Background: Calcium and vitamin D both appear to have antineoplastic effects in the large bowel. Although these nutrients are inter-related metabolically in bone and in the normal intestine, their potential interactions in large-bowel carcinogenesis are not well understood. Methods: We assessed independent and joint effects of calcium supplementation and vitamin D status on adenoma recurrence in 803 subjects in a multi-center, placebo-controlled randomized clinical trial of calcium supplementation for the prevention of colorectal adenoma recurrence. Serum levels of 25-hydroxy [25-(OH) vitamin D and 1,25-dihydroxy [1,25-(OH)2 vitamin D levels were determined, and the Taq I and Fok I polymorphisms in the vitamin D receptor (VDR) gene were analyzed by polymerase chain reaction. Risk ratios (RRs) for any adenoma recurrence were computed for calcium supplementation within groups defined by serum vitamin D levels and for serum vitamin D levels within treatment groups. Associations of VDR polymorphisms with recurrence risk were also evaluated. All statistical tests were two-sided. Results: Among subjects with baseline 25-(OH) vitamin D levels at or below the median (29.1 ng/mL), calcium supplementation was not associated with adenoma recurrence, whereas among those with levels above the median, calcium supplementation was associated with a reduced risk (RR = 0.71, 95% confidence interval [CI] = 0.57 to 0.89, P for interaction = .012). Conversely, serum 25-(OH) vitamin D levels were associated with a reduced risk only among subjects receiving calcium supplements (RR per 12 ng/mL increase of vitamin D = 0.88, 95% CI = 0.77 to 0.99, P for interaction = .006). VDR polymorphisms were not related to adenoma recurrence and did not modify the associations with vitamin D or calcium. Conclusions: Calcium supplementation and vitamin D status appear to act largely together, not separately, to reduce the risk of colorectal adenoma recurrence. VDR genotype does not appear to be associated with risk. [J Natl Cancer Inst 2003;95:1765–71]
1800 Patients not entered into study
223 could not be contacted
1976 were unwilling to participate or ineligible
1 for unknown reasons

2918 apparently eligible patients

1116 entered 3 month placebo run in

188 Patients were unsuitable (<80% compliance)

930 Randomly Assigned

913 subjects completed at least 1 follow-up colonoscopy at Year 1 or Year 4

110 subjects not included in this analysis
73 denied consent for vitamin D assays
18 died before being asked for consent
21 did not have baseline blood specimens available

863 subjects with at least 1 follow-up colonoscopy and baseline vitamin D serum levels included in the present analysis

Table 1. Characteristics of the Calcium Polyp Prevention Study participants included in this analysis*

<table>
<thead>
<tr>
<th>Baseline characteristic</th>
<th>Placebo</th>
<th>Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>405</td>
<td>398</td>
</tr>
<tr>
<td>Males, No. (%)</td>
<td>282 (69.6)</td>
<td>300 (75.4)</td>
</tr>
<tr>
<td>Mean age, years (SD)</td>
<td>60.9 ± 9.0</td>
<td>60.8 ± 9.0</td>
</tr>
<tr>
<td>Median baseline serum 25-OH vitamin D, ng/mL (IQR)†</td>
<td>29.1 (21.5–36.3)</td>
<td>29.1 (21.2–36.2)</td>
</tr>
<tr>
<td>Median baseline serum 1,25(OH)₂ vitamin D, ng/mL, (IQR)‡</td>
<td>41.6 (33.9–50.9)</td>
<td>41.8 (34.2–51.3)</td>
</tr>
<tr>
<td>Race, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>351 (86.7)</td>
<td>327 (82.2)</td>
</tr>
<tr>
<td>Black</td>
<td>30 (7.4)</td>
<td>37 (9.3)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>24 (5.9)</td>
<td>34 (8.5)</td>
</tr>
</tbody>
</table>
| Taq I genotype of the VDR gene, No. (%)$§  
Tt                            | 177 (46.1) | 168 (44.4) |
| Tt                            | 155 (40.4) | 143 (37.8) |
| tt                            | 47 (12.2)  | 60 (15.9) |
| No PCR product            | 5 (1.3)   | 7 (1.9)  |
| Fok I genotype of the VDR gene, No. (%)¶  
FF                            | 171 (44.5) | 164 (43.4) |
| FF                            | 139 (36.2) | 150 (39.7) |
| ff                            | 69 (17.9)  | 58 (15.3) |
| No PCR product            | 5 (1.3)   | 6 (1.6)  |
| Subjects who did not have a year-4 colonoscopy, (%) | 25 (6.2)  | 33 (8.3) |

