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Circulating levels of 25-hydroxyvitamin D and risk of breast cancer: a nested case-control study

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Abstract

Introduction: Experimental evidence suggests a protective role for circulating 25-hydroxyvitamin D (25(OH)D) in breast cancer development, but the results of epidemiological studies have been inconsistent.

Methods: We conducted a case-control study nested within two prospective cohorts, the New York University Women's Health Study and the Northern Sweden Mammary Screening Cohort. Blood samples were collected at enrollment, and women were followed up for breast cancer ascertainment. In total, 1,585 incident breast cancer cases were individually-matched to 2,940 controls. Of these subjects, 678 cases and 1,208 controls contributed two repeat blood samples, at least one year apart. Circulating levels of 25(OH)D were measured, and multivariate odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using conditional logistic regression.

Results: No association was observed between circulating levels of 25(OH)D and overall breast cancer risk (multivariate-adjusted model OR = 0.94, 95% CI = 0.76-1.16 for the highest vs. lowest quintile, p_{trend} = 0.30). The temporal reliability of 25(OH)D measured in repeat blood samples was high (intraclass correlation coefficients for season-adjusted 25(OH)D > 0.70). An inverse association between 25(OH)D levels and breast cancer risk was observed among women who were ≤ 45 years of age (OR$_{Q5-Q1}$ = 0.48, 95% CI = 0.30-0.79, p_{trend} = 0.01) or premenopausal at enrollment (OR$_{Q5-Q1}$ = 0.67, 95% CI = 0.48-0.92, p_{trend} = 0.03).

Conclusions: Circulating 25(OH)D levels were not associated with breast cancer risk overall, although we could not exclude the possibility of a protective effect in younger women. Recommendations regarding vitamin D supplementation should be based on considerations other than breast cancer prevention.

Introduction

Experimental studies support a role for vitamin D in reducing the risk of breast cancer [1,2]. Vitamin D, which is obtained from both dietary sources (food and supplements) and exposure to type B ultraviolet radiation, undergoes two hydroxylation steps before becoming biologically active [3]. 25-hydroxyvitamin D [25(OH)D], produced in the liver from the first hydroxylation, is the precursor of the biologically active form, $1,25(OH)_2D$, which is produced in the kidney as well as in target tissues, including the breast [4]. Circulating 25(OH)D is considered the best marker of vitamin D status because it reflects vitamin D

obtained from both diet/supplements and sun exposure [5] and has a longer half-life than $1,25(OH)_2D$ [6]. Vitamin D acts by binding to the vitamin D receptor (VDR), which is expressed in mammary tissue. The VDR controls the expression of genes regulating cell proliferation, differentiation, and apoptosis [1,7].

The results of epidemiologic studies examining the association between circulating 25(OH)D levels and breast cancer risk have been inconsistent. Seven prospective studies reported no association overall [8-14], whereas three reported a significant or marginally significant inverse association [15-17]. Some significant findings emerged from the results of subgroup analyses, although the subgroups of women for whom these associations were observed were not consistent across studies. The Nurses' Health Study observed a stronger protective effect of

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plasma 25(OH)D on breast cancer risk for women who were 60 years old or older [16], whereas the French E3N cohort observed a stronger effect in women who were less than 53 years old at enrollment [15]. In the Nurses' Health Study II, which consists primarily of pre-menopausal women, no association was observed overall between plasma 25(OH)D levels and breast cancer risk, and a positive, rather than an inverse, association was observed among women who were overweight or obese, the latter of which is defined as a body mass index (BMI) of at least 30 kg/m^2 [13].

Recent reviews have concluded that there is insufficient evidence to recommend vitamin D supplementation for the prevention of breast cancer but that additional research in humans is needed [3,18,19]. The purpose of our study was to examine the association between pre-diagnostic circulating levels of circulating 25(OH)D and breast cancer risk in a case-control study nested within two prospective cohorts: the New York University Women's Health Study (NYUWHS) and the Northern Sweden Mammary Screening Cohort (NSMSC). A unique feature of this study, the largest prospective study to date, was the availability of two 25(OH)D measurements from blood samples donated a minimum of one year apart for a large proportion of the study subjects, allowing us to estimate with good precision the temporal reliability of 25(OH)D, and factors that affected it, in the two cohorts.

Materials and methods
Study population
Descriptions of the NYUWHS and the NSMSC have been provided previously [20,21]. Briefly, the NYUWHS cohort enrolled 14,274 healthy women (34 to 65 years old) at a mammography screening clinic in New York City between 1985 and 1991, and the NSMSC enrolled, between 1995 and 2006, approximately 28,800 women (40 to 69 years old) who are participating in a population-based breast cancer screening program in Västerbotten County, Sweden. After written informed consent was obtained from all study participants, information on demographic and anthropometric variables, medical and reproductive history, family history of breast cancer, and lifestyle factors, including diet, was collected through baseline or subsequent questionnaires or both. Venous blood was collected at enrollment, processed according to standard procedures by the respective cohorts (serum for NYUWHS and plasma for NSMSC), and stored at -80°C. Additional blood samples were collected from women who returned for screening. This study was approved by the Institutional Review Board of the New York University School of Medicine, the Regional Ethics Committee of the University of Umeå, Sweden, and the Swedish Data Inspection Board.

