

## Colonic Epithelial Cell Proliferation Decreases with Increasing Levels of Serum 25-Hydroxy Vitamin D<sup>1</sup>

Peter R. Holt,<sup>2,3</sup> Nadir Arber,<sup>4</sup> Balazs Halmos,<sup>5</sup> Kenneth Forde, Harry Kissileff, Katherine A. McGlynn,<sup>6</sup> Steven F. Moss,<sup>7</sup> K. Fan, Kunhua Yang, and Martin Lipkin

Gastrointestinal Division, St. Luke's-Roosevelt Hospital Center, New York, New York 10025 [P. R. H., N. A., B. H., H. K., S. F. M.]; Department of Medicine [P. R. H., N. A., B. H., H. K., S. F. M.] and Surgery [K. Fo.], College of Physicians and Surgeons, Columbia University, New York, New York 10032, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 [K. A. M.]; and Strang Cancer Prevention Center, New York, New York 10021 [K. Fa., K. Y., M. L.]

### Abstract

Epidemiological evidence suggests a potential role for vitamin D in colon cancer prevention. Vitamin D, absorbed from the intestine or derived from solar ultraviolet light, is metabolized in the liver to 25-hydroxyvitamin D (25-OH D<sub>3</sub>). Previous studies examining effects of vitamin D upon carcinogenesis have focused upon the active metabolite 1,25-dihydroxyvitamin D [1,25-(OH)<sub>2</sub> D<sub>3</sub>], which interacts with nuclear vitamin D receptors in several organs. Until recently, the metabolism of 25-OH D<sub>3</sub> to 1,25-(OH)<sub>2</sub> D<sub>3</sub> was believed to occur only in the kidney, but more recent studies have shown that 25-OH D<sub>3</sub> conversion to 1,25-(OH)<sub>2</sub> D<sub>3</sub> can occur in other tissues. We examined the association between fasting levels of 25-OH D<sub>3</sub>, 1,25-(OH)<sub>2</sub> D<sub>3</sub>, and *BsmI* polymorphism of the *vitamin D receptor (VDR)* gene with indices of colonic epithelial cell proliferation and differentiation in a chemoprevention study, after giving vitamin D or calcium and taking rectal biopsies that were incubated with bromodeoxyuridine. Vitamin D receptor polymorphism was determined by genotyping of the 3' *BsmI* polymorphism in intron eight of the *VDR* gene.

No significant changes in cell proliferation or in differentiation were found in subjects between study start and end. However, fasting serum levels of 25-OH D<sub>3</sub>

showed a highly significant decrease with whole crypt labeling index and the size of the proliferative compartment ( $\phi$  h). There was no correlation between serum levels of 1,25-(OH)<sub>2</sub> D<sub>3</sub> and the proliferative parameters. Calcium supplementation induced a significant effect upon the relationship between serum 25-OH D<sub>3</sub> and rectal epithelial cell labeling index and  $\phi$  h when studied by covariance analysis without a relationship with 1,25-(OH)<sub>2</sub> D<sub>3</sub> levels. *VDR* genotype did not influence the effects of serum 25-OH D<sub>3</sub> or serum 1,25-(OH)<sub>2</sub> D<sub>3</sub> levels upon proliferation.

These data suggest that there might be a local effect of 25-OH D<sub>3</sub> on colonic epithelial cells through conversion of 25-OH D<sub>3</sub> to 1,25-(OH)<sub>2</sub> D<sub>3</sub>. Subsequent studies have demonstrated the presence of 1 $\alpha$ -hydroxylase mRNA in normal colorectal epithelium and in colorectal cancer. Thus, vitamin D may have an important role in determining the effects of calcium on colorectal epithelial proliferation and may explain some of the discrepancies found previously in studies that examine the direct role of calcium on the colorectal epithelium.

### Introduction

Epidemiological evidence suggests a potential role of vitamin D in colon cancer prevention. Colon cancer mortality rates show a marked North to South gradient in the United States with higher rates observed in the North (1). Because this gradient correlates with sunshine exposure and therefore levels of ultraviolet radiation, it has been suggested that low ultraviolet exposure may be a risk factor for colorectal cancer (2). Data from nine population-based cancer registries obtained from the Surveillance, Epidemiology, and End Results program also showed an inverse relationship between amounts of solar radiation and colon cancer incidence rates in males (3). Much of an individual's vitamin D is formed in the skin in response to ultraviolet radiation (4).

Dietary vitamin D intake also has been inversely correlated with colon cancer (5, 6) and with colonic adenoma (7) risk. The vitamin D made in the skin as well as that absorbed from the gastrointestinal tract subsequently is metabolized in the liver to 25-hydroxyvitamin D<sub>3</sub>. Fasting serum levels of 25-OH D<sub>3</sub><sup>8</sup> generally reflect the vitamin D stores of the body (8). Although the major source of circulating vitamin D is 25 OH D<sub>3</sub>, it generally is accepted that 1,25-(OH)<sub>2</sub> D<sub>3</sub> is the active metabolite functioning through its interaction with a nuclear 1,25-(OH)<sub>2</sub> D<sub>3</sub> receptor (9). The kidney is the major source of 1,25-(OH)<sub>2</sub> D<sub>3</sub> through the action of an enzyme, 25-hydroxyvitamin 1 $\alpha$ -OHase, although

Received 4/20/01; revised 11/2/01; accepted 11/8/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This study was supported in part by National Cancer Institute Grant 25439-02 and the Ann Fisher Nutrition Fund.

<sup>2</sup> To whom requests for reprints should be addressed, at Gastroenterology Division, St. Luke's-Roosevelt Hospital Center, Amsterdam Avenue at 114th Street, New York, NY 10025.

<sup>3</sup> Present address: American Health Foundation, New York, NY 10017.

<sup>4</sup> Present address: Tel-Aviv Sourasky Medical Center, 6, Weizmann St., Tel Aviv 64239, Israel.

<sup>5</sup> Present address: Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA 02215.

<sup>6</sup> Present address: NIH, Bethesda, MD 20892.

<sup>7</sup> Present address: Department of Medicine, Brown University, Providence, RI 02912.

<sup>8</sup> The abbreviations used are: 25-OH D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>; 1,25-(OH)<sub>2</sub> D<sub>3</sub>, 1,25-dihydroxy vitamin D<sub>3</sub>; VDR, vitamin D receptor; 1 $\alpha$ -OHase,  $\alpha$ 1-hydroxylase.

Table 1 Fasting serum calcium and vitamin D concentrations<sup>a</sup>

Study subjects	Treatment, daily intake	Serum concentrations		
		Calcium mg/dl	25-OH D <sub>2</sub> ng/ml	1,25-(OH) <sub>2</sub> D <sub>3</sub> pg/ml
Group A (n = 14)	1500 mg CaCO <sub>3</sub>	9.47 ± 0.1	26.2 ± 2.5	30.8 ± 2.5
Group B (n = 12)	1500 mg CaCO <sub>3</sub>	9.52 ± 0.1	37.3 <sup>b</sup> ± 3.7	27.4 ± 2.4
Group C (n = 13)	400 IU Vitamin D 0.5 μg 1,25(OH) <sub>2</sub> D <sub>3</sub>	9.59 ± 0.09	22.0 ± 1.9	39.8 <sup>b</sup> ± 2.3 <sup>b</sup>

<sup>a</sup> Fasting serum taken at the conclusion of a 6-month chemoprevention study in the number (n) of subjects in each group.