*SD = standard deviation; IQR = interquartile range; VDR = vitamin D receptor.

†Five subjects with missing information (two in the placebo group and three in the treatment group).

‡41 subjects with missing information (21 in the placebo group and 20 in the calcium group).


¶Pearson chi-squared test for Hardy–Weinberg equilibrium = 20.8, P < .001.

Recruitment began in November 1988 and ended April 1992; treatment ended in December 1996. Of the 930 subjects who underwent randomization, 913 had at least one follow-up colonoscopy. Of those, 73 denied consent for vitamin D assays, 16 died before being asked for consent, and 21 did not have baseline blood specimens available. Therefore, 803 subjects had follow-up colonoscopy and two sets of vitamin D levels, and were included in this report. The number of subjects included in each analysis varied slightly because of missing data for individual analytes (Fig. 1; Table 1). All participants provided written informed consent and the study was approved by the Human Subjects Committee at each of the involved institutions.

Vitamin D assays were conducted at University of California at Los Angeles (UCLA), Center for Human Nutrition, CNRU Micronutrient Laboratory, using the radioimmunoassay kit from Nichols Institute Diagnostics (Capistrano, CA). The analyses were performed as per the manufacturer’s instructions. The sensitivity of the assay for 25-(OH) vitamin D was 5 ng/mL and for 1,25-(OH)₂ vitamin D was 2.1 pg/mL. To monitor the precision of the assays, serum samples from 182 subjects were split, and the paired aliquots distributed among separate batches shipped to UCLA for analysis. The inter-batch Pearson correlation coefficient was .95 for the 25-(OH) vitamin D measurements and .58 for the 1,25-(OH)₂ vitamin D measurements.

Genotyping of the VDR gene was performed by investigators who were blinded to the subjects’ laboratory and clinical data, including treatment status. Cell pellets obtained by centrifuging baseline serum at 14 000 g in a microcentrifuge were used as the source of genomic DNA. The DNA was purified by phenol/chloroform extraction, retrieved by cold ethanol precipitation, and stored in TE (10 mM Tris, 0.1 mM EDTA, pH 8.0). Polymerase chain reaction (PCR) was performed using primers from GenBank accession number AC004466 [Taq I polymorphism primers 5’-CCT TCT TCT CTA TCC CGG TG-3’ (F) and 5’-GCA GGT CGG GTA GCT TCT-3’ (R) and Fok I polymorphism primers 5’-ACT CTG GCT CTG ACC GTG-3’ (forward) and 5’-TCA TAG CAT TGA AGT GAA AGC-3’ (reverse) at concentrations of 0.25 μM]. The PCR products for these two polymorphisms (Taq I genotype,174 bp and Fok I genotype, 159 bp) were digested with 4 units of each of the restriction enzymes (‘Taq I or Fok I independently) and incubated at 65°C for Taq I and at 37°C for Fok I (New England Biolabs, Hitchin, UK) for 3 hours and separated electrophoretically to identify alleles with...
the presence or absence of the restriction sites (t and f versus T and F, respectively). Negative controls (i.e., with H2O as template) were included in every set of PCRs to ensure that there had been no sample cross-contamination. Controls for completion of digestion were included for every set of PCRs. A sample with known genotype, confirmed by direct sequencing, was also included in each batch of samples. Two data readers independently read the results, and all discrepancies were resolved by conference.