Case ascertainment and control selection
For the NYUWHS, incident cases of invasive breast cancer were identified by mailed questionnaires or follow-up telephone interviews every 2 to 4 years after 1991, supplemented by linkages to state cancer registries in New York, New Jersey, and Florida and the US National Death Index. Medical records were reviewed to confirm self-reported cases. Using a capture-recapture analysis, we estimated that combining active and cancer registry-based follow-up resulted in a breast cancer ascertainment rate of 95% [22]. For the NSMSC, annual linkages to the Swedish National Cancer Registry were used to identify incident cases of breast cancer in the cohort. As of 1 January 2007 for the NYUWHS and 1 January 2010 for the NSMSC, a total of 1,645 incident cases of invasive breast cancer (909 in the NYUWHS and 736 in the NSMSC) had been identified. In the NYUWHS, 16 cases (3%) were excluded because they had a low serum balance. In the NSMSC, 44 cases (6%) were excluded for the following reasons: 26 had low plasma balance, 13 had their plasma reserved because of a subsequent diagnosis of a rarer cancer or other disease, 3 had insufficient volume for laboratory measurement, and 2 had both matched controls excluded for one of the reasons above. The present study included a total of 1,585 incident breast cancer cases (893 from the NYUWHS and 692 from the NSMSC).

Each case was matched to two controls who were selected from the respective cohort by using incidence-density sampling. Matching factors included age at enrollment in the study (± 6 months), date of enrollment/first blood donation (NYUWHS: ± 3 months; NSMSC: ± 1 month), and number (1, 2+) and dates of subsequent blood donations. For the NYUWHS, matching factors also included menopausal status (pre- or post-menopausal) at enrollment and race/ethnicity (Caucasian, African-American, other, or unknown). The vast majority of women in the NSMSC were Caucasian. Initially, women were not matched on menopausal status in this cohort; however, 88% of the cases had at least one control matching on this factor (pre- and peri-menopausal combined or post-menopausal). In total, 2,940 controls were included in the final analysis (1,642 in NYUWHS and 1,298 in NSMSC).

For 678 matched sets (413 in NYUWHS and 265 in NSMSC), two blood samples were analyzed for 25(OH)D. For participants who had donated more than two blood samples, the first and last samples collected before the relevant case's diagnosis were selected.

Laboratory methods
Circulating 25(OH)D was measured by Heartland Assays, Inc. (Ames, IA, USA) by using a direct competitive chemiluminescence immunoassay by using the DiaSorin

LIAISON platform (DiaSorin, Inc., Stillwater, MN, USA) [23]. The assay, which is appropriate for either serum or plasma, is co-specific for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2. All samples, including repeat samples, from a case and her matched controls were analyzed together in the same laboratory batch to minimize laboratory variability. Laboratory personnel were blinded to case-control status of the study samples. Samples from quality-control pools (6% of total samples) were masked and inserted randomly in the batches. The intra- and inter-batch coefficients of variation (CVs) were 9.5% and 11.4%, respectively, for NYUWHS and 7.4% and 9.0%, respectively, for NSMSC.

Estrone was measured by double-antibody radioimmunoassay with reagents from Diagnostic System Laboratories (Webster, TX, USA) at the Laboratory for Hormone Analyses at the International Agency for Research on Cancer, France, for post-menopausal women who were not using hormone replacement therapy (HRT). Intra- and inter-batch CVs were 6.7% and 12.6%, respectively [24,25].

Statistical analysis

We examined the temporal reliability of circulating 25(OH)D by using the intraclass correlation coefficient (ICC). In addition to calculating the overall ICC, we calculated ICCs according to time (years) between sample donations and according to season, for each cohort separately.

Conditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the associations of subject characteristics and circulating 25(OH)D with risk of breast cancer. We conducted analyses separately for each cohort as well as combining them. Because there was no evidence of cohort heterogeneity, most results are presented for the combined cohorts. 25(OH)D concentrations were $\log_2$-transformed to reduce departure from the normal distribution and were included in the model in one of three ways. First, we computed season-adjusted residuals to take into account the known variations of 25(OH)D with season [3]. For each 25(OH)D measurement, the residual was the difference between the observed 25(OH)D value and the value predicted for this day of the year, which was obtained by using a non-parametric local regression (Proc LOESS; SAS Institute Inc., Cary, NC, USA) with 25(OH)D as the dependent variable and day of the year of blood donation as the independent variable [26]. This regression model was run separately for each cohort by using all available 25(OH)D measurements (that is, including repeat samples). We conducted analyses by using cohort-specific season-adjusted quintiles based on the distribution of the residuals in controls. Second, we ran analyses with season-adjusted residuals on the continuous scale. All analyses based on residuals were conducted by using first (that is, baseline) samples only. Finally, we

examined the association of circulating 25(OH)D with breast cancer risk by using pre-specified categories of 25(OH)D levels, which were defined by using cut-points recommended by the Institute of Medicine: less than 50 (inadequate), 50 to 74 (adequate), and at least 75 nmol/L (adequate to high) [3]. These analyses were conducted separately for 'winter' and 'summer', which were defined by examining the unadjusted 25(OH)D levels in controls within each cohort. Winter included the months of January to April, when mean levels were low (48.1 to 51.2 nmol/L), and summer included the months of July to September, when mean levels were high (62.0 to 68.0 nmol/L). There was little variation in mean level from month to month within each of these two seasons. Subjects who had measurements in both winter and summer were included in both season-specific analyses to increase the sample size. Because there was no difference in the main study findings between conditional logistic regression models and unconditional models adjusting for the matching factors and because using conditional regression resulted in the loss of matched sets with samples collected in different seasons for the case and her controls, unconditional logistic regression was used for the season-specific analyses. In analyses by quintiles and pre-specified categories, tests for trend were performed by using an ordered categorical variable. Tests for heterogeneity were carried out by comparing models that included interaction terms to models that excluded them or by using Cochran's Q statistic.