<sup>b</sup> Mean data significantly different from data obtained at study start (P < 0.05).

Table 2 Proliferation and vitamin D receptor genotypes

VDR	Labeling index <sup>a</sup> (%)		phi h <sup>a</sup> (× 10 <sup>-3</sup> )	
	Baseline	Study end	Baseline	Study end
BB n = 10	6.41 ± 0.5	5.5 ± 0.3	9.31 ± 2.8	9.87 ± 2.2
Bb n = 19	5.32 ± 0.3	5.83 ± 0.2	9.1 ± 1.7	7.4 ± 0.7
bb n = 11	6.28 ± 0.5	5.78 ± 0.4	7.32 ± 1.0	4.65 ± 1.0

<sup>a</sup> Data are presented as mean ± SE in the number (n) of subjects having each genotype. There were no significant differences among the three groups of subjects.

nonrenal sources of the enzyme have been reported recently in macrophages (10), keratinocytes (11), and the prostate (12). Prostatic cells, like many other tissues (8), demonstrate the presence of 1,25-OH<sub>2</sub> D<sub>3</sub> receptors (13).

In the present study, we have examined relationships between fasting levels of 25-OH D<sub>3</sub> and 1,25-(OH)<sub>2</sub> D<sub>3</sub> in the serum with indices of rectal epithelial cell proliferation and differentiation. Observations were made in the context of a chemoprevention study that measured effects of vitamin D and calcium on epithelial cells in the colon. The results of this study clearly demonstrate highly significant correlations between increased levels of circulating 25-OH D<sub>3</sub> and several independent indices of cell proliferation, which have been associated with a lower risk for colorectal neoplasia.<sup>9</sup> The findings further document the importance of serum 25-OH D<sub>3</sub> levels in the previously observed beneficial role of calcium in inhibiting colonic epithelial cell proliferation and thereby a putative risk of colorectal neoplasia.

## Materials and Methods

The data presented here were obtained in a prospective blinded randomized study of the administration of either calcium, calcium plus supplemental dietary vitamin D<sub>3</sub>, or 1,25-OH D<sub>3</sub> for 6 months, upon indices of rectal epithelial cell proliferation and differentiation in subjects at increased risk for colonic neoplasia. Potential subjects were chosen from the endoscopic and pathology records of St. Luke's-Roosevelt Hospital Center and Columbia Presbyterian Medical Center, both university hospitals of the College of Physicians & Surgeons of Columbia University in New York City. Eligible subjects had a resected pathologically documented colorectal adenomatous polyp removed within 3 years of entering the study. Excluded were patients with a history of familial cancer syndromes, with a personal history of cancer other than nonmelanoma skin cancer, intestinal malabsorption, inflammatory bowel disease, or prior gastrointestinal surgery except appendectomy. Further exclusions were known abnormalities of calcium metabolism, hy-

perthyroidism or history of milk-alkali syndrome, renal stones, or taking steroid-binding resins. Subjects also were not permitted to have taken >660 mg/day of aspirin or usual daily dosages of nonsteroidal anti-inflammatory drugs within the previous 3 months, or be taking other investigational drugs or medications that might interfere with the study end points. Furthermore, dietary exclusions included the ingestion of more than a total of 1000 mg of calcium or 200 mg of supplemental calcium per day or vitamin D >200 IU/day. Exclusions based upon serological determinations included hypercalcemia (serum level >10.6 mg/dl), current hyperphosphatemia (serum level >6.0 mg/dl), or evidence of significant renal insufficiency (blood urea nitrogen >1.5 times normal).

Subjects were randomized to one of three chemopreventive arms: group A, 1250 mg calcium carbonate equivalent to 500 mg of elemental calcium three times daily; group B, 1250 mg calcium carbonate (500 mg of elemental calcium) orally three times daily with 400 IU vitamin D<sub>3</sub> orally daily; or group C, 0.25 μg 1,25-(OH)<sub>2</sub> D<sub>3</sub> orally twice daily (Table 1) for 6 months. The study was approved by the Institutional Review Boards of both institutions, and all subjects gave informed consent for the study.

At the beginning of the study (baseline) and at study end, rectal biopsies and vitamin D measurements were obtained. Flexible sigmoidoscopy and rectal biopsies were performed between 8 and 11 a.m. in the majority of subjects and between 1 and 2 p.m. in some subjects (at the end of the study, the biopsies in the same individuals were taken at identical times of the day). Sigmoidoscopy was performed without analgesia; the rectal mucosa was examined to 20–25 cm to exclude any inflammation or recurrent polyps. Six to eight biopsies of normal-appearing rectal mucosa were taken (~2 in each quadrant) 10–15 cm from the anal verge. Biopsies were placed in a container with MEM containing bromodeoxyuridine (0.1 mg/ml), cut into small pieces, and flattened on a piece of Metrocell filter. The biopsies then were incubated in a Bellco gas chamber with 95% O<sub>2</sub>, 5% CO<sub>2</sub> and placed in a rocking platform at 10 cycles/minute at 37°C for 1 h. The tissues then were transferred into 95% ethanol and fixed overnight; the fixative was changed once the next day, and then specimens remained in the fixative until processed by standard methods for embedding in paraffin. Three-μm-thick serial tissue sections were prepared for immunohistochemical studies. The sections were organized in ribbons to avoid studying the same crypt more than once.

At baseline and study end, ~10 ml of blood were drawn; serum was separated and then was assayed for calcium, phosphate, creatinine, blood urea nitrogen, and albumin. A tube, protected from light, was analyzed for 25-OH and 1,25-(OH)<sub>2</sub> D<sub>3</sub> levels by a radio receptor method using a Nichols Institute assay kit (15, 16).

**End Point Measurements.** The proliferative kinetics of the rectal mucosa were determined by measuring the relative in-

<sup>9</sup> Presented as an abstract during Digestive Disease Week 2000 (14).