Statistical Analysis

Our primary focus in this analysis was the recurrence of adenomas during the 4-year treatment period. Secondary outcomes were occurrence of adenomas defined by the study pathologist as advanced lesions: tubulovillous adenomas (25–74% villous component), villous adenomas (75% or more villous), those containing advanced dysplasia or invasive cancer, or those larger than 1 cm in diameter (as assessed by the endoscopist).

To evaluate the effect of calcium supplementation on serum vitamin D levels, we computed two-sample and paired t tests. Overdispersed generalized linear models for the Poisson distribution as an approximation to the binomial family were used to compute crude and adjusted risk ratios to assess the risk of at least one new adenoma; covariates were age, sex, study center, smoking status and alcohol intake. The associations of vitamin D levels with adenoma risk were assessed as the risk ratio (RR) per standard deviation of baseline serum levels. To account for the variation of sunlight exposure and its effect on vitamin D serum levels, we also added the month of blood draw as a covariate; the inclusion of this covariate resulted in virtually no change in the relative risk estimates. We divided study subjects at the overall median of baseline 25-(OH) vitamin D or 1,25-(OH)2 vitamin D serum levels and assessed the association of calcium intake with risk in each group to evaluate modification by vitamin D. The interaction between calcium supplementation and vitamin D was also evaluated with product interaction terms and the Wald test. Findings from an analysis using total calcium intake (sum of supplements and dietary calcium intake) were similar to those based on study treatment only and are not presented.

In the analysis of the association of VDR polymorphisms with adenoma recurrence, each genotype was considered separately. Some studies have suggested that the strength of the association between VDR polymorphisms and the risk of colorectal neoplasia may vary with calcium intake and vitamin D concentrations (16,17,22). Therefore, we considered associations of the polymorphisms with adenoma risk among all subjects and also among placebo subjects with low dietary calcium intake or low 25-(OH) vitamin D levels. To assess the effect modification of calcium and vitamin D by VDR genotype, two groups were used (TT versus Tt plus tt and FF versus Ff plus ff). Multiplicative interaction terms and Wald tests were also performed. Tests for Hardy–Weinberg equilibrium were applied using a chi-square test to compare observed and expected allele frequencies. All statistical tests were two-sided.

RESULTS

Table 1 summarizes the baseline characteristics of the participants included in the analysis; there were no substantial differences between the treatment groups. The mean age was 61 years; approximately 73% of the subjects were male, and approximately 84% were white. In both treatment groups combined, the median baseline serum 25-(OH) vitamin D level was 29.1 ng/mL (interquartile range [IQR] = 21.2–36.2 ng/mL) and the median serum 1,25-(OH)2 vitamin D level was 41.8 pg/mL (IQR = 33.9–51.2 pg/mL). The two treatment groups also did not differ statistically significantly in the distribution of VDR genotypes. However, there was deviation from Hardy–Weinberg equilibrium for both genotypes (Table 1).

Study treatment with calcium supplements lowered 1,25-(OH)2 vitamin D serum levels during the trial. In the calcium treatment group, mean serum levels (95% CI) at baseline and at year 4 were 43.3 (41.8 to 44.7) pg/mL and 41.4 (39.8 to 42.9) pg/mL, respectively, with a statistically significant mean decrease of 1.9 pg/mL (95% CI = 0.2 to 3.6; P = .03). In the placebo group, mean levels changed from 42.7 (41.2 to 44.2) pg/mL at baseline to 44.1 (42.6 to 45.5) pg/mL at the end of the study, with a non-statistically significant mean increase of 1.4 pg/mL (95% CI = −0.3 to 3.0; P = .1, P for the difference between groups from paired t test = .006). In contrast, 25-(OH) vitamin D levels were not affected by treatment, decreasing in both groups similarly: from 29.2 (28.0 to 30.4) ng/mL at baseline to 27.6 (26.4 to 28.8) ng/mL at year 4 in the calcium group (mean difference = 1.7 ng/mL, 95%CI = 0.5 to 2.8; P = .004) and from 29.0 (27.9 to 30.1) ng/mL to 27.7 (26.5 to 28.8) ng/mL in the placebo group (mean difference = 1.3 ng/mL, 95% CI = 0.3 to 2.4; P = .01, P for the difference between groups, paired t test = .689). Dairy product consumption also decreased during the trial, by an average of 10% during the treatment period, compared with baseline (data not shown).

