Plasma 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D and Risk of Breast Cancer

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Abstract

Several lines of evidence suggest that vitamin D may reduce incidence of breast cancer, but few epidemiologic studies have addressed the relation of plasma vitamin D metabolites to the risk of this disease. We prospectively examined the relationship between plasma levels of 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] and risk of breast cancer in a case-control study nested within the Nurses' Health Study cohort. Blood samples were collected from study participants in 1989-1990. Breast cancer cases developing between blood collection and June 1, 1996, were matched to cancer-free controls on the basis of age, menopausal status, and other factors. Stored plasma samples from 701 cases and 724 controls were available for metabolite analysis. Cases had a lower mean 25(OH)D level than controls (P = 0.01), but mean 1,25(OH)$_2$D levels were similar (P = 0.49). High levels of both metabolites were associated with a nonsignificant lower risk of breast cancer. Women in the highest quintile of 25(OH)D had a relative risk of 0.73 (95% confidence interval = 0.49-1.07; P_{trend} = 0.06) compared with those in the lowest quintile. For 1,25(OH)$_2$D, the comparable relative risk was 0.76 (95% confidence interval = 0.52-1.11; P_{trend} = 0.39). For both metabolites, the association was stronger in women ages 60 years and older, but results were not statistically significant. Our findings suggest that high levels of 25(OH)D, and perhaps 1,25(OH)$_2$D, may be modestly associated with reduced risk of breast cancer. (Cancer Epidemiol Biomarkers Prev 2005;14(8):1991–7)

Introduction

Results from a variety of studies suggest that vitamin D may reduce risk of breast cancer. Both diet and sunlight contribute to circulating levels of serum vitamin D metabolites. Dietary intake of fortified dairy foods and cereals, some types of fish, multivitamins, and calcium/vitamin D supplements, contribute importantly to vitamin D in elderly populations and those with low ambient sunlight exposure (1-3). In populations with ample sun exposure, cutaneous conversion of 7-dehydrocholesterol to previtamin D after exposure to solar UV radiation provides the greater source. Previtamin D from both diet and cutaneous production is hydroxylated in the liver into 25-hydroxyvitamin D [25(OH)D], the metabolite circulating in the greatest concentration. 25(OH)D is then further hydroxylated to 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] in the kidney nephrons and, as suggested by recent studies, in tissues of the breast, colon, and prostate (4-7). 1,25(OH)$_2$D is the biologically active metabolite that binds to nuclear vitamin D receptors in the intestine and bone, as well as breast and other tissues (8).

Numerous *in vitro* studies have indicated that 1,25(OH)$_2$D can inhibit cell proliferation and promote cell differentiation in breast tumor tissue (5, 9-11), suggesting that high levels of vitamin D metabolites may be protective against breast cancer. In addition, several ecologic studies have reported lower breast cancer incidence and mortality rates in populations with high sunlight exposure, and thus higher vitamin D levels, compared with those with lower ambient sunlight (12-15). However, relatively few epidemiologic studies have directly addressed the relationship between vitamin D and the incidence of breast cancer (15-19) and results have been inconclusive. In the Nurses' Health Study cohort, Shin et al. (16) found that vitamin D intake of ≥500 IU/d was associated with a significant 28% lower risk of breast cancer in premenopausal women, but did not observe a relationship in postmenopausal women. A modest reduction in risk with higher dietary vitamin D intake and/or sun exposure was also observed in the National Health and Nutrition Examination Survey I cohort (15), but not in a case-control study in Canada (19). Only two studies have evaluated how serum vitamin D metabolites may relate to breast cancer (17, 18). Janowsky et al. (17) found mean 1,25(OH)$_2$D levels in plasma samples collected at diagnosis to be significantly lower in breast cancer cases than controls, whereas mean 25(OH)D levels did not differ by disease status. In contrast, Hiatt et al. (18) did not find any association between 1,25(OH)$_2$D levels in blood samples collected between 1964 and 1972 and the incidence of breast cancer (n = 96 cases) over 19 to 27 years of follow-up.

The relationship between specific vitamin D metabolites and the development of breast cancer remains unknown. Although 1,25(OH)$_2$D is the biologically active form that binds to vitamin D receptors in target tissues, its production is tightly regulated (20) and it may thus be a poor predictor of cancer risk. In contrast, 25(OH)D is more sensitive to dietary intake and solar exposure and better reflects overall vitamin D status (1, 2, 20). Furthermore, it is unclear when vitamin D status may be most important in breast cancer etiology and at what point in life metabolite levels should be measured to best predict the risk of disease. To explore these

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issues, we have evaluated the relationship between plasma levels of 25(OH)D and 1,25(OH)$_2$D and risk of breast cancer in a case-control study nested within the Nurses' Health Study cohort.

