Research Article

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Serum 25(OH) Vitamin D and Risk of Breast Cancer: A Nested Case-Control Study from the French E3N Cohort

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Abstract

Background: High 25-hydroxyvitamin D [25(OH)D] serum concentrations have been found to be associated with reduced breast cancer risk. However, few studies have further investigated this relationship according to menopausal status, nor have they taken into account factors known to influence vitamin D status, such as dietary and serum calcium, parathyroid hormone, and estradiol serum levels.

Methods: We designed a nested case-control study within the French E3N cohort. Cases were women diagnosed with incident breast cancer (n = 636). Controls (n = 1,272) were matched with cases on age, menopausal status at blood collection, age at menopause, and center and year of blood collection. Multivariate logistic regression models were established.

Results: We found a decreased risk of breast cancer with increasing 25(OH) vitamin D_3 serum concentrations (odds ratio, 0.73; 95% confidence interval, 0.55-0.96; P trend = 0.02) among women in the highest tertile. We also observed a significant inverse association restricted to women under 53 years of age at blood sampling [odds ratio (T_3 versus T_1), 0.60; 95% confidence interval, 0.37-0.98; P trend = 0.04]. In premenopausal women, the risk was also decreased, although not significantly.

Conclusion: Our findings support a decreased risk of breast cancer associated with high 25(OH) vitamin D_3 serum concentrations, especially in younger women, although we were unable to confirm a direct influence of age or menopausal status.

Impact: Randomized intervention trials with vitamin D supplementation are required to confirm its benefits on breast cancer risk, but the maintenance of adequate vitamin D levels should be encouraged by public health policy. *Cancer Epidemiol Biomarkers Prev; 19(9); 2341–50. ©2010 AACR.*

Introduction

Although the relationship between vitamin D status and breast cancer risk remains unclear, a growing body of evidence suggests that high vitamin D serum concentrations are associated with reduced risk (1, 2). The two naturally occurring vitamin D forms, ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), can be obtained from natural foods, fortified products, or supplements, and vitamin D_3 can be synthesized from 7-dehydrocholesterol in skin exposed to UVB radiation (3). After synthesis in the skin or oral intake, vitamin D is converted into 25 hydroxyvitamin D [25(OH)D] in the liver. 25 Hydroxyvitamin D_3 [25(OH)D3] is the predominant circulating metabolite and correlates with vitamin D status (4). Then, 25(OH)D undergoes renal hydroxylation, tightly regulated by parathyroid hormone (PTH) and calcium concentrations, into the vitamin D hormone calcitriol [1,25-dihydroxyvitamin D; $1,25(OH)_2D$], the biologically active metabolite. A lower proportion of $1,25(OH)_2D$ could also be locally synthesized in tissues, including breast tissue, for local effects (5).

Experimental studies have shown that 25(OH)D (6), calcium (7), and PTH (8) might affect tumor development. High levels of $1,25(OH)_2D$ in the breast might have antitumor effects through the induction of cell differentiation, inhibition of cell growth, and regulation of apoptosis in normal and malignant cells, including human breast cells (9, 10). The actions of $1,25(OH)_2D$ are predominantly mediated by activation of the vitamin D receptor (3), and plays a critical role in regulating intestinal calcium absorption (11); $1,25(OH)_2D$ and extracellular

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Ethical approval: This study was approved by the ethics review board of the Inserm-U1018 research team. Participants provided written consent for the use of their blood samples and all data.

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calcium act jointly as key regulators of cell proliferation, differentiation, and function (12).

Because endogenous production after sun exposure is the main source of vitamin D (13), studies assessing the relationship between 25(OH)D serum concentrations and breast cancer risk are likely to minimize the misclassification of vitamin D exposure than those assessing the relationship between vitamin D dietary sources only. All six existing case-control studies published thus far reported a significant inverse association between serum 25(OH)D and breast cancer risk (14-19). Among six case-control studies nested in cohorts, only a recent study (20) found a statistically significant inverse association, whereas the others failed to find any association (21-25). Studies which analyzed data by menopausal status (14, 15, 17, 20-25) or age (19, 22) suggested that the effect of vitamin D on breast cancer is modulated by the hormonal milieu, a suggestion further supported by the recent finding of an association between circulating 25(OH)D and steroid hormones in young women (26).