In each of the two cohorts, multivariate linear regression analyses were conducted among the controls to explore associations of potential confounders with 25(OH)D. BMI was found to be a negative predictor of 25(OH)D, whereas multivitamin supplement use and past use of HRT were positive predictors in both cohorts. Caucasian race and physical activity were also positive predictors of 25(OH)D in the NYUWHS, and alcohol consumption was a negative predictor in the NSMSC. Covariates in the final multivariate models included the following known breast cancer risk factors: age at menarche (continuous), age at first birth/parity (not more than 20, 21 to 15, 26 to 30, more than 30 years, nulliparous), family history of breast cancer (no or yes), BMI (continuous), past HRT use (never or ever), and alcohol consumption. It is debatable whether to control for outdoor physical activity and multivitamin use, which have been associated with higher levels of circulating 25(OH)D [27,28] since these variables may influence breast cancer risk through their effect on 25(OH)D levels. However, these factors may affect breast cancer risk through other mechanisms [29], in which case they could act as possible confounders in analyses of 25(OH)D and breast cancer risk. Therefore, in addition to presenting models adjusting for the factors listed above, we

present results adjusting for physical activity and multi-vitamin use (no or yes). In the NYUWHS, physical activity was expressed as metabolic equivalent of task-hours per week (MET-hours/week) from walking and vigorous exercise, and women were classified into ter-tiles. In the NSMSC, women were classified as inactive, moderately active, or active by combining data on physi-cal activity at work and frequency of walking, biking, and exercising. Baseline data were used for all variables except HRT in the NYUWHS, which represented use up to the date of diagnosis (or index date for controls).

We performed multiple imputation by using fully con-ditional specification [30] for the following covariates with missing data: alcohol consumption (23%), physical activity (23%), multivitamin use (18%), HRT use (6%), age at menarche, parity, age at first full-term pregnancy, and BMI (all with not more than 2% missing data). We compared analyses including all subjects and imputed data for covariates to analyses including only subjects with no missing data (complete case method). Because results from both analyses were similar, we present only the analyses that included all subjects and imputed data.

We conducted stratified analyses by using conditional logistic regression for the following variables: age at enroll-ment, lag-time between blood donation and diagnosis, and estrogen receptor (ER) status. In order not to lose the matched sets in which a case and her controls were discor-dant, unconditional logistic regression, adjusted for cohort and age at blood sampling, was performed for the follow-ing variables: menopausal status, BMI, circulating estrone levels (for post-menopausal women only), and insulin-like growth factor 1 (IGF-I) levels. Tertiles were used for the IGF-1 analysis because of the limited number of women for whom IGF-1 had been measured (193 cases and 269 controls from the NYUWHS only). Finally, we performed an analysis limited to Caucasians (90% of subjects). All sig-nificance testing was two-sided, and a P value of less than 0.05 was considered statistically significant.

Results

Descriptive statistics for the breast cancer cases and their matched controls are presented in Table 1. Mean age at enrollment was 54 years for both cases and controls. Cases were diagnosed an average of 8.7 years after blood donation. Established risk factors for breast cancer - including younger age at menarche, nulliparity, older age at first full-term pregnancy, and having a first-degree family history of breast cancer - occurred more com-monly in cases. Cases were more likely to report having used HRT. BMI was significantly different between cases and controls in post-menopausal women, among whom a greater proportion of cases were overweight and obese. Among the 77% of cases for which receptor status was known, 78% of tumors were ER-positive.

For women who donated more than one blood sample, the average time between sample donations was 2.1 years in the NYUWHS and 4.4 years in the NSMSC. Overall, the temporal reliability of 25(OH)D was good (ICC = 0.65, 95% CI 0.61 to 0.69 for NSMSC and 0.78, 95% CI 0.76 to 0.80 for NYUWHS) and improved for NSMSC when season-adjusted residuals were used (ICC = 0.71, 95% CI 0.67 to 0.74) (Table 2). The ICC for the NYUWHS was not changed by seasonal adjustment, because women in the NYUWHS generally returned to the screening center and donated a blood sample at the same time each year. We observed that the ICC decreased as time increased between sample dona-tions, although this trend did not appear to extend beyond the first 8 years. For the NSMSC, the season-adjusted ICCs were 0.56 (95% CI = 0.42 to 0.68) for samples collected 5 to 8 years apart (n = 113 subjects) and 0.63 (95% CI = 0.51 to 0.74) for samples collected between 8 and 11.7 years apart (n = 106 subjects). For both cohorts, the ICC was substantially lower when one sample was donated in the winter and the other one in the summer months (ICC = 0.47, 95% CI = 0.29 to 0.61 for NSMSC, n = 92; ICC = 0.66, 95% CI = 0.50 to 0.77 for the NYUWHS, n = 68).