Materials and Methods

Study Population. The Nurses' Health Study is a cohort of 121,700 U.S. female registered nurses who responded to a mailed questionnaire in 1976. Participants were 30 to 55 years old at the time of the initial mailing and provided information on their medical history and health-related behaviors, such as use of oral contraceptives and smoking status. Cohort members have completed questionnaires every 2 years thereafter to update information on various risk factors and to identify new diagnoses of cancer and other diseases. The protocol for this study was approved by the Human Research Committees of Harvard Medical School and Brigham and Women's Hospital (Boston, MA).

In 1989-1990, we asked participants who had not previously been diagnosed with cancer to provide a blood sample, which were ultimately received from 32,826 study members ages 43 to 69. The details of collection have been previously described in detail (21). Briefly, we provided participants with a kit of blood collection supplies and asked them to have their blood drawn and returned to us via overnight courier. We also provided an ice pack to keep the sample cool during mailing. Participants also completed a short questionnaire providing information on the day and time of collection, their current body weight, and current use of medications including hormone replacement therapy. Ninety-seven percent of samples were received by our laboratory within 26 hours of draw. Upon receipt, samples were centrifuged; separated into plasma, red cell, and white cell components; and archived at $-130°C$ or colder in continuously monitored liquid nitrogen freezers. As of May 31, 1996, the follow-up rate of participants contributing blood samples was 99%.

Breast Cancer Nested Case-Control Study. Eligible cases for the present study included all participants providing blood samples who reported an incident diagnosis of breast cancer on a biennial Nurses' Health Study questionnaire between the date of return of the blood sample and the end of the study on June 1, 1996. New diagnoses were reported by 735 women, ~20% of whom were premenopausal and 70% were postmenopausal at the time of blood collection; the remaining 10% were perimenopausal or of uncertain menopausal status. The duration of follow-up between blood collection and breast cancer diagnosis ranged from <1 to 82 months. We asked women reporting a diagnosis for permission to review their medical records to confirm the diagnosis and identify tumor location, histologic type, subtype, and invasiveness.

Controls were chosen from participants who provided blood samples but who did not report a diagnosis of any cancer before June 1, 1996. One control was matched to each breast cancer case on the basis of age (±2 years), menopausal status (premenopausal versus postmenopausal versus unknown), use of postmenopausal hormones within 3 months of blood collection (yes versus no), month of blood collection, time of day of blood collection (±2 hours), and fasting status at the time of collection (<10 hours or unknown versus ≥10 hours since last meal). Most case-control matches were exact, although we relaxed the criteria for some factors when necessary.

Analysis of Vitamin D Metabolites. For the analysis of 25(OH)D, plasma was available from 701 cases and 724 controls; if sufficient plasma was not available for one member of a case-control pair, we analyzed the sample anyway. Samples were analyzed in three batches. Dr. Michael F. Holick (Boston University School of Medicine, Boston, MA) analyzed samples from batch 1 (178 cases and 184 controls) between November 1993 and July 1994 and batch 2 (279 cases and 286 controls) between October 1999 and June 2000. Batch 3 samples (244 cases and 254 controls) were analyzed between June and September 2003 by Dr. Bruce W. Hollis (Medical University of South Carolina, Charleston, SC).

For all assays, case-control pairs were analyzed together and were ordered randomly within pair to mask the laboratory to disease status. We also included masked split samples of plasma from three plasma pools (two pools for batch 3) along with each batch to evaluate laboratory precision and assess between-batch variation. The assays used for measurement of plasma 25(OH)D have been previously described in detail (22, 23). Briefly, plasma samples assayed by Chen et al. (22) were extracted with absolute ethanol, and the extract was then treated with a protein-binding assay with a high affinity for 25(OH)D. Plasma samples assayed by Hollis (23) were done by RIA with prior acetonitrile extraction. Mean coefficients of variation for 25(OH)D were 17.6% (batch 1), 16.4% (batch 2), and 8.7% (batch 3). We evaluated the correlation between the two assays used to measure 25(OH)D using plasma samples from 20 control women. For all samples, the Pearson correlation coefficient between assays was 0.36 (P = 0.12); after the exclusion of two outliers, the correlation was 0.73 (P = 0.0005). For the analysis of 1,25(OH)$_2$D, plasma was available from 634 cases and 640 controls. Samples were analyzed in a single batch by Dr. Hollis between March and July 2001 by RIA using radioiodinated tracers (23). The mean coefficient of variation for 1,25(OH)$_2$D was 7.3%.