Table 3 Correlation between colonic epithelial proliferation and serum vitamin D<sub>3</sub> levels

Total group	Baseline				Study end			
	Proliferation	Slope	r <sup>2</sup>	P	Proliferation	Slope	r <sup>2</sup>	P
25-OH D <sub>3</sub>								
Epithelial cell	56.1 ± 5.0	+0.06	0.102	0.437	57.6 ± 5.7	+0.08	0.027	0.337
Labeled cell	3.24 ± 0.9	-0.01	0.024	0.346	3.28 ± 0.6	-0.22	0.165	0.014
Labeling index %	5.79 ± 1.6	-0.03	0.040	0.226	5.73 ± 1.2	-0.45	0.208	0.005
phi h × 10 <sup>-2</sup>	8.22 ± 7.	+0.96	0.024	0.346	6.98 ± 4.8	-0.14	0.120	0.038
Lh × 10 <sup>-1</sup>	2.79 ± 2.67	+0.16	0.005	0.684	2.48 ± 2.6	-0.67	0.160	0.016
1,25-(OH) <sub>2</sub> D <sub>3</sub>								
Epithelial cell	56.1 ± 5.0	+0.03	0.003	0.737	57.6 ± 5.67	+0.16	0.084	0.087
Labeled cell	3.24 ± 0.94	+0.01	0.006	0.654	3.28 ± 0.62	+0.02	0.093	0.070
Labeling index	5.80 ± 1.6	+0.01	0.005	0.666	5.73 ± 1.16	+0.02	0.028	0.335
phi h × 10 <sup>-2</sup>	8.22 ± 7.02	-0.03	0.002	0.787	6.98 ± 4.81	+0.09	0.036	0.277
Lh × 10 <sup>-1</sup>	2.79 ± 2.69	+0.06	0.001	0.885	2.48 ± 2.59	+0.47	0.062	0.144

Table 4 Correlations between AE1 cytokeatin and acidic mucin expression and serum vitamin D<sub>3</sub> levels at study end

	Cytokeratin AE1		Acidic mucins	
	r <sup>2</sup>	P	r <sup>2</sup>	P
25-OH D <sub>3</sub>				
Group A + B + C	0.008	0.586	0.010	0.437
Group A + B	0.000	0.942	0.003	0.824
Group A	0.387	0.018	0.012	0.416
1,25-(OH) <sub>2</sub> D <sub>3</sub>				
Group A + B + C	0.102	0.047	0.035	0.201
Group A + B	0.049	0.279	0.075	0.163
Group A	0.092	0.293	0.100	0.248

corporation of bromodeoxyuridine into nuclear DNA (16). Twenty half-crypt columns or more were recorded from each biopsy for individual measurements. Crypt columns were measured as the crypt lumen visible from the opening to the base, lined up with one layer of cells. The numbers of cells and the number of labeled cells in each half crypt were determined from the midpoint between two crypts at the surface to the midpoint of the deepest part of the crypt. The labeling index of the total crypt was determined from the number of labeled cells divided by the total number of cells in the crypt. The changes in the distribution of proliferating cells within the rectal mucosa also were determined and analyzed by reference to five equal zones along the rectal crypt from mouth to base (17). A primary end point for proliferative cell distribution was a determination of phi h, which represents the percentage of proliferating (labeled) cells in the upper 40% of the crypt as a ratio of the total number of labeled cells in the crypt. Preliminary data in this laboratory using two separate biopsies from the same subject showed that the within-subject variation was small (~15%).

Indices of cell differentiation that were analyzed were cytokeatin AE1 (18) and acidic mucins (18). AE1 expression was demonstrated immunohistochemically using the avidin-biotin-peroxidase technique. The monoclonal antibody to AE1 that was used was purchased from Signet Laboratories, Inc. (Dedham, MA). A semiquantitative scoring method was used to evaluate AE1 expression based on the positive area of staining. To study acid mucins cytochemically, a high-iron diamine-Alcian blue stain was used. With this technique, sulfomucins and sialomucins presented in different colors in the cytoplasm. A semiquantitative measurement was used to evaluate the ratio of number of cells with sulfomucin to sialomucin. These markers had been determined by immunohistochemical methods

previously for evaluating the effects of potential chemopreventive agents upon cell differentiation properties (18).

Vitamin D receptor polymorphism was determined on DNA extracted from whole blood. Genotyping of the 3' *BsmI* polymorphism in intron 8 of the *VDR* gene was performed by an established method (19) in the laboratory of Dr. Katherine A. McGlynn.

**Statistical Methods.** To ensure that no differences existed between groups prior to randomization, an ANOVA on the group effect was performed on baseline means for each dependent variable. Then the effects of the group assignment on each dependent variable were analyzed in two ways: (a) mean change in each dependent variable was calculated and tested for significance by paired *t* test for each group separately and for all three groups combined to determine whether any variables changed significantly from baseline; and (b) the mean change in each dependent variable was compared between groups by means of ANOVA. The results of statistical analyses are described as statistically significant when *P* < 0.05. Levels of serum of 25-OH D<sub>3</sub> and 1,25-(OH)<sub>2</sub> D<sub>3</sub> were further correlated with individual indices of cell proliferation and differentiation. Correlation and linear regression studies were further performed with a MiniTab statistics program to correlate the serum levels of 25-OH D<sub>3</sub> and 1,25-(OH)<sub>2</sub> D<sub>3</sub> with cell proliferation and differentiation measurements.

To determine whether calcium intake influenced the relationship between labeling indices and phi h and levels of serum 25-OH D<sub>3</sub> and 1,25-(OH)<sub>2</sub> D<sub>3</sub>, an analysis of covariance was performed with labeling indices and phi h as the dependent variable, calcium treatment as the independent variable, and levels of serum vitamin D<sub>3</sub> as covariates.

## Results

The study was started in January 1996, and the last patient completed the study in January 1998. Of the 45 subjects enrolled into the study, there were 6 dropouts; therefore, 39 subjects completed the study. Dropouts were divided similarly between the study groups. There were 22 males and 17 females (mean age, 61.5 years) who completed the study. Twenty-nine subjects were Caucasian, 7 were black, and 2 were Hispanic. The mean overall compliance from pill counts was 86.9% and did not differ amongst the study groups.

The daily intake of dietary components did not differ at baseline or study end among the three groups. Mean calcium intake was ~650 mg/day, and mean dietary vitamin D intake was ~8 μg/day at baseline. The calcium intake of group A subjects from dietary and supplemental sources totaled a mean