There was clear evidence that vitamin D status modified the effect of calcium supplementation on adenoma recurrence (Table 2). Among subjects with 25-(OH) vitamin D levels at or below the overall median (29.1 ng/mL), calcium had no effect on the risk of one or more recurrent adenomas (RR = 1.05; 95% CI = 0.85 to 1.29). In contrast, calcium supplementation was associated with a statistically significantly reduced risk among subjects with baseline 25-(OH) vitamin D levels above the median (RR = 0.71; 95% CI = 0.57 to 0.89; P for interaction = .012). Similar findings were seen when serum vitamin D levels were considered in tertiles or quartiles (data not shown). Furthermore, the interaction between calcium treatment and 25-(OH) vitamin D serum levels as a continuous measurement was highly statistically significant (P = .006). A similar pattern also held for advanced adenomas, although with the smaller number of endpoints, statistical significance was lost (Table 2). In contrast to the results with 25-(OH) vitamin D, risk ratios for calcium treatment did not differ across 1,25-(OH)2 vitamin D groups (Table 2).

There was no substantial overall association between 25-(OH) or 1,25-(OH)2 vitamin D levels and adenoma recurrence (Table 3). However, just as vitamin D status modified the effect of calcium supplementation, calcium supplementation modified the associations of vitamin D with adenoma risk. In the calcium group, there was an inverse association between levels of serum 25-(OH) vitamin D and the risk of recurrence of 1 or more adenomas (adjusted risk ratio per 12 ng/mL increase in vitamin D = 0.88; 95% CI = 0.77 to 0.99) but not in the placebo group (adjusted risk ratio per 12 ng/mL increase in vitamin D = 1.09; 95% CI = 0.97 to 1.22; P for interaction = .006) (Table 3). Changes in levels of 1,25-(OH)2 vitamin D, by contrast, had
little association with adenoma recurrence either overall or in the individual treatment groups (Table 3). In addition to modifying the association of 25-(OH) vitamin D with adenoma recurrence overall, calcium modified the association of these levels with advanced adenomas, although the interaction was of marginal statistical significance (P for interaction = .048).

We analyzed the association of both the Taq I and Fok I polymorphisms of the VDR gene with risk of adenoma recurrence overall and found no such association for either polymorphism (Table 4). We obtained similar results when we restricted the analysis to placebo subjects in the lower half of baseline calcium intake or in the lower half of baseline 25-(OH) vitamin D serum levels (data not shown). Neither VDR genotype was associated with a substantial modification of the effect of calcium on risk of adenoma recurrence or of the association of either form of vitamin D with adenoma risk (Table 4).

**DISCUSSION**

In this randomized, placebo-controlled clinical trial, vitamin D status strongly modified the effect of calcium supplementation...
Vitamin D obtained from the diet or sunlight-induced cutaneous synthesis is hydroxylated first in the liver (forming 25-(OH) vitamin D) and subsequently in the kidney (forming 1,25-(OH)\textsubscript{2} vitamin D). Serum levels of 25-(OH) vitamin D reflect overall vitamin D status from combined dietary and skin sources, but the levels of 1,25-(OH)\textsubscript{2} vitamin D are so tightly regulated that this more active form does not reflect vitamin D status except in the case of clear deficiency or excess (29). Many studies focused on serum levels of 25-(OH) vitamin D have found a fairly consistent inverse link with the risk of colorectal cancer (20,30,31), but associations with 1,25-(OH)\textsubscript{2} vitamin D have generally not been striking (32,33). Our findings, for both adenomas in general and advanced adenomas in particular, are consistent with this pattern.