Measurement of Covariates. We evaluated other factors that could potentially be associated with vitamin D metabolites and/or breast cancer incidence. Body mass index [BMI, calculated as weight in kilograms/(height in meters)^2], menopausal status, and menopausal hormone use at blood draw were assessed using the questionnaire participants completed at the time of blood collection. We assessed levels of other factors at approximately the time of blood draw using information provided on previous Nurses' Health Study questionnaires, including height, age at menarche, parity and age at first birth, weight at age 18, age at menopause, family history of breast cancer in participant's mother or sister, history of benign breast disease, alcohol intake, and smoking status. We used the semiquantitative food frequency questionnaire administered as part of the main study questionnaire in 1990 to assess dietary intake of various nutrients, including vitamins A, C, and E; retinol; carotenoids; folate; and calcium. Finally, because plasma samples from these breast cancer cases and controls had been assayed previously for other hormonal and dietary factors, we were able to assess plasma carotenoids and retinol, and, among postmenopausal women not using hormone replacement therapy, plasma estradiol (24).

Data Analysis. We divided participants into quintiles of 25(OH)D and 1,25(OH)$_2$D based on the distribution of these metabolites in the control group. Because the mean and SD of 25(OH)D differed between batches, and identical quality control samples included in each batch varied in the same manner, quintile cut points were calculated separately for each batch (see footnotes to Table 2 for quintile cut points). We first assessed the relationship between each vitamin D metabolite and risk of breast cancer with simple conditional logistic regression, adjusted only for matching factors. Odds ratios were used to estimate the relative risk (RR) of cancer for quintiles Q_2 through Q_5 compared with the lowest quintile. We calculated 95% confidence intervals (95% CI) and used the Mantel extension test to evaluate the presence of linear trend across quintiles; in the test for trend, the median level of each quintile [batch-specific quintile for 25(OH)D] was modeled as a continuous variable in the regression model.

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We then used multivariable conditional logistic regression to control for the effects of other factors. Variables were included in the multivariable regression model if they confounded the vitamin D–breast cancer relationship or had been previously associated with breast cancer risk in our population. Our final model included BMI at age 18, parity/age at first birth, family history of breast cancer, history of benign breast disease, age at menarche, age at menopause, plasma α-carotene level, and intakes of alcohol. Variable categories are presented in the footnotes to Table 2. In postmenopausal women, we also controlled for plasma estradiol (in nonusers of postmenopausal hormones; quartile cut points ≤5, 6-7, 8-11, and ≥12 pg/mL), age at menopause, and total duration of postmenopausal hormone use. Additional factors, including BMI at blood collection, oral contraceptive use and duration, physical activity (as metabolic equivalent task-hours per week), plasma retinol levels, and dietary intake of other nutrients (such as vitamins A, C, and E; retinol; calcium; and folate) were not associated with breast cancer incidence and/or vitamin D metabolites, and were therefore not included in the final model.

In addition to an analysis including all cases, we evaluated the effect of the timing of sample collection by conducting a subanalysis excluding cases diagnosed within 2 years of blood draw and their matched controls. Furthermore, we used unconditional multivariable logistic regression to evaluate the vitamin D–breast cancer relationship to include those cases whose matched control did not have plasma available for metabolite analysis and vice versa. We also used unconditional regression for analyses conducted in subgroups of our population to maximize our statistical power.

To determine if the relationship between vitamin D metabolites and breast cancer risk varied over levels of other factors, we stratified our population by level of the following at blood collection: age (<60 versus ≥60 years), BMI (<25 versus ≥25 kg/m^2), smoking status (never versus past versus current), menopausal status at blood collection and at diagnosis (premenopausal versus postmenopausal), postmenopausal hormone use at blood draw (in postmenopausal women; ever versus never), parity (nulliparous versus parous), time of year of blood collection (May-October versus November-April), plasma retinol levels (≤batch-specific median versus >median), and laboratory analysis method [for 25(OH)D; protein-binding assay versus RIA]. We then compared the RR between levels of these factors; multiplicative interactions were considered statistically significant if the Wald two-sided P value for the interaction term in the multivariable regression model was <0.05. In addition, to control further for differences in 25(OH)D levels by the timing of blood collection, we divided participants into quintiles using cut points specific to the season of blood draw (February-April, May-July, August-October, November-January) as well as to batch and repeated our analyses.