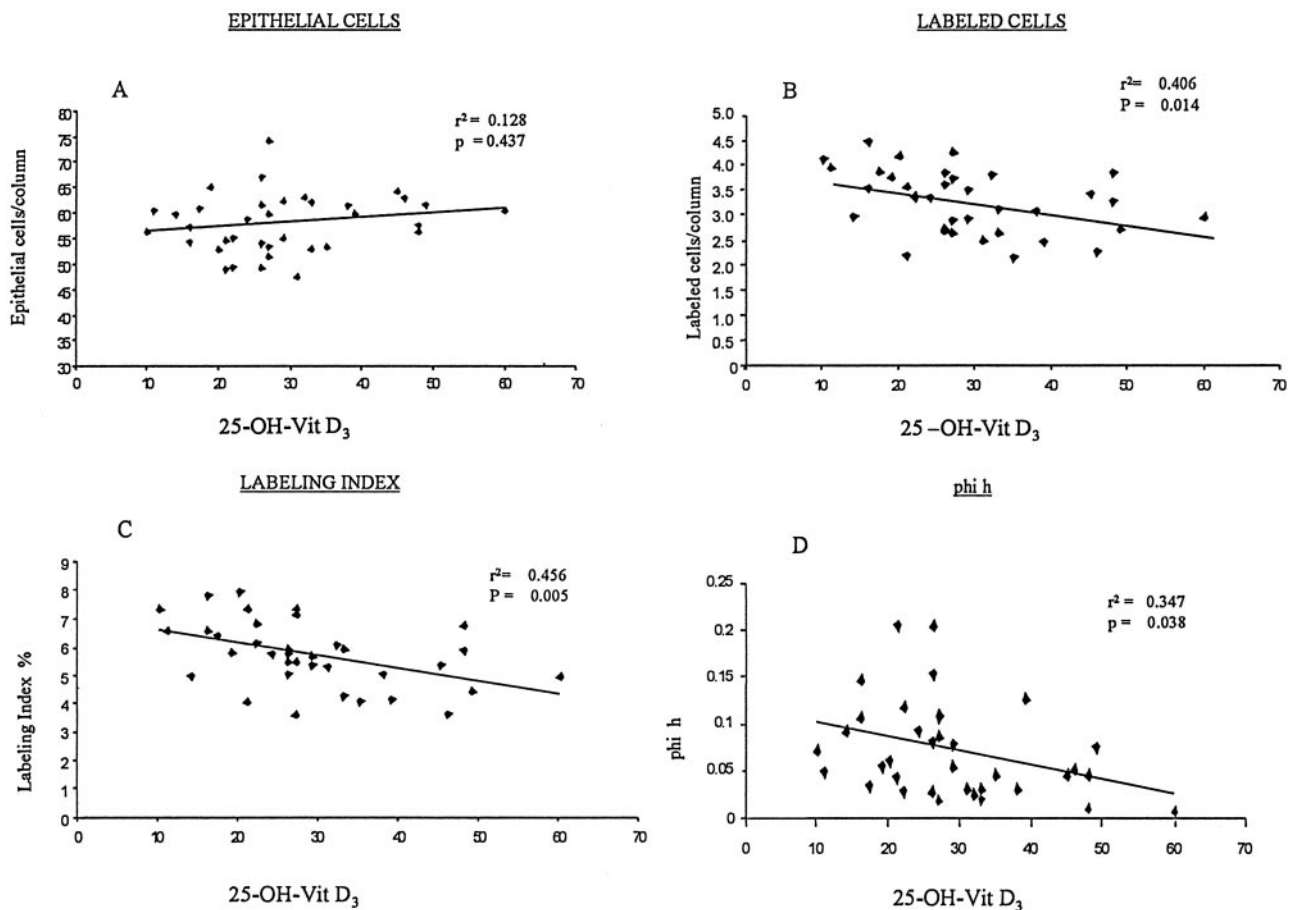


Fig. 1. Correlation between serum levels of 25-OH vitamin D and cell proliferation. Data are shown in ng/ml at study end from the total group of 39 subjects that completed the study. Correlation ( $r^2$ ) and Ps shown on each figure. Top left, total epithelial cell number per crypt column; top right, labeled cells/crypt column; bottom left, labeling index (%); bottom right, phi h value.

of 1922 mg/day, and group B subjects received a total of 1925 mg/day. The vitamin D intake of group B subjects totaled 19.8  $\mu$ g/day during the study. Standard laboratory determinations of serum phosphorus, alkaline phosphatase, and liver and renal function were within normal limits and did not change between baseline and study end in any study group.

At baseline, serum levels of calcium, 25-OH D<sub>3</sub>, and 1,25-(OH)<sub>2</sub> D<sub>3</sub> were within normal limits and did not differ among the groups. Serum calcium and vitamin D concentrations at the completion of the study are shown in Table 1. There were no significant changes in blood levels of 25-OH D<sub>3</sub> nor 1,25-(OH)<sub>2</sub> D<sub>3</sub> during the study period, except in group B subjects receiving vitamin D supplements, who increased their 25-OH D<sub>3</sub> level by 44% ( $P < 0.001$ ) without significant changes in 1,25-(OH)<sub>2</sub> D<sub>3</sub> levels, and in group C subjects, who received 1,25-(OH)<sub>2</sub> D<sub>3</sub> where there were no changes in 25 OH D<sub>3</sub> levels and a modest (14%) but significant rise in serum 1,25-(OH)<sub>2</sub> D<sub>3</sub> levels.

The proliferative indices did not change between study start and study end within any group.<sup>10</sup> Furthermore, the VDR BsmI genotypes of the study groups did not affect proliferative indices (as shown in Table 2) for total crypt labeling index and

phi h. We then studied the relationship between fasting serum levels of 25-OH D<sub>3</sub> or 1,25-(OH)<sub>2</sub> D<sub>3</sub> and crypt cell proliferation kinetics (Table 3) and differentiation markers (Table 4) at the onset and end of the study. At baseline, no correlations were found between the serum levels of either 25-OH or 1,25-(OH)<sub>2</sub> D<sub>3</sub> and the proliferation cell kinetics or differentiation markers (cytokeratin AE1 or acidic mucin scores). This may have occurred because there was relatively little variation in the blood levels of serum 25-OH D<sub>3</sub> or 1,25-(OH)<sub>2</sub> D<sub>3</sub> metabolites before supplemental calcium or vitamin D was administered. In striking contrast, there were significant correlations between serum 25-OH D<sub>3</sub> levels and epithelial cell proliferative kinetics at the 6-month end point of the study (Fig. 1), whereas no correlation were found between serum 1,25-(OH)<sub>2</sub> D<sub>3</sub> vitamin levels and either whole crypt labeling index or phi h (Fig. 2).

To evaluate these correlations at study end more closely, we determined the relationship between proliferative indices and vitamin D levels in subjects in groups A, B, and C separately. In group C subjects receiving 1,25-(OH)<sub>2</sub> D<sub>3</sub> for 6 months, there were no significant correlations with any of the individual parameters studied. In contrast, despite the small number of subjects examined, cell proliferation decreased as blood levels of vitamin 25-OH D<sub>3</sub> increased in subjects in groups A and B who were taking supplemental calcium ( $P = 0.005$  and 0.08; Fig. 3).

<sup>10</sup> Unpublished data.