Table 3 reports ORs and 95% CIs for breast cancer risk according to season-adjusted quintiles of 25(OH)D. There was no association between circulating vitamin D and breast cancer risk overall (adjusted model OR_{Q5-Q1} = 0.94, 95% CI = 0.76 to 1.16, P_{trend} = 0.30) or within either cohort. Results with 25(OH)D on the continuous scale were similar. Adjusting for physical activity and multivita-min use, in addition to the other confounders, did not materially affect the results (OR_{Q5-Q1} = 0.94, 95% CI = 0.76 to 1.16, P_{trend} = 0.27).

In analyses using pre-specified categories of circulating 25(OH)D (not adjusted for season), we observed no asso-ciation with breast cancer risk for samples taken either in the winter, when more than half of the subjects had levels below 50 nmol/L, or in the summer, when more of the subjects had levels more than 75 nmol/L (Table 4). A suggestive protective effect was observed for women in the NYUWHS who donated blood in the summer months (OR = 0.69, 95% CI = 0.45 to 1.07, P_{trend} = 0.10 for con-centrations of at least 75 versus less than 50 nmol/L), but no such association was observed in the NSMSC.

Table 5 shows the results of subgroup analyses. Higher circulating 25(OH)D was associated with a decreased risk of breast cancer among women who were pre-menopausal at blood donation (OR_{Q5-Q1} = 0.67, 95% CI = 0.48 to 0.92, P_{trend} = 0.03) but not among those who were post-meno-pausal (OR_{Q5-Q1} = 1.21, 95% CI = 0.92 to 1.58, P_{trend} = 0.67, $P_{interaction}$ = 0.05). A similar protective effect was observed for women who were not more than 45 years old at blood donation (OR_{Q5-Q1} = 0.48, 95% CI = 0.30 to 0.79, P_{trend} = 0.01, $P_{interaction}$ = 0.08). There was no evidence of effect modification by ER status of the tumor, lag-time

Table 1 Characteristics of breast cancer cases and matched controls

Characteristic	Case subjects Number (percentage)	Control subjects Number (percentage)	P value[a]
Age at enrollment, years			
≤ 45	301 (19%)	575 (20%)	
46-54	557 (35%)	1,005 (34%)	Matched
≥ 55	727 (46%)	1,360 (46%)	
Age at diagnosis, years			
≤ 55	393 (25%)		
56-64	511 (32%)		
≥ 65	681 (43%)		
Race			
Caucasian	1,370 (90%)	2,484 (90%)	
African-American	94 (6%)	163 (6%)	Matched
Other	51 (4%)	118 (4%)	
Missing	70	175	
Education			
Some high school or less	288 (25%)	582 (26%)	
Completed high school	415 (36%)	820 (37%)	0.09
College or higher	439 (38%)	801 (36%)	
Missing	443	737	
Menopausal status at enrollment			
Pre- or peri-menopausal	637 (40%)	1,134 (39%)	Matched[b]
Post-menopausal	948 (60%)	1,806 (61%)	
Age at menarche, years			
< 12	285 (18%)	477 (17%)	
12	397 (26%)	670 (23%)	
13	439 (28%)	803 (28%)	0.005
> 13	438 (28%)	926 (32%)	
Missing	26	64	
Nulliparous	327 (21%)	530 (18%)	0.029
Missing	46	63	
Age at first full-term pregnancy, years			
≤ 20	171 (14%)	409 (18%)	
21-25	523 (44%)	1,016 (44%)	
26-30	318 (27%)	596 (26%)	0.006
> 30	172 (15%)	281 (12%)	
Missing	28	45	
Ever user of oral contraceptives	677 (46%)	1219 (45%)	0.45
Missing	121	209	
Ever user of hormone replacement therapy	445 (30%)	685 (25%)	< 0.001
Missing	99	176	
First-degree family history of breast cancer	299 (19%)	437 (15%)	0.0005
Body mass index, kg/m^2			
Pre- and peri-menopausal			
< 20.0	53 (8%)	104 (9%)	
20.0-24.9	339 (54%)	614 (55%)	0.24
≥ 25.0	237 (38%)	409 (36%)	
Missing	8	7	
Post-menopausal			
< 20.0	31 (3%)	78 (4%)	
20.0-24.9	396 (43%)	848 (48%)	

Table 1 Characteristics of breast cancer cases and matched controls (Continued)

≥ 25.0	498 (54%)	835 (48%)	0.0009
Missing	23	45	
Multivitamin user	515 (41%)	956 (39%)	0.49
Missing	332	509	
Ever smoker	642 (50%)	1,264 (52%)	0.52
Missing	299	524	
Alcohol, drinks per day			
0	501 (43%)	928 (41%)	
< 1	552 (47%)	1,161 (51%)	
≥ 1	123 (10%)	187 (8%)	0.80
Missing	409	664	
Physical activity			
NYUWHS, MET-hours per week			
< 5.5	249 (31%)	412 (28%)	
5.5-21.5	271 (34%)	506 (35%)	0.12
≥ 21.5	274 (35%)	548 (37%)	
Missing	99	176	
Mammary Screening Cohort, activity level			
Inactive	55 (14%)	107 (13%)	
Moderately active	145 (37%)	305 (37%)	
Active	192 (49%)	408 (50%)	0.84
Missing	300	478	
Dietary vitamin D in IU/day, median (10th-90th percentile)	141 (55, 272)	145 (55, 275)	0.60
Missing	340	526	
Circulating 25(OH)D in nmol/L, median (10-90th percentile)	53.0 (31.2, 81.0)	54.2 (30.7, 82.6)	0.27

[a]P value from conditional logistic regression (P value from trend test for ordered categories). [b]Menopausal status (pre- or post-menopausal) was a matching factor for all sets in the New York University Women's Health Study (NYUWHS). For Northern Sweden Mammary Screening Cohort (NSMSC), women were not initially matched on this factor, and 88% of the cases had at least one control matched on menopausal status (pre- and peri-menopausal combined or post-menopausal). 25(OH)D, 25-hydroxyvitamin D; MET, metabolic equivalent of task.