Because women using calcium or vitamin D supplements might be under treatment for bone loss and correspondingly have a low risk of breast cancer (25), we repeated our main analysis after excluding supplement users. Finally, to evaluate whether an association with vitamin D metabolites might vary by characteristics of the breast tumors, we looked separately at the relationship of the metabolites and invasive versus *in situ* tumors, ductal versus lobular tumors, tumors with no node involvement versus node involvement, and tumors by estrogen receptor (ER) and progesterone receptor (PR) status. We then used a polytomous logistic regression procedure to assess whether results differed significantly for ER+/PR+ versus ER+/PR− versus ER−/PR− tumors [there were too few ER−/PR+ cases (n = 17) to evaluate separately]; heterogeneity of RRs was assessed with a likelihood ratio test.

Results

Baseline characteristics of breast cancer cases and matched controls are presented in Table 1. The mean age in both groups was ~57 years. Compared to controls, cases had a slightly lower BMI at age 18 and slightly greater age at first birth. History of benign breast disease and family history of breast cancer was more common in cases than controls, and cases were more likely to be ever smokers and to consume seven or more alcoholic drinks per week. Cases had a lower mean 25(OH)D level than controls (P = 0.01), whereas mean 1,25(OH)$_2$D levels were similar (P = 0.49).

In the simple conditional regression model adjusted for matching factors only, women in the highest quintile of 25(OH)D had a RR of 0.73 (95% CI, 0.52-1.05) compared with those in the lowest quintile (Table 2), with evidence of a linear trend across quintiles (P_{trend} = 0.04). After adjustment for other breast cancer risk factors, women in the highest three quintiles of 25(OH)D were at similarly lower risk, although results were not statistically significant; RRs in Q_3 through Q_5 versus Q_1 were 0.74, 0.77, and 0.73, respectively. When evaluated as a continuous variable, each 1 ng/mL increase in 25(OH)D was associated with a 1.3% lower risk of breast cancer (RR, 0.987; 95% CI, 0.976-0.998; P = 0.02).

We did not observe evidence of a linear relationship between plasma 1,25(OH)$_2$D level and risk of breast cancer (P_{trend} = 0.39). Compared to those in the first quintile, women in Q_2 through Q_5 had multivariable RR of 0.68, 0.73, 0.78, and 0.76, respectively. Results from unconditional regression analysis were essentially unchanged; for example, the RR for the highest versus lowest quintiles for 25(OH)D and 1,25(OH)$_2$D were 0.73 (95% CI, 0.51-1.04) and 0.80 (95% CI, 0.56-1.14), respectively. Additional adjustment of 25(OH)D for 1,25(OH)$_2$D and vice versa did not substantially alter the findings for either metabolite (results not shown). Risk was also lower in women in the highest quintile of both metabolites (31 cases and 38 controls) compared with those in the lowest quintile of both (47 cases and 37 controls; RR, 0.72; 95% CI, 0.35-1.46), although power for this comparison was low.

The associations between 25(OH)D and breast cancer was somewhat stronger in our analysis excluding cases diagnosed in the first 2 years of follow-up (Table 2). Women in the highest quintile of 25(OH)D had a 35% lower risk of disease compared with those in the lowest quintile (RR, 0.65; 95% CI, 0.40-1.06; P_{trend} = 0.06). Results for 1,25(OH)$_2$D, excluding these cases, were similar with those of the main analysis.

Table 1. Characteristics of breast cancer cases and their matched controls at blood collection (1989-1990) among women in the Nurses' Health Study

Characteristic	Cases (n = 701)	Controls (n = 724)
	Mean (SD)	
Age (y)	57.2 (7.0)	57.1 (7.0)
Age at menarche (y)	12.4 (2.0)	12.5 (1.5)
BMI at age 18 (kg/m^2)	21.1 (2.7)	21.4 (2.8)
BMI at blood collection (kg/m^2)	25.5 (4.8)	25.3 (4.6)
Parity* (no. pregnancies >6 mo)	3.2 (1.5)	3.3 (1.6)
Age at first birth* (y)	24.7 (4.5)	24.2 (4.8)
Age at menopause† (y)	48.1 (8.0)	47.8 (8.0)
Plasma 25(OH)D (ng/mL)	31.5 (12.1)	33.1 (12.7)
Plasma 1,25(OH)$_2$D (ng/mL)‡	33.5 (6.9)	33.2 (6.1)
	Percentage (%)	
Postmenopausal	68.3	67.5
Family history of breast cancer	16.8	10.2
History of benign breast disease	35.4	25.7
Ever smokers	56.6	51.4
≥7 alcoholic drinks/wk	14.7	13.3

*Among parous women (n = 654 cases and 656 controls).
†Among postmenopausal women (n = 554 cases and 567 controls).
‡Sufficient plasma for assay available for 634 cases and 640 controls.