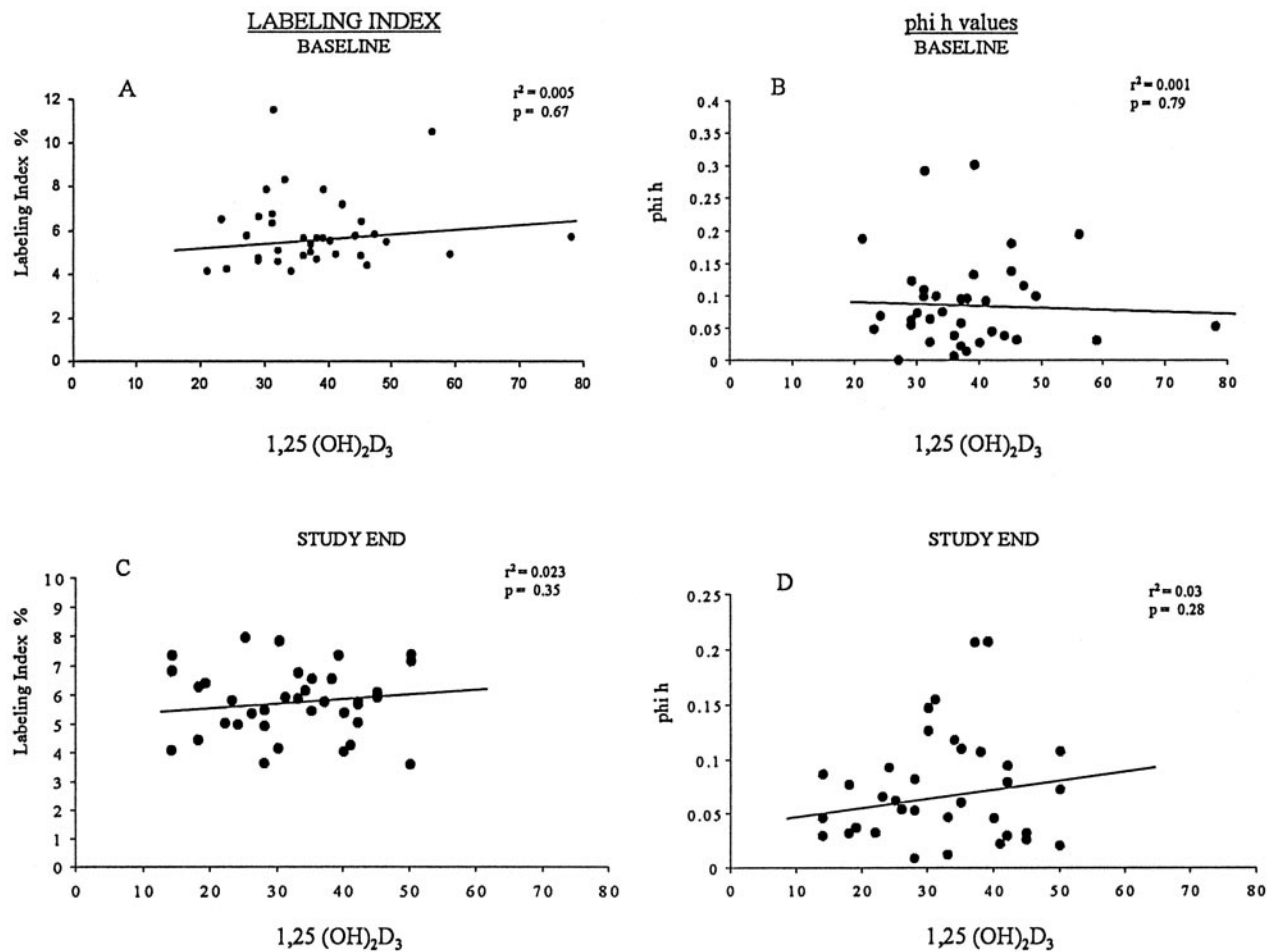


Fig. 2. Correlation between serum levels of 1,25-(OH)<sub>2</sub> vitamin D and cell proliferation. Data are shown in pg/ml from the total group of subjects that completed the study. Correlations and *P*s as in Fig. 1. *Top left*, labeling index (%) at baseline; *bottom left*, labeling index (%) at study end; *top right*, phi h at baseline; *bottom right*, phi h at study end.

We then evaluated the effect of calcium supplementation upon the relationships between serum 25-OH D<sub>3</sub> and epithelial cell labeling index and phi h at the study end. This was done using an analysis of covariance for the slopes in groups A + B subjects (who were taking supplemental calcium) and the slope for group C subjects (no supplemental calcium) for 25-OH D<sub>3</sub> with individual parameters of proliferation. There was a significant difference between the slopes for labeling index (significance of covariance, *P* = 0.03) and a trend for phi h (*P* = 0.08). In contrast, there was no effect of calcium upon 1,25-(OH)<sub>2</sub> D<sub>3</sub> for either labeling index (significance of covariance, *P* = 0.7) or for phi h (*P* = 0.59). Thus, supplemental calcium increased the effects of 25-OH D<sub>3</sub> upon epithelial cell proliferation.

We further determined relationships between serum 25-OH D<sub>3</sub> and 1,25-(OH)<sub>2</sub> D<sub>3</sub> levels with VDR genotypes without finding any significant correlations with proliferation indices. Furthermore, there were no significant correlations between the acidic mucin scores or cytokeratin AE1 scores and serum concentration of either 25-OH D<sub>3</sub> or of 1,25-(OH)<sub>2</sub> D<sub>3</sub> (Table 4).

## Discussion

In the present prospective randomized study of the effects of the oral administration of calcium and/or vitamin D as calciferol or as

1,25-(OH)<sub>2</sub> D<sub>3</sub> on indices of rectal epithelial proliferation and differentiation, we had the opportunity to determine relationships of proliferative parameters to circulating 25-OH D<sub>3</sub> and 1,25-(OH)<sub>2</sub> D<sub>3</sub>. Vitamin D activity in epithelial cells involves the conversion of 25-OH D<sub>3</sub> to 1,25-(OH)<sub>2</sub> D<sub>3</sub> by a 1 $\alpha$  hydroxylase enzyme and the interaction of 1,25-(OH)<sub>2</sub> D<sub>3</sub> with nuclear receptors. These receptors are a thousand-fold more responsive to 1,25-(OH)<sub>2</sub> D<sub>3</sub> than to 25-OH D<sub>3</sub> and determine vitamin D action on a variety of end organs. Vitamin D is well known to have profound effects upon small intestinal cells upregulating calbindin, the cytosolic calcium binding protein (20), as well as calcium pumps situated on the lateral basolateral membrane of small intestinal epithelial cells (21). These cellular effects are believed to greatly enhance the transport of calcium from the lumen of the small intestine and colon into the bloodstream (22, 23). Because the colon is also a site for calcium absorption, it has been assumed that 1,25-(OH)<sub>2</sub> D<sub>3</sub> similarly affects cellular transport in colonic epithelial cells. Furthermore, until recently, it has been believed that virtually all 1,25-(OH)<sub>2</sub> D<sub>3</sub> is formed from 25-OH D<sub>3</sub> in the kidney, based upon the observation that nephrectomy almost completely depletes circulating 1,25-(OH)<sub>2</sub> D<sub>3</sub> (24).

However, in pathological states, such as sarcoidosis, the body is known to be very sensitive to administration of calciferol (25).