Table 2 Intraclass correlation coefficients by time between sample donation (years) and by season (NSMSC and NYUWHS)

	NSMSC		NYUWHS	
	Number of subjects	ICC (95% CI)	Number of subjects	ICC (95% CI)
All[a]	700	0.65 (0.61, 0.69)	1,168	0.78 (0.76, 0.80)
All[b]	700	0.71 (0.67, 0.74)	1,168	0.78 (0.76, 0.80)
Time between samples[b], years				
≤ 1			393	0.80 (0.76, 0.83)
> 1-2	146	0.79 (0.71, 0.84)	325	0.81 (0.77, 0.84)
> 2-3	139	0.81 (0.75, 0.86)	208	0.80 (0.75, 0.84)
> 3-5	196	0.71 (0.63, 0.73)	214	0.74 (0.67, 0.79)
> 5-8	113	0.56 (0.42, 0.68)		
> 8-11.7	106	0.63 (0.51, 0.74)		
Seasons[a]				
Both samples in winter	148	0.74 (0.66, 0.81)	266	0.78 (0.72, 0.82)
Both samples in summer	53	0.79 (0.67, 0.88)	210	0.81 (0.76, 0.85)
One sample in winter, one in summer	92	0.47 (0.29, 0.61)	68	0.66 (0.50, 0.77)

[a]Log$_2$-transformed 25(OH)D values. [b]Season-adjusted 25(OH)D residual values. 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; ICC, intraclass correlation coefficient; NSMSC, Northern Sweden Mammary Screening Cohort; NYUWHS, New York University Women's Health Study.

Table 3 Odds ratios and 95% confidence intervals for breast cancer risk according to season-adjusted circulating levels of 25(OH)D (by quintiles and as a continuous variable)

	Quintiles						Continuous	
	1	2	3	4	5	P_{trend}	OR (95% CI)[a]	P value
Both cohorts								
Cases/controls	311/589	362/588	309/588	315/588	288/587			
Unadjusted model	1.0 (ref)	1.18 (0.97, 1.43)	0.99 (0.81, 1.21)	1.02 (0.84, 1.24)	0.93 (0.76, 1.14)	0.18	0.94 (0.84, 1.06)	0.31
Adjusted model[b]	1.0 (ref)	1.16 (0.95, 1.42)	1.00 (0.81, 1.22)	1.04 (0.85, 1.27)	0.94 (0.76, 1.16)	0.30	0.95 (0.84, 1.08)	0.44
Adjusted model[c]	1.0 (ref)	1.16 (0.95, 1.42)	0.99 (0.81, 1.21)	1.03 (0.84, 1.27)	0.94 (0.76, 1.16)	0.27	0.95 (0.84, 1.07)	0.40
NYUWHS								
Cases/controls	191/329	190/328	176/329	170/328	166/328			
Unadjusted model	1.0 (ref)	0.99 (0.76, 1.29)	0.92 (0.71, 1.20)	0.89 (0.69, 1.16)	0.86 (0.66, 1.12)	0.18	0.91 (0.79, 1.05)	0.21
Adjusted model[b]	1.0 (ref)	0.98 (0.75, 1.28)	0.94 (0.72, 1.23)	0.92 (0.70, 1.21)	0.88 (0.67, 1.16)	0.31	0.93 (0.80, 1.08)	0.34
Adjusted model[c]	1.0 (ref)	1.00 (0.76, 1.31)	0.95 (0.73, 1.24)	0.93 (0.71, 1.22)	0.90 (0.68, 1.19)	0.37	0.94 (0.81, 1.09)	0.42
NSMSC								
Cases/controls	120/260	172/260	133/259	145/260	122/259			
Unadjusted model	1.0 (ref)	1.47 (1.09, 1.97)	1.10 (0.81, 1.49)	1.22 (0.90, 1.64)	1.03 (0.75, 1.40)	0.62	1.00 (0.82, 1.23)	0.99
Adjusted model[b]	1.0 (ref)	1.48 (1.08, 2.01)	1.09 (0.79, 1.49)	1.26 (0.92, 1.72)	1.05 (0.76, 1.46)	0.74	1.02 (0.82, 1.26)	0.89
Adjusted model[c]	1.0 (ref)	1.46 (1.07, 1.99)	1.08 (0.79, 1.48)	1.24 (0.91, 1.70)	1.04 (0.75, 1.45)	0.71	1.01 (0.81, 1.26)	0.94

[a]Odds ratio (OR) for a doubling in 25(OH)D level. [b]Adjusted for age at menarche (continuous), age at first birth/parity (≤ 20, 21-25, 26-30, > 30 years, nulliparous), family history of breast cancer (no or yes), body mass index (continuous), hormone replacement therapy use (never or ever), and alcohol consumption (continuous). [c]Adjusted for physical activity and multivitamin use in addition to variables in footnote a. 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; NSMSC, Northern Sweden Mammary Screening Cohort; NYUWHS, New York University Women's Health Study; ref, reference.