In a case-control study nested in the French E3N (Etude Epidémiologique auprès des femmes de l'Education Nationale) prospective cohort, we investigated the risk of breast cancer in women according to baseline 25 (OH)D3 serum concentrations as markers of vitamin D status, taking into account serum calcium, PTH, and steroid hormone concentrations; we also analyzed breast cancer risk according to age and menopausal status.

Materials and Methods

Study cohort

The E3N cohort includes 98,995 French women born between 1925 and 1950, and insured by a health insurance plan mainly covering teachers. Participants, who gave written informed consent for external health follow-up through the health insurer, completed self-administered questionnaires, sent every 2 to 3 years since baseline in 1990, on medical and gynecologic history, menopausal status, and a variety of lifestyle characteristics. In each questionnaire, participants were asked whether a cancer had been diagnosed, and if so, pathology reports were requested from the attending physicians. The study was approved by the French National Commission for Data Protection and Privacy. The usual diet was assessed through a validated 208-item dietary history questionnaire sent out between June 1993 and June 1995 (27). Responders to the dietary questionnaire constituted the French component of the European Prospective Investigation into Cancer and Nutrition study. Blood samples were collected between 1995 and 1998 among 24,505 E3N participants, aliquoted into plasma, serum, lymphocytes and erythrocytes, and stored in liquid nitrogen (28). Along with blood samples, we collected information on fasting status, smoking, body mass index (BMI), use of medication in the preceding 12 hours, and menopausal status. Menopausal status at the date of blood collection was also confirmed by information requested in each questionnaire until July 2005. We defined the date of menopause as the date preceding 12 consecutive months of amenorrhea (excluding hysterectomy), the date of bilateral oophorectomy or, if not available (in decreasing order of priority), the self-reported date of menopause, the date when menopausal hormone therapy use began, the date when menopausal symptoms began, or an imputed date corresponding to age 47 if menopause was due to oophorectomy and age 51 otherwise (median ages for surgical and natural menopause in the cohort, respectively).

Population for analysis

For this case-control study nested within the E3N cohort, we selected women who completed the dietary questionnaire and who had available information on age at blood collection, date of collection, center of collection, menopausal status at collection, and fasting status at collection. This left us with a subcohort of 17,540 subjects among whom, during a follow-up period of up to 10 years from blood collection until July 2005, we identified 636 cases of incident invasive breast cancer (58 in premenopause and 578 in postmenopause). Two controls per case (n = 1,272) were selected (96 premenopause and 1,176 in postmenopause), matched on age (±2 years), menopausal status (premenopausal or postmenopausal) at blood collection, age at menopause (±2 years), study center (same among the 40 centers of collection), and date of blood collection (same year).

Analysis of 25(OH)D, calcium, PTH, estradiol, and progesterone serum concentrations

Serum samples were divided into batches of nine samples corresponding to three cases and their matched controls in random order. Analyses were done by the biochemistry laboratory of Bichat Hospital (Paris), which was blinded to the case-control status of the samples. Serum from intact human 25(OH)D3, intact human PTH 1-84, estradiol, and progesterone were measured on an Elecsys Analyser (Roche Diagnostic) by chemiluminescence immunoassay. This method very specifically determined 25(OH)D3, the predominant circulating metabolite in blood serum without interference by 25(OH) D2, and which has been found to provide results similar to those of the DiaSorin Liaison method (29), often used in studies assessing serum 25(OH)D concentrations (23, 25). Serum calcium was routinely determined on a Hitachi 911 Roche autoanalyzer. The optimal level of vitamin D and the threshold below which a person could be viewed as being deficient remains controversial. Low levels of vitamin D led to a corresponding increase in PTH levels to maintain calcium homeostasis. Because a threshold of 30 ng/mL has been suggested as being necessary to minimize deleterious health consequences in terms of both bone health and other diseases (30), 25(OH)D3 at <30 ng/mL was considered insufficient and 20 ng/mL as deficient (3) in the present article.

Statistical analysis

Seventy-five serum samples could not be used for any biological measurements either because samples were hemolyzed ($n = 7$), the volumes were insufficient ($n = 45$), or they could not be retrieved from the blood repository ($n = 23$); thus, they were all placed in a separate category.