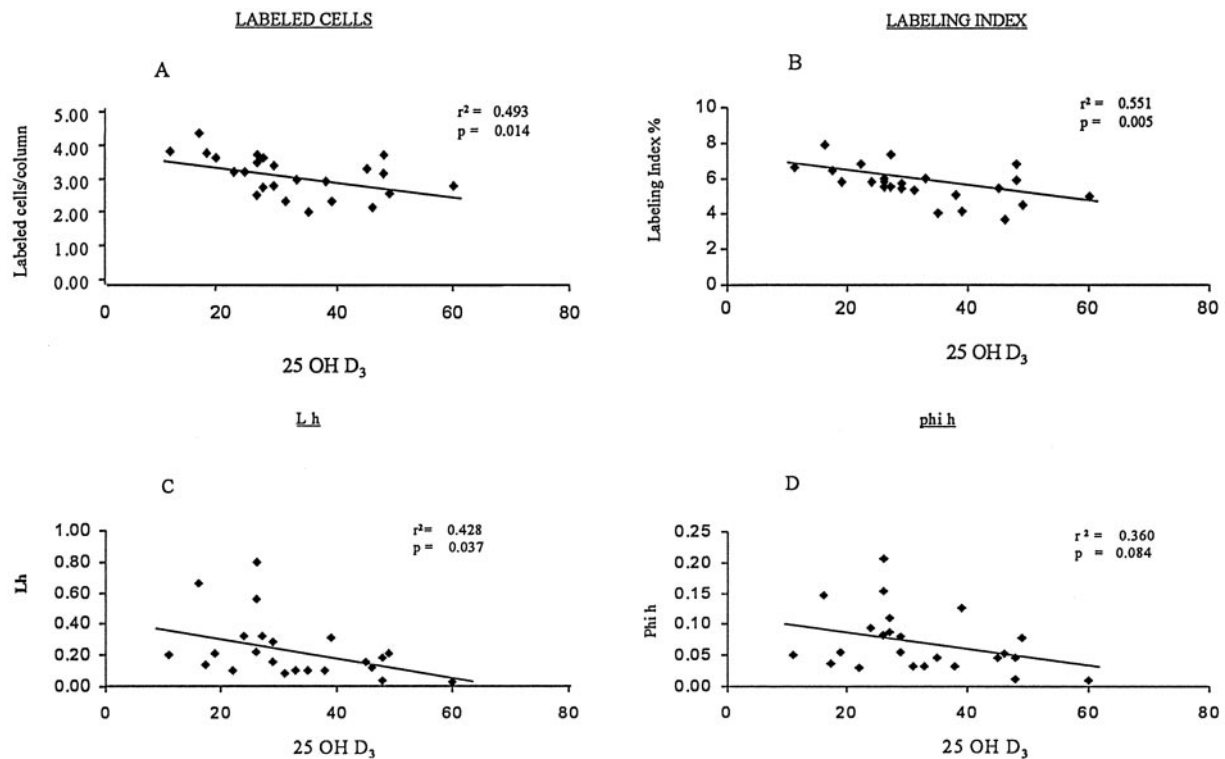


Fig. 3. Correlation between serum levels of 25-OH vitamin D and cell proliferation. Data are shown in ng/ml at study end from subjects in groups A + B given calcium  $\pm$  vitamin D who completed the study. Correlations and *P*s as in Fig. 1. *Top left*, labeled cells/crypt; *top right*, labeling index (%); *bottom left*, Lh value; *bottom right*, phi h value.

This has been shown to result from formation of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in monocytes because of the presence in these cells of a 25-hydroxyvitamin D 1 $\alpha$ -OHase (26, 27). Very recently, extrarenal conversion of 25-OH D<sub>3</sub> to 1,25-(OH)<sub>2</sub>D<sub>3</sub> has been demonstrated to occur through the action of such a 1 $\alpha$ -OHase enzyme in keratinocytes (11) and prostatic cancer cells *in vitro* (12). Furthermore, prostatic cells have been shown to have VDRs that respond to the addition of 1,25-(OH)<sub>2</sub>D<sub>3</sub> to an incubation medium (14). It has been proposed very recently that vitamin D may be a chemopreventive or chemotherapeutic agent for prostate cancer.

VDRs have been demonstrated to be present in colon cancer cells (28, 29). Such colon cancer cells respond to incubation with 1,25-(OH)<sub>2</sub>D<sub>3</sub> at concentrations between  $1 \times 10^{-9}$  and  $1 \times 10^{-7}$  M by inhibition of proliferation (30, 31) and, in some situations, enhanced differentiation (32). Furthermore, administration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> to experimental animals deficient in vitamin D has resulted in a series of different cellular changes within the colon (33–35). Because of these observations, it has been suggested that noncalcemic derivatives of 1,25-(OH)<sub>2</sub>D<sub>3</sub>, which can interact with colonic VDRs, might be useful in the treatment or prevention of colorectal neoplasia (36).

The present study clearly shows significant correlations between circulating levels of 25-OH D<sub>3</sub> with indices of colorectal epithelial cell proliferation. These observations led us to suggest that there might be 1 $\alpha$ -OHase enzyme present in normal colonic mucosa that could convert circulating 25-OH D<sub>3</sub> to 1,25-(OH)<sub>2</sub>D<sub>3</sub> in epithelial cells. Indeed, very recent preliminary observations in our laboratory have demonstrated the presence of the mRNA 1 $\alpha$ -OHase in extracts of normal colorectal mucosa and greatly increased levels in some adjacent human colon cancers (37). Since the comple-

tion of this report, Zehnder *et al.* (38) have described extrarenal 1 $\alpha$ -OHase in several tumors including the colorectum using *in situ* hybridization and immunohistochemical analysis. Cross *et al.* (39) also have demonstrated very recently 1 $\alpha$ -OHase mRNA in colonic tumors and normal adjacent colonic tissue. It therefore now appears that there can be local autocrine or paracrine control of 1,25-(OH)<sub>2</sub>D<sub>3</sub> formation from 25-OH D<sub>3</sub> in colorectal epithelia, which thereby alters colonic cell proliferation.

Our data also suggest that there is an important interaction between administration of supplemental dietary calcium and the levels of circulating 25-OH D<sub>3</sub>, which has an effect upon colorectal epithelial cell proliferation. Thus, some of the variation in colorectal cell proliferation occurring after calcium administration reported in several previous studies (40–42) might have been attributable to lower serum 25-OH vitamin D levels. Vitamin D may have a further important role in mediating a calcium inhibitory effect on abnormal colorectal cell development (43, 44) and in determining an intracellular calcium gradient in mammalian colonic crypts (45). Future studies of the chemopreventive action of calcium and vitamin D must take into account the levels of circulating 25-OH D<sub>3</sub> and probably the activity of the colonic enzyme that converts 25-OH D<sub>3</sub> to 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

#### Acknowledgments

We thank Dr. John O'Connor for performing the vitamin D assays, Ann Washington for excellent technical assistance, Jodi Gilman for nutritional evaluation of the subjects, and Dr. Donald McMahon for data management. Furthermore, we thank the many physicians who allowed their patients to be studied and, above all, the forbearance of the volunteer subjects.