Table 4 Odds ratios and 95% confidence intervals for breast cancer risk according to pre-specified categories of circulating 25(OH)D concentration by season[a]

		Pre-specified categories, nmol/L				Continuous[b]	P value
		< 50	50-74	75+	P_{trend}		
Winter (Jan., Feb., March, April)							
Both cohorts[c]	Cases/controls	391/704	244/472	65/116			
	OR (95% CI)	1.0 (ref)	0.94 (0.77, 1.15)	1.06 (0.76, 1.48)	0.95	0.97 (0.82, 1.15)	0.74
NYUWHS	Cases/controls	193/331	105/217	35/65			
	OR (95% CI)	1.0 (ref)	0.82 (0.61, 1.10)	0.91 (0.58, 1.44)	0.35	0.90 (0.73, 1.13)	0.37
NSMSC	Cases/controls	198/373	139/255	30/51			
	OR (95% CI)	1.0 (ref)	1.10 (0.83, 1.46)	1.25 (0.76, 2.06)	0.34	1.11 (0.85, 1.47)	0.44
Summer (July, Aug., Sept.)							
Both cohorts[c]	Cases/controls	113/186	217/348	99/212			
	OR (95% CI)	1.0 (ref)	1.02 (0.75, 1.38)	0.76 (0.53, 1.09)	0.14	0.82 (0.64, 1.05)	0.12
NYUWHS	Cases/controls	77/121	125/190	67/148			
	OR (95% CI)	1.0 (ref)	1.07 (0.73, 1.56)	0.69 (0.45, 1.07)	0.10	0.80 (0.60, 1.07)	0.14
NSMSC	Cases/controls	36/65	92/158	32/64			
	R (95% CI)	1.0 (ref)	0.99 (0.59, 1.67)	0.89 (0.46, 1.70)	0.72	0.88 (0.52, 1.47)	0.61

[a]Adjusted for age at menarche (continuous), family history of breast cancer (no or yes), age at first birth/parity (≤ 20, 21-25, 26-30, > 30 years, nulliparous), body mass index (continuous), hormone replacement therapy use (never or ever), and alcohol consumption (continuous). [b]Odds ratio (OR) for a doubling in 25(OH)D level. [c]Adjusted for cohort in addition to all factors in footnote a. 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; NSMSC, Northern Sweden Mammary Screening Cohort; NYUWHS, New York University Women's Health Study; ref, reference.

between blood sampling and diagnosis, BMI, or circulating estrone levels. Results of the analysis limited to Caucasians were similar to those of the analysis that included all subjects.

When we examined the association of 25(OH)D with breast cancer risk by IGF-1 levels at baseline, the ORs for the third tertile were 0.62 (95% CI = 0.30 to 1.28) in the below-median stratum and 0.79 (95% CI = 0.39 to 1.62) in the above-median stratum. The test for interaction between IGF-1 and 25(OH)D on the continuous scale was not statistically significant (P = 0.61).

Table 5 Stratified odds ratios and 95% confidence intervals for breast cancer risk according to quintiles of season-adjusted residual values of circulating 25(OH)D concentration at enrollment