Comparison of characteristics between cases and controls was done using χ^2 tests for categorical variables and Student's test for continuous variables. We also ran principal component analyses and correlation tests to assess variables associated with 25(OH)D3 serum concentrations. Serum 25(OH)D3 was then considered in tertiles determined from distribution among controls. We created a fourth category for missing 25(OH)D3 serum concentrations. Cases and controls were first compared with conditional logistic regression for the whole population. Odds ratios (OR) estimated the relationship between breast cancer risk and each tertile of 25(OH)D3 serum concentration in comparison with the lowest. To evaluate the crude association, we created a first model, which included only matching covariates [i.e., age at blood collection, menopausal status, age at menopause, date (same year), and center of blood collection].

Next, as potential confounders, we included BMI at blood collection (kg/m^2, continuous), use of menopausal hormone therapy in postmenopausal women (current/past/never) at blood collection, and variables estimated from the last questionnaire filled out before blood collection: personal history of mammography (yes/no), history of breast benign disease (yes/no), family history of breast cancer (yes/no), number of children (0, 1, 2, 3+), smoking status (never, past, current), use of oral contraceptives (ever/never), age at menarche (year, continuous), and physical activity [Metabolic Equivalent Task-Hour per week (METS-h/w), continuous]. We further added to the models' variables estimated from the dietary questionnaire sent in 1993: alcohol consumption (in grams of daily ethanol intake, continuous), total energy intake without alcohol (kcal/d, continuous), calcium and vitamin D dietary intakes (mg/d, continuous), and vitamin D and calcium supplement intakes at blood collection (yes/no). Models were also run with calcium (mmol/L, continuous) and PTH (pg/mL, continuous) serum concentrations because they were closely involved in the regulation of vitamin D metabolism, and also with estradiol (pmol/L, continuous) and progesterone (nmol/L, continuous) serum levels, found to be confounding factors, as they were associated both with 25(OH)D3 and breast cancer risk in our population.

We also conducted unconditional logistic regressions stratified by menopausal status at the time of both breast cancer diagnosis and blood collection, and by age at blood collection (<53, 53-60, and 60+ years, ages corresponding with tertile cutoff points in our population). For these two series of analyses, we created a first model adjusted for age at blood collection, menopausal status (only in the age-stratified analysis), age at menopause, and season of blood collection. To take into account both latitude and sun exposure of each region at the date of blood collection, we used mean daily UV dose exposure (continuous variables in kJ/m^2) in unconditional logistic regressions for parsimony of models, which was estimated among the 40 centers for blood collection and the year of sampling using the UV mapping algorithm (31). The three other models were computed by adding the same covariates as in the conditional regression analyses. We also ran models stratified on BMI (women with BMI ≤25 versus >25 kg/m^2) and on calcium intake (daily calcium intake <1,000 versus ≥1,000 mg/d plus women using calcium supplements at blood collection).

Tests for linear trends across tertiles of 25(OH)D3 serum concentration were done using median concentrations in each tertile excluding missing values. All statistical tests were two-sided; $P < 0.05$ was considered statistically significant. SAS statistical software (version 9.1; SAS Institute, Inc.) was used for all analyses. Results were presented as mean (SD) for continuous variables and N (%) for categorical variables.

Results

Selected characteristics of cases and controls are presented in Table 1. High alcohol consumption, familial history of breast cancer, and personal history of benign breast disease were more common in cases than in controls. Cases had both higher estradiol and progesterone serum concentrations in premenopause and postmenopause [399.3 pmol/L (SD, 234.3) and 10.1 nmol/L (SD, 15.2) in premenopause and 125.6 pmol/L (SD, 217.5) and 4.0 nmol/L (SD, 9.7) in postmenopause, respectively] than in controls [349.2 pmol/L (SD, 202.2) and 9.0 nmol/L (SD, 13.8) in premenopause and 105.8 pmol/L (SD, 205.5) and 3.2 nmol/L (SD, 7.7) in postmenopause]. The 25(OH)D3 serum concentration was lower for cases than for controls [24.4 ng/mL (SD, 10.9) and 25.1 ng/mL (SD, 11.0), respectively]; 75% of women had 25(OH)D3 serum concentrations lower than 30 ng/mL and 37.5% had serum concentrations lower than 20 ng/mL.