## References

- Garland, C. F., and Garland, F. C. Do sunlight and vitamin D reduce the likelihood of colon cancer? *Int. J. Epidemiol.*, *9*: 227–231, 1980.
- Garland, C. F., Garland, F. C., and Gorham, E. D. Can colon cancer incidence and death rates be reduced with calcium and vitamin D? *Am. J. Clin. Nutr.*, *54*: 193S–201S, 1991.
- Emerson, J. C., and Weiss, N. S. Colorectal cancer and solar radiation. *Cancer Causes Control*, *3*: 95–99, 1992.
- Holick, M. F., MacLaughlin, J. A., Clark, M. B., Holick, S. A., and Potts, J. T. Photosynthesis of previtamin D in human skin and the physiologic consequences. *Science (Wash DC)*, *212*: 203–205, 1980.
- Garland, C., Shekelle, R. B., Barrett-Connor, E., Criqui, M. H., Ross, A. H., and Paul, O. Dietary vitamin D and calcium and risk of colorectal cancer: a 19-year prospective study in men. *Lancet*, *i*: 307–309, 1985.
- Tangrea, J., Helzlsouer, K., Pietinen, P., Taylor, P., Hollis, B., Virtamo, J., and Albanes, D. Serum levels of vitamin D metabolites and the subsequent risk of colon and rectal cancer in Finnish men. *Cancer Causes Control*, *8*: 615–625, 1997.
- Platz, E. A., Hankinson, S. E., Hollis, B. W., Colditz, G. A., Hunter, D. J., Speizer, F. E., and Giovannucci, E. Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and adenomatous polyps of the distal colorectum. *Cancer Epidemiol. Biomark. Prev.*, *9*: 1059–1065, 2000.
- Holick, M. F. Chapter 18: Vitamin D. *In*: M. E. Shils, J. A. Olson, M. Shike, and A. C. Ross. *Modern Nutrition in Health and Disease*, pp. 329–346. Baltimore, MD: Lippincott Williams and Wilkins, 1999.
- Reichel, H., Koefler, H. P., and Norman, A. W. The role of the vitamin D endocrine system in health and disease. *N. Engl. J. Med.*, *320*: 981–991, 1989.
- Adams, J. S., Sharma, O. P., Gacad, M. A., and Singer, F. R. Metabolism of 25-hydroxyvitamin D<sub>3</sub> by cultured alveolar macrophages in sarcoidosis. *J. Clin. Investig.*, *72*: 1856–1860, 1983.
- Bikle, D. D., Nemanic, M. F., Gee, E., and Elias, P. 1,25(OH)<sub>2</sub>D<sub>3</sub> production by human keratinocytes. Kinetics and regulation. *J. Clin. Investig.*, *78*: 557–566, 1986.
- Peehl, D. M., Skowronski, R. J., Leung, G. K., Wong, S. T., Stamey, T. A., and Feldman, D. Antiproliferative effects of 1,25-dihydroxyvitamin D<sub>3</sub> on primary cultures of human prostatic cells. *Cancer Res.*, *54*: 805–810, 1994.
- Miller, G. J., Stapleton, G. E., Ferrara, J. A., Lucia, M. S., Pfister, S., Hedlund, T. E., and Upadhyay, P. The human prostatic carcinoma cell line Lncap expresses biologically active, specific receptors for 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. *Cancer Res.*, *52*: 515–520, 1992.
- Holt, P. R., Arber, N., O'Connor, J., Halmos, B., McGlynn, C., Moss, S. F., Pou, R., Yang, K., Fan, K., Newmark, H., and Lipkin, M. Serum 25-hydroxyvitamin D<sub>3</sub> inhibits proliferation of colonic epithelial cells in subjects at high risk for colon neoplasia. *Gastroenterology*, *118*: A276, 2000.
- Hollis, B. W. Assay of circulating 1,25-dihydroxyvitamin D involving a novel single cartridge extraction and purification procedure. *Clin. Chem.*, *32*: 2060–2063, 1986.
- Hollis, B. W., Kamerud, J. Q., Kurkowsky, A., Beaulieu, J., and Napoli, J. L. Quantification of circulating 1,25-dihydroxyvitamin D by radioimmunoassay with <sup>125</sup>I-labeled tracer. *Clin. Chem.*, *42*: 586–592, 1996.
- Lipkin, M., Enker, W. E., and Winawer, S. J. Tritiated-thymidine labeling of rectal epithelial cells in “non-prep” biopsies of individuals at increased risk for colonic neoplasia. *Cancer Lett.*, *87*: 153–161, 1987.
- Yang, K., Fan, K., Newmark, H., Leung, D., Lipkin, M., Steele, V. E., and Kelloff, G. J. Cytokeratin, lectin, and acidic mucin modulation in differentiating colonic epithelial cells of mice after feeding Western-style diets. *Cancer Res.*, *56*: 4644–4648, 1996.
- Morrison, N. A., Qi, J. C., Tokita, A., Kelly, P. J., Crofts, L., Nguyen, T. V., Sambrook, P. N., and Eisman, J. A. Prediction of bone mineral density from vitamin D receptor alleles. *Nature (Lond.)*, *367*: 284–287, 1994.
- Christakos, S., Gabrielides, C., and Rhoten, W. B. Vitamin D-dependent calcium binding proteins: chemistry, distribution, functional considerations and molecular biology. *Endocrine Rev.*, *10*: 3–26, 1989.
- Ghijzen, W. E. J. M., and van Os, C. H. 1 $\alpha$ , 25-dihydroxyvitamin D<sub>3</sub> regulates ATP-dependent calcium transport in basolateral plasma membranes of rat enterocytes. *Biochim. Biophys. Acta*, *689*: 170–172, 1982.
- Wasserman, R. H., Brindak, M. E., Meyer, S. A., and Fullmer, C. S. Evidence for multiple effects of vitamin D<sub>3</sub> on calcium absorption: response of rachitic chicks, with or without partial vitamin D<sub>3</sub> repletion, to 1,25-dihydroxyvitamin D<sub>3</sub>. *Proc. Natl. Acad. Sci. USA*, *79*: 7939–7943, 1982.
- Favus, M. J., Kathpalia, S. C., Coe, F. L., and Mond, E. Effects of diet calcium and 1,25-dihydroxyvitamin D<sub>3</sub> on colon calcium active transport. *Am. J. Physiol.*, *238*: G75–G78, 1980.
- Fraser, D. R., and Kodicek, E. Unique biosynthesis of a biologically active vitamin D metabolite. *Nature (Lond.)*, *228*: 764–766, 1970.
- Harrell, G. T., and Fisher, S. Blood chemical changes in Boeck's sarcoid. *J. Clin. Investig.*, *18*: 687–693, 1939.
- Adams, J. S., Singer, F. R., Gacad, M. A., Sharma, O. P., Hayes, M. J., Vouros, P., and Holick, M. F. Isolation and structural identification of 1,25-dihydroxyvitamin D<sub>3</sub> produced by cultured alveolar macrophages in sarcoidosis. *J. Clin. Endocrinol. Metab.*, *60*: 960–966, 1985.
- Adams, J. S., and Gacad, M. A. Characterization of 1 $\alpha$ -hydroxylation of vitamin D<sub>3</sub> sterols by cultured macrophages from patients with sarcoidosis. *J. Exp. Med.*, *161*: 755–765, 1985.
- Brehier, A., and Tomassett, M. Human colon cell line HT-29: characterization of 1,25-dihydroxyvitamin D<sub>3</sub> receptors in established human cancer cell lines in culture. *Cancer Res.*, *42*: 1116–1119, 1982.
- Giuliano, A. R., Franceschi, T. R., and Wood, R. J. Characterization of the vitamin D receptor from the Caco-2 human colon carcinoma cell line: effect of cellular differentiation. *Arch. Biochem. Biophys.*, *285*: 261–269, 1991.
- Thomas, M. G., Tebbutt, S., and Williamson, R. N. Vitamin D and its metabolites inhibit cell proliferation in human rectal mucosa and a colon cancer cell line. *Gut*, *33*: 1660–1663, 1992.
- Cross, H. S., Pavelka, M., Slavik, J., and Peterlik, M. Growth control of human colon cancer cells by vitamin D and calcium *in vitro*. *J. Natl. Cancer Inst.*, *84*: 1355–1357, 1992.
- Shabahang, M., Buras, R. R., Davoodi, F., Schumaker, L. M., Nauta, R. J., and Evans, S. R. T. 1,25-Dihydroxyvitamin D<sub>3</sub> receptor as a marker of human colon carcinoma cell line differentiation and growth inhibition. *Cancer Res.*, *53*: 3712–3728, 1993.
- Wali, R. K., Baum, C. L., Sitrin, M. D., Bolt, M. J. G., Dudeja, P. K., and Brasitus, T. A. Effect of vitamin D status on the rapid actions of 1,25 dihydroxycholecalciferol in rat colonic membranes. *Am. J. Physiol.*, *262*: 945–953, 1992.
- Bolt, M. J. G., Bissonnette, B. M., Wali, R. K., Hartmann, S. C., Brasitus, T. A., and Sitrin, M. D. Characterization of phosphoinositide-specific phospholipase C in rat colonocyte membranes. *Biochem. J.*, *292*: 271–276, 1993.
- Khare, S., Bolt, M. J. G., Wali, R. K., Skarosi, S. F., Roy, H. K., Niedziela, S., Scaglione-Sewell, B., Aquino, B., Abraham, C., Sitrin, M. D., Brasitus, T. A., and Bissonnette, M. 1,25-Dihydroxyvitamin D<sub>3</sub> stimulates phospholipase c in rat colonocytes: role of c-Src in PLC- $\gamma$  activation. *J. Clin. Investig.*, *99*: 1831–1841, 1997.
- Wali, R. K., Bissonnette, M., Khare, S., Hart, J., Sitrin, M. D., and Brasitus, T. A. 1 $\alpha$ ,15-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol, a non-calcemic analogue of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, inhibits azoxymethane-induced colonic carcinogenesis. *Cancer Res.*, *55*: 3050–3054, 1995.
- Tangpricha, V., Flanagan, J. N., Whitlatch, L. W., Tseng, C. C., Holt, P. R., Lipkin, M. S., Holick, M. F. The presence of 25-hydroxyvitamin D-1 $\alpha$  hydroxylase in normal and malignant colon tissue and colon cancer cell lines. *Lancet*, *357*: 1673–1674, 2001.
- Zehnder, D., Bland, R., Williams, M. C., McNinch, R. W., Howie, A. J., Stewart, P. M., and Hewison, M. Extrarenal expression of 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase. *J. Clin. Endocrinol. Metabol.*, *86*: 888–894, 2001.
- Cross, H. S., Bareis, P., Hofer, H., Bischof, M. G., Bajna, E., Kriwanek, S., Bonner, E., and Peterlik, M. 25-Hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase and vitamin D receptor gene expression in human colonic mucosa is elevated during early cancerogenesis. *Steroids*, *66*: 287–292, 2001.
- Bostick, R. M. Human studies of calcium supplementation and colorectal epithelial cell proliferation. *Cancer Epidemiol. Biomark. Prev.*, *6*: 971–980, 1997.
- Ahnen, D. J., and Byers, T. Proliferation happens. *JAMA*, *280*: 1095–1096, 1998.
- Holt, P. R. Dairy foods and prevention of colon cancer: human studies. *JACN*, *18*: 380S–391S, 1999.
- Holt, P. R., Attilasoy, E. O., Gilman, J., Guss, J., Moss, S. F., Newmark, H., Fan, K., Yang, K., and Lipkin, M. Modulation of abnormal colonic epithelial cell proliferation and differentiation by low fat dairy foods. *JAMA*, *280*: 1074–1079, 1998.
- Baron, J. A., Beach, M., Mandel, J. S., et al. Calcium supplements for the prevention of colorectal adenomas. *N. Engl. J. Med.*, *340*: 101–108, 1999.
- Brenner, B. M., Russell, N., Albrecht, S., and Davies, R. J. The effect of dietary vitamin D<sub>3</sub> on the intracellular calcium gradient in mammalian colonic crypts. *Cancer Lett.*, *127*: 43–53, 1998.