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Table 2. RRs and 95% CIs of breast cancer by quintile of plasma 25(OH)D and 1,25(OH)$_2$D

	Cases/controls	All cases and controls		Excluding cases diagnosed 1990-1992
		Simple RR* (95% CI)	Multivariable RR† (95% CI)	Multivariable RR‡ (95% CI)
25(OH)D				
Quintile				
1	159/138	1.00	1.00	1.00
2	149/129	1.00 (0.71-1.39)	0.95 (0.66-1.36)	0.87 (0.56-1.36)
3	125/141	0.75 (0.54-1.06)	0.74 (0.51-1.06)	0.66 (0.43-1.03)
4	144/152	0.80 (0.58-1.11)	0.77 (0.54-1.11)	0.75 (0.48-1.16)
5	124/141	0.73 (0.52-1.05)	0.73 (0.49-1.07)	0.65 (0.40-1.06)
P_{trend}§		0.04	0.06	0.06
1,25(OH)$_2$D				
Quintile				
1	138/127	1.00	1.00	1.00
2	111/121	0.79 (0.55-1.13)	0.68 (0.46-1.02)	0.65 (0.40-1.07)
3	117/122	0.84 (0.59-1.20)	0.73 (0.50-1.07)	0.64 (0.40-1.03)
4	123/122	0.87 (0.61-1.24)	0.78 (0.53-1.14)	0.70 (0.44-1.11)
5	129/126	0.89 (0.63-1.26)	0.76 (0.52-1.11)	0.76 (0.47-1.21)
P_{trend}§		0.91	0.39	0.28

NOTE: For 25(OH)D, quintile cut points for batch 1 were ≤20, 21 to 28, 29 to 33, 34 to 39, and ≥40 ng/mL; for batch 2, ≤28, 29 to 34, 35 to 39, 40 to 47, and ≥48 ng/mL; and for batch 3, ≤18, 19 to 24, 25 to 29, 30 to 36, and ≥37 ng/mL. For 1,25(OH)$_2$D, quintile cut points were ≤28.5, 28.6 to 31.5, 31.6 to 34.3, 34.4 to 38.1, and ≥38.2 ng/mL.
*Conditional logistic regression, adjusted for matching factors (age, fasting status at blood draw, month of blood collection, time of day of blood collection, menopausal status, and current use of postmenopausal hormones).
†Conditional logistic regression, adjusted for matching factors, BMI at age 18 (<19, 19-20.9, 21-22.9, 23-24.9, ≥25 kg/m^2), parity/age at first birth (nulliparous, 1-4 children/age at first birth <25 years, 1-4 children/age at first birth 25-29 years, 1-4 children/age at first birth ≥30 years, ≥5 children/age at first birth <25years, ≥5 children/age at first birth ≥25 years), family history of breast cancer (no family history, history in mother or sister), history of benign breast disease (no, yes), postmenopausal hormone use (continuous, in months), age at menarche (<12, 12, 13 or ≥14 years), age at menopause (<45, 45-49, 50-55, >55 years), alcohol intake (0, 1-2, 3-6, 7-13, ≥14 drinks per week), and plasma α-carotene (batch-specific quintiles).
‡For 25(OH)D, analyses includes 469 cases and their matched controls. For 1,25(OH)$_2$D, analyses includes 443 cases and their matched controls. Adjusted for all factors in multivariable model above.
§Two-sided P_{trend} over quintiles, calculated by using the median value of each quintile as a continuous variable in the multivariable model.

The association between both vitamin D metabolites and breast cancer risk seemed to differ by age at blood collection (Table 3) although multiplicative interactions were not statistically significant. Among women ≥60 years old, participants in the highest quintile of 25(OH)D had a RR of 0.57 (95% CI, 0.31-1.04; P_{trend} = 0.03) compared with those in the lowest quintile. In comparison, 25(OH)D level was not associated with reduced risk of breast cancer in younger women (RR for Q_5 versus Q_1, 0.92; 95% CI, 0.57-1.48; P_{trend} = 0.88). Comparable RRs for 1,25(OH)$_2$D were 0.72 (95% CI, 0.40-1.32; P_{trend} = 0.23) for women ≥60 years old and 0.88 (95% CI, 0.56-1.40; P_{trend} = 0.91) for women <60 years ($P_{interaction}$ = 0.41). Age-stratified results additionally adjusted for plasma estradiol level in postmenopausal women not using hormone replacement were similar compared with results not adjusted for estradiol. For example, after adjustment, the RR for the highest versus lowest quintile in older women was 0.61 (95% CI, 0.33-1.11) for 25(OH)D and 0.73 (95% CI, 0.40-1.34) for 1,25(OH)$_2$D. In younger women, comparable RR were 0.96 (95% CI, 0.59-1.56) for 25(OH)D and 0.91 (95% CI, 0.57-1.45) for