Women ages 53 years or under had a similar mean 25(OH)D3 serum concentration [25.3 (SD, 11.0)] as women between 53 and 60 [25.4 (SD, 11.0)], but a higher concentration than those over 60 [24.5 (SD, 10.9); $P = 0.04$]. Lower 25(OH)D3 serum concentrations were observed in women with a BMI of >30 kg/m^2 [22.1 ng/mL (SD, 8.8)] than in women with a BMI of <25 kg/m^2 [25.5 ng/mL (SD, 11.1); $P = 0.01$; data not shown].

The 25(OH)D3 serum concentration was correlated with the calcium serum concentration ($\rho = 0.13$, $P < 0.0001$ in the whole population; $\rho = 0.17$, $P = 0.0009$ in premenopausal women; and $\rho = 0.12$, $P < 0.0001$ in postmenopausal women) and negatively correlated with the PTH serum concentration ($\rho = -0.11$, $P = 0.0002$; $\rho = -0.16$, $P = 0.002$; and $\rho = -0.12$, $P < 0.0001$, respectively). No correlation was found between the 25(OH)D3 serum concentration and either the estradiol or progesterone serum concentration. In women who were premenopausal

Table 1. Selected characteristics of breast cancer cases and their matched controls at blood collection (1995-1998) among women in the E3N cohort (n = 1,908)

Baseline characteristics	Cases (n = 636)	Controls (n = 1,272)
	Mean (SD)	
Age (y)	56.9 (6.4)	56.9 (6.4)
Age at menarche (y)	12.7 (1.3)	12.9 (1.4)
BMI	23.8 (3.6)	23.8 (3.8)
Age at menopause	50.7 (3.7)	50.7 (3.6)
Number of children	1.9 (1.2)	2.1 (1.2)
Recreational physical activity (METS-h/wk)	51.0 (31.0)	50.3 (22.6)
Alcohol intake (g/d)*	12.0 (15.0)	10.9 (13.8)
Serum 25(OH)D_3 (ng/mL)	24.4 (10.9)	25.1 (11.0)
Serum calcium (mmol/L)	2.29 (0.1)	2.29 (0.1)
Serum PTH (pg/mL)	26.7 (11.2)	27.2 (19.1)
Serum estradiol (pmol/L)		
Premenopausal	399.3 (234.3)	349.2 (202.2)
Postmenopausal	125.6 (217.5)	105.8 (205.5)
Serum progesterone (nmol/L)		
Premenopausal	10.1 (15.2)	9.0 (13.8)
Postmenopausal	4.0 (9.7)	3.2 (7.7)
Calcium intake (mg)*	1,017.5 (273.0)	1,044.7 (289.9)
Vitamin D intake (μg)*	2.4 (1.2)	2.4 (1.2)
Total energy intake without alcohol (kcal)*	21.2 (5.7)	20.7 (5.6)
Mean UVB dose exposure (kJ/m^2 by day)	1.5 (0.2)	1.5 (0.2)
	N (%)	
Postmenopausal	489 (77.2)	990 (77.2)
Family history of breast cancer		
No	504 (79.3)	1,104 (86.8)
Yes	132 (20.7)	168 (13.2)
History of benign breast disease		
No	243 (38.2)	407 (32.0)
Yes	393 (61.8)	865 (68.0)
Personal history of mammography		
No	16 (2.5)	51 (4.0)
Yes	620 (97.5)	1,221 (96.0)
Smoking status		
Never	350 (55.0)	696 (54.7)
Past	217 (34.1)	436 (34.2)
Current	69 (10.9)	140 (11.1)
OC use		
No	383 (60.2)	761 (59.8)
Yes	253 (39.8)	511 (40.2)
Use of postmenopausal MHT (among postmenopausal women)	354 (72.4)	687 (69.5)
Season		
Spring	160 (25.2)	279 (21.9)
Summer	112 (17.6)	250 (19.6)
Autumn	188 (29.6)	370 (29.1)
Winter	176 (27.7)	373 (29.3)
Current use of calcium supplement		
No	589 (92.6)	1,183 (93.0)
Yes	47 (7.4)	89 (7.0)
Current use of vitamin D supplement		
No	602 (94.6)	1,210 (95.1)
Yes	34 (4.4)	62 (4.9)

NOTE: Assessed at the time of blood collection except where indicated.