A possible protective effect of vitamin D on colorectal cancer risk was first proposed about 20 years ago, on the basis of an inverse ecologic association between colon cancer rates and solar radiation (23). In vitro and in vivo studies have shown that vitamin D and vitamin D analogs can inhibit cell proliferation, induce differentiation, and promote apoptosis (24–27). Many animal studies (11,12) and epidemiologic reports (5,13,14) also support an antineoplastic effect of vitamin D intake, although null results have been reported as well (8,28).

Vitamin D obtained from the diet or sunlight induces cutaneous synthesis is hydroxylated first in the liver (forming 25-(OH) vitamin D) and subsequently in the kidney (forming 1,25-(OH)\textsubscript{2} vitamin D). Serum levels of 25-(OH) vitamin D reflect overall vitamin D status from combined dietary and skin sources, but the levels of 1,25-(OH)\textsubscript{2} vitamin D are so tightly regulated that this more active form does not reflect vitamin D status except in the case of clear deficiency or excess (29). Many studies focused on serum levels of 25-(OH) vitamin D have found a fairly consistent inverse link with the risk of colorectal cancer (20,30,31), but associations with 1,25-(OH)\textsubscript{2} vitamin D have generally not been striking (32,33). Our findings, for both adenomas in general and advanced adenomas in particular, are consistent with this pattern.

Some—although far from all—observational data suggest that calcium is associated with a reduced risk of large-bowel neoplasia (5,6,20,28), an effect that has been confirmed in clinical trials (9,10). However, the mechanisms underlying the antitumorogenic effects of calcium in the large bowel are not clear. One hypothesis is based on its capability to bind to and precipitate bile acids and soluble fatty acids, rendering them relatively inert in the bowel lumen (4,34). Recent studies suggest that extracellular calcium can affect cell proliferation and differentiation through the calcium sensing receptor, a cell surface receptor that is expressed in normal colon cells and colon cancer cell lines (35–37).

A potential interaction between calcium and vitamin D on colorectal carcinogenesis has been addressed in only a few previous studies, with conflicting findings. One experimental study in rodents found that calcium and vitamin D supplementation together resulted in a smaller protective effect than either supplement alone (38). However, most studies have reported that vitamin D has a stronger association with reduced risk of neoplasia in animals fed a diet relatively high in calcium (2,12). Much of the published human epidemiologic data regarding this question does not indicate substantial deviations from independent effects (7,8,20,39). However, in one case–control study (31), the inverse association of serum 25-(OH) vitamin D levels with colorectal adenoma was stronger among subjects with calcium intake at or above the median than in those with calcium intake below the median. Two careful cohort studies also found evidence of a positive interaction between calcium and vitamin D in lowering the risk of colon cancer (5,6). In view of the measurement error associated with these risk factors, however, perhaps it is not surprising that a clear picture of interactive effects does not emerge from observational studies.

The mechanistic basis for an interaction between calcium and vitamin D is not clear. Calcium supplementation has the potential to decrease 1,25-(OH)\textsubscript{2} vitamin D levels, which could conceivably have a detrimental effect on cancer prevention. Conversely, vitamin D supplementation might decrease luminal calcium in the large bowel by increasing its absorption in the small bowel, thereby interfering with intra-luminal antineoplastic effects of calcium. Additional clues about the interaction between these nutrients may be provided by recent studies suggesting that vitamin D controls intracellular calcium gradients within colonic crypts (40,41) and increases expression of the calcium sensing receptor (42), both effects that would be expected to have a strong impact on carcinogenesis.