		Quintiles					P_{trend}	P_{het}[a]
		1	2	3	4	5		
Caucasians[b]								
	Cases/controls	209/399	298/449	270/469	282/473	244/471	0.46	0.31
	OR (95% CI)	1.00	1.28 (1.01, 1.62)	1.10 (0.87, 1.38)	1.17 (0.92, 1.48)	0.97 (0.76, 1.24)		
Age at enrollment, years[b]								
≤ 45	Cases/controls	72/101	54/103	62/103	61/110	52/136		
	OR (95% CI)	1.00	0.67 (0.42, 1.07)	0.78 (0.49, 1.25)	0.73 (0.46, 1.14)	0.48 (0.30, 0.79)	0.01	0.08
45-54	Cases/controls	118/220	116/220	97/201	117/209	109/186		
	OR (95% CI)	1.00	0.92 (0.65, 1.29)	0.82 (0.58, 1.17)	0.99 (0.70, 1.40)	1.04 (0.73, 1.48)	0.73	
≥ 55	Cases/controls	121/268	192/265	150/284	137/269	127/265		
	OR (95% CI)	1.00	1.76 (1.30, 2.38)	1.28 (0.94, 1.73)	1.28 (0.94, 1.75)	1.20 (0.87, 1.66)	0.96	
Lag-time to diagnosis[b]								
≤ 8 years	Cases/controls	123/261	174/240	144/258	140/253	141/252		
	OR (95% CI)	1.00	1.62 (1.19, 2.22)	1.23 (0.90, 1.69)	1.26 (0.92, 1.73)	1.18 (0.85, 1.63)	0.99	0.30
> 8 years	Cases/controls	188/328	188/348	165/330	175/335	157/335		
	OR (95% CI)	1.00	0.92 (0.71, 1.20)	0.86 (0.66, 1.13)	0.91 (0.70, 1.19)	0.82 (0.62, 1.08)	0.20	
ER status[b]								
ER⁺	Cases/controls	173/355	219/347	178/358	200/355	78/352		
	OR (95% CI)	1.00	1.31 (1.00, 1.70)	1.04 (0.80, 1.35)	1.22 (0.93, 1.58)	1.10 (0.83, 1.44)	0.79	0.79
ER⁻	Cases/controls	53/99	60/99	56/111	49/93	54/95		
	OR (95% CI)	1.00	1.03 (0.63, 1.70)	0.99 (0.60, 1.64)	1.00 (0.61, 1.66)	1.08 (0.64, 1.85)	0.85	
Menopausal status[c]								
Pre-menopausal	Cases/controls	150/229	128/228	122/218	123/221	114/238		
	OR (95% CI)	1.00	0.80 (0.59, 1.09)	0.79 (0.58, 1.08)	0.80 (0.58, 1.09)	0.67 (0.48, 0.92)	0.03	0.05
Post-menopausal	Cases/controls	161/360	234/360	187/370	192/367	174/349		
	OR (95% CI)	1.00	1.48 (1.15, 1.90)	1.16 (0.89, 1.50)	1.22 (0.94, 1.58)	1.21 (0.92, 1.58)	0.67	
BMI[c], kg/m²								
< 25	Cases/controls	115/245	153/312	164/323	195/361	192/403		
	OR (95% CI)	1.00	1.01 (0.75, 1.36)	1.05 (0.78, 1.40)	1.10 (0.83, 1.47)	0.97 (0.73, 1.29)	0.99	0.56
25+	Cases/controls	187/331	201/267	142/260	115/215	90/171		
	OR (95% CI)	1.00	1.31 (1.01, 1.70)	0.93 (0.70, 1.22)	0.91 (0.68, 1.22)	0.93 (0.68, 1.27)	0.16	
Estrone, pg/mL[c, d]								
< 23.25	Cases/controls	32/90	42/85	40/94	44/97	33/102		
	OR (95% CI)	1.00	1.38 (0.79, 2.41)	1.19 (0.68, 2.09)	1.36 (0.78, 2.36)	0.95 (0.53, 1.70)	0.83	0.41
23.25-32.45	Cases/controls	43/83	59/82	48/91	53/81	47/74		
	OR (95% CI)	1.00	1.40 (0.84, 2.33)	1.03 (0.61, 1.73)	1.33 (0.78, 2.25)	1.29 (0.75, 2.22)	0.49	
> 32.45	Cases/controls	48/83	80/94	56/80	37/82	41/52		
	OR (95% CI)	1.00	1.70 (1.05, 2.76)	1.36 (0.81, 2.27)	0.87 (0.50, 1.50)	1.66 (0.94, 2.95)	0.82	

[a]Cochran's Q statistic was used to test for heterogeneity according to lag-time and estrogen receptor (ER) status. [b]Adjusted for age at menarche (continuous), family history of breast cancer (yes or no), age at first birth/parity (≤ 20, 21-25, 26-30, > 30 years, nulliparous), body mass index (BMI) (continuous), hormone replacement therapy use (ever or never), and alcohol consumption (continuous). [c]Unconditional logistic regression analyses adjusted for cohort and age at sampling in addition to all factors in footnote b. [d]Measured in post-menopausal women only. 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval; OR, odds ratio.

Discussion

In this case-control study nested within two cohorts, we did not observe an association between circulating 25(OH)D and breast cancer risk overall. We observed an inverse association between 25(OH)D and breast cancer risk in the subgroups of women who were not more than 45 years old or pre-menopausal at blood donation, although

the test for interaction was significant only for menopausal status. Because of substantial overlap, we were not able to sort out whether younger age or pre-menopausal status was driving the association.

Epidemiologic studies on vitamin D and breast cancer risk have been reviewed recently [31,32]. Traditional case-control studies [33-39] with blood samples collected

after breast cancer diagnosis have found inverse associations between circulating 25(OH)D and breast cancer risk. However, changes in lifestyle, particularly physical activity, following breast cancer diagnosis and treatment may affect circulating 25(OH)D, and associations observed in these studies therefore may not reflect pre-diagnosis associations [32]. Among the 10 prospective studies published to date, eight reported no association overall [8-14], one reported a marginally significant inverse association [16], and one reported a significant inverse association [15].

In regard to younger and pre-menopausal women, data from prospective studies are more limited since eligibility in some of the largest studies was restricted to older women [8-10]. Among the five prospective studies that reported results in younger or pre-menopausal women or both, four reported no association [11-13,16]. In the largest of the four studies, the Nurses' Health Study II, which collected blood in relatively young (age range of 32 to 54 years), mostly pre-menopausal women, a large number of whom were still pre-menopausal at diagnosis (294 cases), the multivariate-adjusted OR associated with the top quintile of 25(OH)D was 1.19 (95% CI = 0.77 to 1.84, P_{trend} = 0.51). The French E3N cohort, though, reported a significant inverse association in women who were younger (< 53 years old) at blood donation, results consistent with ours, and also observed a non-significant protective association in the smaller subgroup of women who were pre-menopausal at diagnosis. The investigators suggested that vitamin D may act by inhibiting the tumor growth-stimulating effects of IGF-1 [15]. Because IGF-1 levels decrease with age, a stronger anticarcinogenic effect of vitamin D would be expected in younger/pre-menopausal women. However, our analysis stratifying directly by IGF-1 level did not support this hypothesis, although the sample size was limited.