Abbreviations: OC, oral contraceptive; MHT, menopausal hormone therapy.

*Assessed at the time of dietary questionnaire (1993). Calcium and vitamin D intakes estimated with residual methods.

at diagnosis, we observed a positive correlation between the 25(OH)D3 serum concentration and the mean daily UV dose in the 40 areas of blood collection (ρ = 0.18, P = 0.02). In women under 53 years of age at the time of blood collection, the correlation was lower (ρ = 0.07, P = 0.08). However, unconditional logistic regressions without mean daily UV dose adjustments did not affect point estimates (P for homogeneity between the fully adjusted model and the model without UV dose = 0.73). No other statistically significant correlation with 25(OH)D3 was found. It was the case in particular to 25(OH)D3 and vitamin D dietary intake, and for calcium serum concentrations and dietary calcium intakes.

Table 2 shows the results from conditional logistic regression analyses run on the whole population. Risk of breast cancer decreased with increasing 25(OH)D3 serum concentration; associations reached statistical significance in the full model with dietary covariates and serum biomarkers (calcium, PTH, estradiol, and progesterone); the OR for the uppermost (concentrations over 27.0 ng/mL) versus the lowest tertile (<19.8 ng/mL) was 0.73; the 95% confidence interval (95% CI) was 0.55 to 0.96; and P for trend across tertiles was 0.02.

We tested the hypothesis of a differential association of 25(OH)D3 serum concentration with breast cancer risk according to menopausal status (Table 3), by stratifying on menopausal status at blood collection and at

diagnosis. We observed a stronger inverse association between breast cancer risk and vitamin D concentrations for premenopausal breast cancer (OR, 0.37; 95% CI, 0.12-1.15) for the upper versus lower tertile than for postmenopausal breast cancer, whether the blood collection had been premenopausal or postmenopausal. However, the test for an interaction between menopausal status at diagnosis and 25(OH)D3 was not statistically significant ($P = 0.59$).

Associations between high 25(OH)D3 serum concentration and breast cancer risk seemed to be heterogeneous across age categories ($P = 0.06$). We then explored the effect of age on the relationship between 25(OH)D3 and breast cancer risk, using 53 and 60 years as cutoff points which corresponded to tertiles (Table 4). Significant decreases in breast cancer risk were limited to the youngest women, within the model which included all variables, an OR of 0.60; 95% CI, 0.37 to 0.98, in the last tertile of 25(OH)D3 (concentration higher than 27.0 ng/mL) compared with the first (<19.8 ng/mL), and a significant trend toward decreasing risk across tertiles ($P = 0.04$).

No statistically significant interactions were found between breast cancer risk, serum 25(OH)D3 levels, dietary calcium intake ($P = 0.75$), and BMI ($P = 0.42$). However, the significant negative association between serum 25(OH)D3 and breast cancer risk in our population was restricted to women with dietary calcium intake values of <1,000 mg/d (OR for the upper tertile of vitamin D serum concentration = 0.58; 95% CI, 0.39-0.86) and to women with a BMI of <25 kg/m^2 (corresponding OR, 0.70; 95% CI, 0.51-0.95; data not shown). Sensitivity analyses excluding breast cancer cases occurring in the first year after blood collection ($n = 80$) showed a stronger association between serum 25(OH)D3 and breast cancer risk (OR, 0.76; 95% CI, 0.59-0.99 and OR, 0.71; 95% CI, 0.55-0.93 for the second and third tertiles, respectively). Another sensitivity analysis, which excluded women who were taking vitamin D supplements ($n = 95$) and their matched controls, did not modify the association (OR, 0.82; 95% CI, 0.63-1.05 and OR, 0.74; 95% CI, 0.57-0.97) for the second and third tertiles, respectively, in the fully adjusted model.