Table 3. RRs and 95% CIs for breast cancer by quintile of 25(OH)D and 1,25(OH)$_2$D, stratified by participant's age at blood collection

	<60 y		≥60 y	
	Case/controls	Multivariable RR* (95% CI)	Cases/controls	Multivariable RR* (95% CI)
25(OH)D				
Quintile				
1	97/94	1.00	62/47	1.00
2	84/86	0.96 (0.62-1.49)	65/49	1.07 (0.60-1.92)
3	77/87	0.80 (0.51-1.26)	48/57	0.64 (0.35-1.16)
4	90/102	0.85 (0.55-1.32)	54/55	0.68 (0.38-1.24)
5	70/76	0.92 (0.57-1.48)	54/71	0.57 (0.31-1.04)
P_{trend}†		0.88		0.03
$P_{interaction}$		0.20		
1,25(OH)$_2$D				
Quintile				
1	87/81	1.00	60/49	1.00
2	58/71	0.76 (0.47-1.25)	55/53	0.76 (0.42-1.38)
3	70/87	0.72 (0.46-1.15)	50/38	1.00 (0.53-1.88)
4	89/78	1.17 (0.75-1.84)	39/47	0.61 (0.32-1.15)
5	79/79	0.88 (0.56-1.40)	53/51	0.72 (0.40-1.32)
P_{trend}†		0.91		0.23
$P_{interaction}$		0.41		

*RR from unconditional logistic regression adjusted for age, fasting status at blood draw, month of blood collection, time of day of blood collection, BMI at age 18, parity/age at first birth, family history of breast cancer, benign breast disease, age at menarche, plasma α-carotene level, and intake of alcohol. See footnotes to Table 2 for variable categories. Results for postmenopausal women also adjusted for quartile of plasma estradiol (in nonusers of postmenopausal hormones), age at menopause, and total duration of postmenopausal hormone use.
†Two-sided P_{trend} over quintiles, calculated by using the median value of each quintile as a continuous variable in the multivariable model.

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$1,25(OH)_2D$. Results stratified by menopausal status at blood collection did not suggest a relationship between vitamin D metabolites in premenopausal women. In postmenopausal women, we saw some evidence that high levels of 25(OH)D were more protective in never-users of postmenopausal hormones than in users, but our power for this comparison was relatively low and differences were not statistically significant.

Results stratified by smoking status at blood draw suggested an inverse association between 25(OH)D vitamin D and breast cancer risk in never smokers (RR for Q_5 versus Q_1, 0.57; 95% CI, 0.33-1.00; P_{trend} = 0.10) but not in past or current smokers (RR for Q_5 versus Q_1, 0.91; 95% CI, 0.56-1.50; P_{trend} = 0.52; $P_{interaction}$ = 0.14). In contrast, risk estimates for $1,25(OH)_2D$ were similar in direction and magnitude ($P_{interaction}$ = 0.92).

Analyses stratified by other factors did not suggest that the relationship between vitamin D metabolites and breast cancer varied by laboratory analysis method, menopausal status at diagnosis, BMI, parity, plasma retinol levels, or season of blood collection. Results for 25(OH)D using season-specific quintile cut points were virtually identical to those of the main analysis (results not shown). When we excluded women reporting use of calcium and/or vitamin D supplements at the time of blood collection, the relationship between 25(OH)D and breast cancer risk did not change; the RR in the highest versus lowest quintile was 0.60 (95% CI, 0.36-1.00).

Finally, we evaluated whether the effect of vitamin D differed by characteristics of the breast tumors. Results varied slightly for tumors with different ER and PR status, although these differences were not statistically significant ($P_{heterogeneity}$ = 0.18). Whereas relatively few of our cases had ER−/PR− breast tumors (n = 88 cases) and our power for this analysis was low, we observed an inverse association between these tumors and 25(OH)D level. RRs for women in Q_2 through Q_5 versus Q_1 were 1.34, 0.89, 0.78, and 0.58 (95% CI, 0.26-1.34), respectively, with a marginally significant P_{trend} (P = 0.08). In contrast, 25(OH)D did not seem related to incidence of more common ER+/PR+ tumors (n = 305 cases), for which the RR for women in Q_2 to Q_5 versus Q_1 were 0.88, 0.81, 0.67, and 0.90 (95% CI, 0.57-1.41; P_{trend} = 0.30), respectively. Plasma 25(OH)D was also not linearly associated with risk of ER+/PR− tumors (n = 78 cases), which had RR for Q_2 to Q_5 versus Q_1 of 1.25, 0.79, 0.95, and 0.72 (95% CI, 0.30-1.70; P_{trend} = 0.33), respectively. We did not observe any variation in the relationship between $1,25(OH)_2D$ and breast cancer risk by tumor receptor status. For both metabolites, results were similar for invasive (n = 592) and *in situ* tumors (n = 109), ductal (n = 491) and lobular tumors (n = 76), and tumors with node involvement (n = 152) and no node involvement (n = 420; results not shown).