A protective effect of vitamin D on breast cancer would also be expected to be stronger in pre-menopausal women if vitamin D acts by inhibiting estrogen-stimulated breast cell proliferation [40], since estrogen levels are much higher before menopause. However, we found no evidence that the effect of vitamin D varies according to estrone levels in post-menopausal women, in spite of the strong positive association between estrone and breast cancer risk in our study. Moreover, 25(OH)D was not associated with either ER-positive or ER-negative breast cancer, and this is consistent with results from other prospective studies of 25(OH)D that found no difference by ER status [10,13].

Too few subjects had very high concentrations of 25(OH)D for us to be able to examine the association of concentrations of at least 100 nmol/L with breast cancer risk. However, the lack of dose response in the less than 50 to at least 75 nmol/L range (Table 4) suggests that a true association would have to be of the threshold type, a hypothesis for which there is little biological support. A linear dose-response association at levels of not more than 75 nmol/L has been observed for colorectal cancer, the one type of cancer for which there is consistent evidence of a protective effect of vitamin D [41].

Several factors that are associated with breast cancer risk are also associated with circulating 25(OH)D and therefore could confound the 25(OH)D-breast cancer association [42]. Dark skin, higher BMI, and lower physical activity have been repeatedly found to be associated with lower levels of 25(OH)D, whereas associations between 25(OH)D and current use of HRT, vitamin supplements, and alcohol have been less consistent [28,42,43]. The importance of taking into account these lifestyle factors was demonstrated in the Women's Health Initiative study, in which a significant inverse association of 25(OH)D with breast cancer risk was attenuated and became non-significant after adjustment for BMI and physical activity [42]. In our study, we matched on race/ethnicity (a surrogate for dark skin) and also conducted analyses limited to Caucasians, and this gave results similar to the analyses that included all races. As shown in Tables 3, 4, and 5, adjusting for BMI, HRT use, physical activity, and multivitamin use had very little effect on the ORs. Residual confounding is possible, particularly by physical activity, for which we classified women in one of three categories and data were missing for 11% in the NYUWHS and 38% in the NSMSC. However, an analysis limited to the subjects for whom physical activity was available showed very similar results, as did an analysis adjusting for MET-hours per week as a continuous variable in the NYUWHS (data not shown). Another potential source of confounding is breast cancer screening frequency, as screening visits that are more frequent could result in earlier diagnosis of breast cancer or correlate with other health-conscious behaviors leading to higher 25(OH)D status. However, because blood donations occurred at the time of mammographic screening visits, number of blood donations can be considered a proxy for mammographic screening frequency. We matched on number of blood donations in the NYUWHS, whereas in the NSMSC, although we did not match on this variable, 59% of cases had at least one control who matched exactly on the number of blood donations, and 89% of cases had at least one control who matched within ± 1 blood donation. We therefore believe that confounding by screening history is unlikely in our study.

We used residuals obtained by local regression to take into account seasonal variation. This method has been used only rarely in studies of 25(OH)D [44], although it has been used in epidemiologic analyses of other

biomarkers with temporal variation (for instance, hormones known to vary during pregnancy) [45,46]. In our study, when the residual method was used, the exposure value for each woman was the difference between the absolute level observed for this woman and the projected mean of 25(OH)D for this day of the year (reference day). A positive residual indicated that a woman had a higher-than-average level at this time of the year, whereas a negative residual indicated a lower-than-average level. The projected mean was calculated by using all samples collected in the same cohort on the reference day, as well as samples taken on neighboring days, with progressively decreasing weights given to samples collected further away from the reference day. This method, therefore, seems well suited to take into account the gradual changes observed during the shoulder seasons, when levels progressively increase (spring) or decrease (fall).

Strengths of this study include its prospective design, inclusion of two cohorts with different diet and sun exposure, and large sample size. This is also the first study to include repeat blood samples on a large number of women. The repeat samples enabled us to assess temporal reproducibility and gave an indication of the potential impact of ignoring seasonal variation when studying the association of circulating vitamin D with disease risk. The lower ICCs observed when samples were collected in different seasons, compared with the same season, highlight the importance of taking season into account in the study design or analysis or both, as other studies have concluded [47]. The ICC of 0.63 for samples collected 8.0 to 11.7 years apart in the NSMSC compares well with the ICCs of other biomarkers that have been linked to breast cancer risk, such as post-menopausal sex hormones. However, the ICC decreased with increasing time between blood donations, although this trend did not seem to extend beyond the first 8 years. This observation underlines that a single measurement of 25(OH)D is an imperfect reflection of vitamin D status over the long time period during which breast cancer develops. Thus, the association of vitamin D status with breast cancer risk may have been underestimated because of random error in measurement of the true exposure of interest (that is, the long-term average level of 25(OH)D). Another limitation of our study is the relatively small number of subjects with very high levels of circulating 25(OH)D (≥ 100 nmol/L).

Conclusions
This large prospective study does not support a relationship between circulating 25(OH)D and risk of breast cancer, except possibly in younger women. These results add to a growing body of evidence from prospective studies and randomized trials that suggests that higher vitamin D levels do not reduce breast cancer risk. Recommendations in regard to vitamin D supplementation should be based on considerations other than breast cancer prevention, such as bone health.

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