Discussion

In this case-control study nested in a large cohort of French women, we found evidence of a significant inverse association between 25(OH)D3 serum concentrations and breast cancer risk. Our results show a more pronounced decreased breast cancer risk in younger women than in older women. Although not significant, our findings also suggested a stronger decrease in breast cancer risk in premenopausal women than in postmenopausal women. When adjusting for serum calcium and PTH, which are correlated with 25(OH)D3 serum concentrations, and for estradiol and progesterone concentrations, which were found to be confounding factors, the association was strengthened.

To our knowledge, this is the first case-control study, nested in a large prospective cohort of women and designed to analyze baseline 25(OH)D3 serum concentrations and subsequent breast cancer risk, which takes into account important potential confounders. In particular, we adjusted for the effect of seasonal and latitude effects

Table 2. Multivariate OR and 95% CI for breast cancer incidence by serum 25(OH)D_3 concentration, nested case-control study in the E3N cohort ($n = 1,908$)

Tertile of serum 25(OH)D_3 (ng/mL)	No. cases/no. controls	OR* (95% CI)	OR† (95% CI)	OR‡ (95% CI)	OR§ (95% CI)
All‖	636/1,272				
<19.8	226/404	1	1	1	1
19.8-27	198/402	0.87 (0.68-1.10)	0.84 (0.66-1.08)	0.84 (0.66-1.08)	0.81 (0.63-1.04)
>27	191/412	0.80 (0.62-1.03)	0.80 (0.62-1.04)	0.81 (0.62-1.06)	0.73 (0.55-0.96)
P trend		0.09	0.10	0.12	0.02

*ORs and CIs from conditional logistic regression matched on age (±2 y), menopausal status (premenopausal or postmenopausal) at blood collection, age at menopause (±2 y), study center (same geographic localization in France among the 40 collection centers), and date of blood collection (same year).

†Conditional logistic regression adjusted for the same variables as in * plus BMI at the time of blood collection, physical activity, age at menarche, number of children, tobacco status, previous use of oral contraceptives, MHT use (among postmenopausal women only), personal history of mammography, benign breast disease, and previous family history of breast cancer.

‡Conditional logistic regression adjusted for the same variables as in † plus alcohol consumption, total energy intake without alcohol, calcium and vitamin D dietary and supplement intakes assessed from the dietary questionnaire.

§Conditional logistic regression adjusted for the same variables as in ‡ plus serum calcium, PTH, estradiol, and progesterone concentrations.

‖A separate fourth category for missing values (cases, $n = 21$; controls, $n = 54$) was considered; OR were not significant.

Table 3. Multivariate OR and 95% CI for breast cancer incidence by serum 25(OH)D_3 concentration, nested case-control study in the E3N cohort (n = 1,908) stratified by menopausal status at blood collection and diagnosis

Tertile of serum 25(OH)D_3 (ng/mL)	No. cases/no. controls	OR* (95% CI)	OR† (95% CI)	OR‡ (95% CI)	OR§ (95% CI)
All‖	636/1,272				
Premenopausal at blood collection and premenopausal at diagnosis					
<19.8	20/25	1	1	1	1
19.8-27	20/31	0.69 (0.30-1.62)	0.55 (0.21-1.42)	0.42 (0.15-1.18)	0.43 (0.14-1.25)
>27	14/34	0.41 (0.16-1.08)	0.37 (0.14-1.04)	0.35 (0.12-1.03)	0.37 (0.12-1.15)
P trend		0.07	0.06	0.07	0.11
Premenopausal at blood collection and postmenopausal at diagnosis					
<19.8	40/61	1	1	1	1
19.8-27	22/65	0.52 (0.27-0.99)	0.54 (0.27-1.05)	0.50 (0.25-0.99)	0.50 (0.25-1.02)
>27	27/54	0.79 (0.41-1.50)	0.76 (0.39-1.50)	0.76 (0.38-1.52)	0.72 (0.35-1.45)
P trend		0.8	0.5	0.5	0.4
Postmenopausal at blood collection and postmenopausal at diagnosis					
<19.8	166/318	1	1	1	1
19.8-27	156/306	0.97 (0.74-1.28)	0.96 (0.73-1.26)	0.96 (0.72-1.27)	0.91 (0.69-1.21)
>27	150/324	0.88 (0.67-1.16)	0.87 (0.66-1.15)	0.87 (0.66-1.16)	0.80 (0.60-1.07)
P trend		0.3	0.3	0.3	0.12

*ORs and CIs from unconditional logistic regression adjusted for age at blood collection, age at menopause, and mean daily UV dose exposure among the 40 centers of blood collection, and season of blood collection (same year).

