for megalin-mediated endocytosis of GC-bound vitamin D metabolites, which may have implications for colon cancer prevention.

The strongest evidence from epidemiologic studies of a relationship between low vitamin D status and cancer is for increased risk of colorectal neoplasia (21, 43, 44). Low circulating 25(OH)D concentration has been associated with adenoma incidence and recurrence (20, 26), as well as increased colon cancer incidence (4, 45, 46). However, though the associations are consistent, there are observations in some populations that indicate a potential role for unaccounted factors. For example, historically, age-adjusted rates of colorectal neoplasia are lower among Hispanic populations compared with non-Hispanic, while circulating 25(OH)D concentrations are lower (47). There is also an observed paradox for African American populations with low circulating 25(OH)D level, yet reduced risk of health outcomes such as fractures (48). The GC–megalin interaction could also provide a potential mechanism for this relationship as bone is one tissue in which megalin-mediated endocytosis has been demonstrated (49, 50). This further supports the hypothesis that the increased affinity of 1F isotype for vitamin D metabolites, though rare in White populations, may have evolved to protect from catabolism and deliver limited stores of vitamin D metabolites more efficiently to cells. Interestingly, the 2 isotype demonstrated relatively higher activation at the lower concentration of 25(OH)D compared with 1,25(OH)2D. This could mean that in White individuals with the 2 isotype, who demonstrate lower circulating 25(OH)D concentrations, the GC is able to deliver and release the metabolite more efficiently at the cellular level, whereupon it is metabolized to 1,25(OH)2D. This hypothesis was supported by the results of the current work in colon cancer cells.

The results of the current work identified differences in activation of a VDR-based luciferase system by GC isotype and vitamin D metabolite concentration in colon cancer cells. At the highest concentration of 1,25(OH)2D tested, the 1F, 1S and 1F isotypes demonstrated increased activation, whereas the 1F, 2F or 2 isotypes did not activate the system as well. Arnaud and colleagues demonstrated that the 1F isotype had the highest affinity for both 25(OH)D and 1,25(OH)2D, whereas the 1F or 2F isotypes did not activate the system as well. Arnaud and colleagues demonstrated that the 1F isotype had the highest affinity for both 25(OH)D and 1,25(OH)2D compared with 1S and 2 isotypes (14). This may indicate that at low circulating vitamin D metabolite concentrations, 1F binds more tightly to help maintain a store of these lipophilic molecules, but at the cellular interface additional factors likely play a role in delivery within certain tissues. For example, because 1F has the highest affinity constant, and thus lowest dissociation constant, it may be the interaction of GC with proteins such as megalin and cubilin that actively mediate uptake in place of diffusion alone when metabolite concentrations are low. This hypothesis supports further testing of GC isotypes to more clearly determine their role in cancer risk in both the circulation and once they reach the tissue and cellular levels.

There were strengths as well as limitations of the current work. For the epidemiologic analysis, the samples size was...
sufficient to detect the large difference in 25(OH)D concentration by GC isotype. However, the sample was not large enough to appropriately account for population stratification by race/ethnicity. Furthermore, the 1F_1F isotype is more common in African American populations and the next step will be to examine the associations between GC isotype, circulating vitamin D metabolites concentrations, and risk for colorectal neoplasia in large, diverse populations. For the colon cell-based experiments, the strengths included testing the associations in two different, but complementary, versions of the luciferase assay system as well as across multiple doses of 25(OH)D and 1,25(OH)2D. However, future studies should incorporate testing of purified GC with interactions between megalin and cubilin as well as compare the mechanism of vitamin D uptake in normal colon with colon cancer cells. Furthermore, variation is common in the luciferase assay and testing the ranking of GC isoatypes using additional techniques, such as quantitative PCR, would be beneficial. Nonetheless, these novel results provide quantitative evidence for the effect of variation in GC on the vitamin D endocrine system in the colon; and further analysis and experimentation are warranted.

We have presented evidence for the effect of Gc-globulin isotype on vitamin D status at the population level as well as a potential mechanism for variation in vitamin D metabolite uptake in colon cancer cells. This translational research supports the hypothesis that circulating 25(OH)D concentration alone is not the only factor of importance in evaluating risk for disease, including cancer. Factors that affect delivery of vitamin D metabolites at the tissue level, including GC, and potentially tissue-specific variation in that mechanism, may alter the risk of neoplasia in that tissue.

We thus suggest that studying GC isotype, in addition to circulating vitamin D metabolite concentrations, may identify heterogeneity in associations between circulating vitamin D metabolite concentration and colorectal neoplasia risk, specifically. Identifying genetic variation that modifies colorectal neoplasia risk could ultimately help identify individuals at risk for cancer or potential recipients for targeted chemoprevention.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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