Discussion

Findings from our study suggest that high plasma level of 25(OH)D, and perhaps $1,25(OH)_2D$, may be associated with a lower risk of breast cancer, especially in older women, although results were marginally significant. Women ages 60 and older in the highest quintile of 25(OH)D had a 43% lower risk of breast cancer compared with those with the lowest levels, whereas those with the highest levels of $1,25(OH)_2D$ had a 28% lower risk. Neither 25(OH)D nor $1,25(OH)_2D$ was associated with a lower risk of breast cancer in younger women.

In the main analysis, we found that women in the third through fifth quintiles of 25(OH)D had a lower risk of developing breast cancer over 82 months of follow-up than women in the lower two quintiles. The similarity in risk between the lowest two (i.e., 1.00 and 0.95) and highest three quintiles (i.e., 0.74, 0.77, and 0.73) raises the possibility that a threshold level of 25(OH)D exists above which women experience protection against breast cancer, but that having plasma levels much higher than this cutoff does not further reduce risk. In our analysis of $1,25(OH)_2D$ levels, breast cancer risk was lower in women in the second through fifth quintiles, and there was no evidence of a dose-response relationship. Overall, these data suggest the possibility that women who may be deficient in 25(OH)D, and perhaps $1,25(OH)_2D$, may be at increased risk for breast cancer. In our study, women in the lowest quintile of 25(OH)D had metabolite levels low enough to be considered vitamin D deficient (<20 ng/mL; ref. 26).

We observed an association between vitamin D metabolites and breast cancer risk in women ages 60 years and older but not in younger women, although we did not find significant variation in these relationships by menopausal status at blood collection or cancer diagnosis. These findings differed somewhat from those of an analysis of dietary vitamin D in our population, which observed a significant reduction in the incidence of breast cancer with high vitamin D intake in premenopausal but not in postmenopausal women (16). However, a recent study of plasma vitamin D metabolites and risk of colorectal cancer in our cohort also observed modification of the vitamin D−cancer association by age (27); high plasma 25(OH)D was associated with a significantly lower risk of colorectal cancer in women ages 60 and older, but not in younger women. The likelihood of vitamin D deficiency increases with age, as the cutaneous production of vitamin D decreases, and with estrogen deficiency, which seems to reduce activation of vitamin D and expression of the vitamin D receptor (5, 28). Consequently, older women and postmenopausal women may be at increased risk (5, 29). This did not seem to be the case in our population; among study participants not using vitamin D supplements in 1990, mean 25(OH)D and $1,25(OH)_2D$ levels did not vary substantially by age [mean in women <60 versus ≥60 years: for 25(OH)D, 30.3 versus 31.5 ng/mL, P = 0.18; for $1,25(OH)_2D$, 33.9 versus 33.5 ng/mL, P = 0.45]. Differences in the association between vitamin D and breast cancer by age may instead reflect differences in vitamin D metabolism and/or the interaction of $1,25(OH)_2D$ with vitamin D receptors in breast tissue occurring with age.

We also found some evidence that a beneficial effect of 25(OH)D was limited to never smokers. Several studies have observed significantly lower 25(OH)D and $1,25(OH)_2D$ levels in smokers compared with nonsmokers (30, 31). It is possible that smoking may interfere with vitamin D metabolism and/or the effect of $1,25(OH)_2D$ on breast tissue.