†From unconditional logistic regression adjusted for the same variables as in * plus BMI at the time of the blood collection, physical activity, age of menarche, number of children, tobacco status, previous use of oral contraceptives, MHT use (among postmenopausal women only) at blood collection, personal history of mammography, benign breast diseases, and previous family history of breast cancer.

‡From unconditional logistic regression adjusted for the same variables as in †, plus alcohol consumption, total energy intake without alcohol, calcium and dietary vitamin D assessed from the dietary questionnaire, and supplement intakes. In the premenopausal subgroup, supplement intakes were removed due to lack of convergence (only one case taking vitamin D supplement).

§From unconditional logistic regression adjusted for the same variables as in ‡ plus serum calcium, PTH, estradiol, and progesterone concentrations.

‖A separate fourth category for missing values (cases, n = 21; controls, n = 54) was considered; ORs were not significant.

on 25(OH)D3 synthesis via the date, the center, or the mean daily UV dose for the center at the time of blood collection; in addition, we adjusted for calcium and PTH serum concentrations. We also took into account estradiol and progesterone serum concentrations, which have been reported to be associated with vitamin D status (12, 26). Moreover, we controlled for both dietary and supplement intakes.

One pooled analysis (2), and a recent meta-analysis (1), examined the relationship between 25(OH)D serum concentration and risk of breast cancer. According to the pooled analysis (2) of the Nurses' Health Study (NHS; ref. 22) and of a British case-control study (18), women with 25(OH)D serum concentrations of >52 ng/mL had a significant 50% lower risk of breast cancer than those with levels of <13 ng/mL. If we presume the linearity of the dose-response gradient (as suggested in our study and in this pooled analysis), this estimate is consistent with ours, which showed a 27% lower risk of breast cancer for women with 25(OH)D serum concentrations higher than 27 ng/mL compared with those with serum concentrations lower than 19.8 ng/mL. The authors of that meta-analysis (1) found a pooled OR of 0.58 and 95% CI of 0.50 to 0.66 for the highest quartile of 25(OH)D in comparison to the lowest, similar to ours, although the cutoff points in the studies varied by as much as 27 ng/mL (23) to 60 ng/mL (18) for the highest, and 13 to 20 ng/mL for the lowest. This disparity between quartile cutoff points might be explained by sun exposure and the latitudes of the studies, but also by differences in vitamin D food fortification between Europe and the United States because the fortification of dairy foods and margarines has long been common in the United States (32), whereas it is restricted to very few products in France.

More studies found an inverse association between 25(OH)D and breast cancer risk at premenopause [two (14, 20) out of five studies (14, 17, 20-22)] than at postmenopause [three, significant (15, 17) or not (22), out of

eight studies (20, 21, 23-25)]. Despite the low power and borderline significance, our study was consistent with the two premenopausal studies in that it showed decreased risk both in women who were still premenopausal at the end of follow-up and in our youngest group of women. Investigators from the NHS (33) and the Women's Health Study (34) reported a lower risk of developing premenopausal breast cancer associated with higher vitamin D intakes (both from diet and supplements).

To our knowledge, only the NHS (22) evaluated the influence of age and estrogen/progesterone deficiency using steroid blood concentrations; however, those results suggested a stronger decrease in risk in the oldest group of women (>60 years of age). An explanation for the discrepancy with our study, despite a similar design, might lie in the different mean serum concentrations of 25(OH)D (~25 and 33 ng/mL in the E3N and the NHS, respectively) and in a distinct percentile distribution (highest cutoff point of 27 and 48 ng/mL in the E3N and NHS, respectively).