### Correction

Re: Holt, *et al*: Colonic Epithelial Cell Proliferation Decreases with Increasing Levels of Serum 25-Hydroxy Vitamin D. *11*: 113–119, January 2002.

Dr. Kurihara's initials would also be listed with Strang Cancer Prevention Center, New York, New York. The authors names should read as follows:

Peter R. Holt,<sup>2,3</sup> Nadir Arber,<sup>4</sup> Balazs Halmos,<sup>5</sup> Kenneth Forde, Harry Kissileff, Katharine A. McGlynn,<sup>6</sup> Steven F. Moss,<sup>7</sup> Naoto Kurihara, Kunhua Fan, Kan Yang, and Martin Lipkin

Re: Dietrich, *et al*: Antioxidant Supplementation Decreases Lipid Peroxidation Biomarker F<sub>2</sub>-isoprostanes in Plasma of Smokers. *11*: 7–13, 2002.

Footnote 1 on page 7 should have included the following:

Supported by the University of California Tobacco-Related Disease Research Program (TRDRP:6RT-0008 and 7RT-0160), by the NIH Grant P30 ES01896, and in part by the German Academic Exchange Service, DAAD, Postdoctoral Fellowship for Dr. Marion Dietrich. Work in J. D. M.'s laboratory is supported by NIH Grants GM15431, DK26657, and DK48831. J. D. M. is the recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.



# BLOOD CANCER DISCOVERY

## Colonic Epithelial Cell Proliferation Decreases with Increasing Levels of Serum 25-Hydroxy Vitamin D

Peter R. Holt, Nadir Arber, Balazs Halmos, et al.

*Cancer Epidemiol Biomarkers Prev* 2002;11:113-119.

**Updated version** Access the most recent version of this article at:  
<http://cebp.aacrjournals.org/content/11/1/113>

**Cited articles** This article cites 38 articles, 14 of which you can access for free at:  
<http://cebp.aacrjournals.org/content/11/1/113.full#ref-list-1>

**Citing articles** This article has been cited by 18 HighWire-hosted articles. Access the articles at:  
<http://cebp.aacrjournals.org/content/11/1/113.full#related-urls>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://cebp.aacrjournals.org/content/11/1/113>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.