To some extent, our findings for 25(OH)D and $1,25(OH)_2D$ differed and suggested that plasma levels of 25(OH)D may better predict breast cancer risk than $1,25(OH)_2D$ levels. The majority of studies of the health effects of vitamin D have focused on $1,25(OH)_2D$, as this is the biologically active metabolite and its concentration is closely regulated by a variety of hormones, including calcitonin, parathyroid hormone, estrogen, growth hormone, and insulin (20). In contrast, 25(OH)D levels are more sensitive to changes in diet and sunlight exposure and may not reflect the level of $1,25(OH)_2D$ ultimately available to target tissue. However, several recent studies have suggested that 25(OH)D also may be hydroxylated to form $1,25(OH)_2D$ at extrarenal sites, including breast tissue (4-7). $1,25(OH)_2D$ metabolized through this mechanism seems to act only as an autocrine or paracrine hormone, does not enter the general circulation, and may not be measurable by standard plasma assay (5). If the amount of $1,25(OH)_2D$ produced in target tissue constitutes a substantial percentage of available intracellular $1,25(OH)_2D$, a measurement of its precursor 25(OH)D may better reflect the total amount of vitamin D ultimately available to breast cells than circulating plasma $1,25(OH)_2D$ levels. In our population, the correlation between 25(OH)D and $1,25(OH)_2D$ was relatively low (Pearson's r = 0.11), and the simultaneous inclusion of both in regression models did not alter the findings for either

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metabolite, suggesting that the effects of each metabolite on breast cancer risk may be independent. To some extent, this finding offers support for a second mechanism of action of 25(OH)D on breast tissue in addition to renal hydroxylation to $1,25(OH)_2D$.

Few previous studies have evaluated the relationship between plasma vitamin D metabolites and breast cancer risk. To some extent, these studies have been limited by the timing of their collection of blood samples in relation to cancer diagnosis. Results from our analysis of 25(OH)D, excluding cases diagnosed during the first 2 years of follow-up, suggest that metabolite levels measured within a few years of diagnosis may not be as predictive of breast cancer risk as levels measured several years earlier. Also of concern is whether the presence of a tumor itself may affect circulating vitamin D levels, either by altering 25(OH)D metabolism (5) or by altering an individual's dietary intake of vitamin D or exposure to sunlight before diagnosis. Differences in the timing of blood sample collection may explain the inconsistency of our findings concerning 25(OH)D with those of Janowsky et al. (17). The authors evaluated metabolite levels collected at the time of diagnosis in 156 breast cancer cases and 184 controls, and did not observe a relationship between 25(OH)D and risk, although high levels of $1,25(OH)_2D$ were associated with reduced risk. Blood sample collection many years before diagnosis may also not reflect vitamin D levels that are etiologically relevant to the development of breast cancer. Hiatt et al. (18) did not find an association between $1,25(OH)_2D$ and breast cancer risk using blood samples from 96 matched case-control pairs collected on average 15 years before diagnosis.

To our knowledge, our study is the first to examine whether ER and PR expression of breast tumors may modify the relationship between vitamin D metabolites and cancer risk. Although power for these comparisons was low and results were not statistically significant, our findings suggested that 25(OH)D was inversely associated with risk of ER−/PR− negative tumors, but not with ER+/PR+ and ER+/PR− tumors. *In vitro* studies have suggested that the action of $1,25(OH)_2D$ on breast tumors may be through pathways other than the disruption of estrogen signaling (32), and that cells derived from ER− tumors may undergo regression through apoptosis after exposure to $1,25(OH)_2D$. Further exploration of potential differences in the relationship between vitamin D metabolites and incidence of ER− breast tumors may be warranted.

Although our study assessed 25(OH)D and $1,25(OH)_2D$ levels at only a single point in time, plasma vitamin D metabolite levels seem to remain relatively consistent over several years. In an analysis of plasma vitamin D metabolites in blood samples drawn ~3 years apart from 144 middle-aged men, correlations for 25(OH)D and $1,25(OH)_2D$ were 0.70 ($P < 0.0001$) and 0.50 ($P < 0.0001$), respectively.[8] However, in future studies, it would be beneficial to compare breast cancer risk associated with vitamin D metabolites collected at multiple times during participant follow-up.

One strength of our study was the ability to adjust risk estimates for the effect of a large number of other breast cancer risk factors. In addition to controlling for reproductive factors, such as parity, age at first birth, menopause status, and postmenopausal hormone use, we were able to account for possible confounding by other nutrients, including vitamins A, C, and E; folate; calcium; and plasma α-carotene. Furthermore, in our analyses of postmenopausal women and older women, we were able to adjust for plasma estradiol, a significant predictor of breast cancer risk (24). The addition of these variables to the regression model did not substantially changed the risk estimates for $1,25(OH)_2D$ and breast cancer.

[8] E. Giovannucci, personal communication.

In summary, our findings suggest that high plasma levels of 25(OH)D, and perhaps $1,25(OH)_2D$, may be modestly associated with a reduced risk of breast cancer in older women. The possibility that risk of breast cancer may be influenced by the level of local conversion of 25(OH)D to $1,25(OH)_2D$ in breast tissue, as well as by circulating $1,25(OH)_2D$ level, should be explored in greater detail.

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