The VDR, a nuclear ligand–activated receptor, is thought to mediate most vitamin D effects. Several polymorphisms of the VDR gene have been identified, including three at the 3’ end that are in strong linkage disequilibrium (Taq I, Bsm I and Apa I) (43). These polymorphisms may have functional significance (44,45). For example, individuals who are homozygous for the T (wild type) allele of the Taq I polymorphism appear to have higher calcium absorption in the small intestine, an effect that is consistent with a corresponding association of this genotype with increased bone mass (45). Also, variant alleles of these 3’ polymorphisms have been related to a reduced risk of colon cancer (15,16), predominantly among people with low calcium or vitamin D intake. The Fok I polymorphic site at the 5’ end of the VDR gene has not been related to risk of colorectal adenomas (17,31) but was associated with risk of large adenomas in individuals with low calcium and vitamin D intake (17). These findings suggest that effects of the VDR genotype may depend on vitamin D or calcium status. In our study, however, we found little evidence of an impact of either the Taq I or the Fok I polymorphisms on adenoma recurrence. This lack of effect persisted even when the analysis was restricted to placebo subjects in the lower half of baseline calcium intake or of baseline 25-(OH) vitamin D serum levels, suggesting that there is also no association even at low calcium intake or suboptimal vitamin D status.

There was deviation from Hardy–Weinberg equilibrium for both of the VDR gene polymorphisms that we assayed. There are several possible reasons for such deviation from expected proportions, including laboratory error and population stratification. In this study, the most likely explanation is the extensive inclusion/exclusion criteria applied to an admixed population, which prevented the study sample from reproducing the genotype patterns of the source population.

In our study, calcium supplementation was associated with reduced 1,25-(OH)\textsubscript{2} vitamin D levels, presumably because the high calcium intake suppressed 1α-hydroxylase activity in the kidney, inhibiting the formation of 1,25-(OH)\textsubscript{2} vitamin D (46). The decrease in 25-(OH) vitamin D levels in both study groups may well have been a consequence of the reduced intake of dairy products, the main source of dietary vitamin D, during the trial. This reduction could have been caused by one of the steps we took to prevent hypercalcemia among trial participants: we periodically monitored dairy product consumption and occasion-
ally counseled some participants about the importance of avoiding excessive calcium intake.

Our study has several strengths. The uniform follow-up and blinded pathology review by a single pathologist assured unbiased assessment of adenoma recurrence. In analyses of calcium effects, the randomized design provided protection from confounding and enabled us to avoid the measurement error associated with dietary estimation of calcium intake. We used serum 25-(OH) vitamin D levels to assess vitamin D status, an approach that is also less subject to measurement error than the use of estimated dietary intake. Also, a single pathologist evaluated all lesions, assuring uniform unblinded endpoint review.

On the other hand, this study also has some limitations. One is measurement error. The split-sample correlation was not as high for 1,25-(OH)2 vitamin D as for 25-(OH) vitamin D, although this may be a predictable finding for an analyte that circulates in picogram concentrations. The sensitivity of the assay at that range may make it difficult to detect biologically significant variations in serum levels, and the resulting measurement errors could have obscured some associations. Furthermore, not all the subjects of the original clinical trial were included in this analysis, either because we did not have consent for the assays or because we lacked a suitable blood specimen. In addition, all subjects had at least one previous adenoma, and it is possible that risk factors for adenoma recurrence differ from those for occurrence of any adenoma or for colorectal cancer.

Overall, the associations we describe provide a strong indication that vitamin D and calcium have a joint antineoplastic effect in the large bowel. Further investigation is needed to understand the mechanistic basis of the vitamin D/calcium interaction and to clarify the amount of intake of each nutrient required for optimum protective effects. Nevertheless, these data clearly suggest the potential for important chemopreventive effects from calcium and vitamin D.

**References**


NOTES

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