Our results, demonstrating a more pronounced decreased risk in younger and premenopausal women, may be explained by the joint relationship of calcium, vitamin D, and insulin-like growth factors (IGF; ref. 35). *In vitro* studies have suggested that calcium and vitamin D exert anticarcinogenic effects on breast cancer cells that express IGF-I and IGF-binding protein 3. In addition, vitamin D inhibits the IGF-I–stimulated growth of breast cancer cells (36). Because circulating levels of IGF-I and/or IGF-binding protein 3 decline with age (37), the interaction between IGF pathways and calcium and vitamin D are likely to be stronger for younger women than for postmenopausal women, possibly leading to higher risk reduction in young women (38). In addition, the elderly have been shown to have a decreased capacity for vitamin D synthesis in the skin with similar sun exposure (39). Renal production of $1,25(OH)_2D$, the metabolically active form of vitamin D, is also reduced with aging (40), concomitantly with lower 25(OH)D3 mean serum concentrations in older women compared with women

Table 4. Multivariate OR and 95% CI for breast cancer incidence by serum $25(OH)D_3$ concentration, nested case-control study in the E3N cohort (n = 1,908) stratified by age at blood collection <53 y (n = 618), 53 to 60 y (n = 653), and >60 y (n = 637)

Tertile of serum $25(OH)D_3$ (ng/mL)	No. cases/no. controls	OR* (95% CI)	OR† (95% CI)	OR‡ (95% CI)	OR§ (95% CI)
All‖	636/1,272				
<53 y					
<19.8	77/125	1	1	1	1
19.8-27	68/140	0.78 (0.52-1.18)	0.76 (0.49-1.16)	0.77 (0.50-1.18)	0.78 (0.50-1.20)
>27	50/128	0.61 (0.39-0.97)	0.60 (0.37-0.96)	0.64 (0.40-1.03)	0.60 (0.37-0.98)
P trend		0.04	0.03	0.07	0.04
53-60 y					
<19.8	76/131	1	1	1	1
19.8-27	70/129	0.93 (0.62-1.41)	0.95 (0.62-1.46)	0.92 (0.60-1.41)	0.85 (0.55-1.31)
>27	69/156	0.77 (0.51-1.15)	0.81 (0.53-1.23)	0.77 (0.50-1.19)	0.71 (0.46-1.10)
P trend		0.2	0.3	0.2	0.1
>60 y					
<19.8	73/148	1	1	1	1
19.8-27	60/133	0.91 (0.60-1.39)	0.94 (0.61-1.45)	0.96 (0.62-1.49)	0.94 (0.34-1.49)
>27	72/128	1.11 (0.74-1.67)	1.15 (0.75-1.77)	1.15 (0.75-1.78)	1.09 (0.70-1.71)
P trend		0.3	0.5	0.5	0.6

*ORs and CIs from unconditional logistic regression adjusted for age at blood collection, menopausal status (premenopausal or postmenopausal), age at menopause, mean daily UV dose exposure among the 40 centers of blood collection, and season of blood collection (same year).

†From unconditional logistic regression adjusted for the same variables as in * plus BMI at the time of the blood collection, physical activity, age of menarche, number of children, tobacco status, previous use of oral contraceptives, MHT use (among postmenopausal women only), personal history of mammography, benign breast diseases, and previous family history of breast cancer.

‡From unconditional logistic regression adjusted for the same variables as in †, plus alcohol consumption, total energy intake without alcohol, calcium and dietary vitamin D assessed from the dietary questionnaire, and supplement intakes.

§From unconditional logistic regression adjusted for the same variables as in ‡ plus serum calcium, PTH, estradiol, and progesterone concentrations.

‖A separate fourth category for missing values (cases, n = 21; controls, n = 54) was considered; ORs were not significant.

ages 53 or under in our analysis. However, our study did not enable us to confirm whether it was age or menopausal status which was the true modifier of the relationship between 25(OH)D and breast cancer risk.

According to another hypothesis, higher 25(OH)D serum concentrations could reduce subsequent breast cancer risk in premenopausal women, as shown by the recent finding of reduced progesterone and estradiol serum concentrations with higher circulating 25(OH)D levels (26). Estrogen deficiency also seems to reduce vitamin D activation and Vitamin D Receptor (VDR) expression, suggesting that older and postmenopausal women might be at an increased risk (12), and that higher vitamin D concentrations would be necessary to achieve the same benefit in postmenopausal as in premenopausal women.

In our study, the significant negative association between 25(OH)D3 serum concentration and breast cancer risk restricted to women with low to medium daily dietary calcium intake is in agreement with a hypothesis previously described for prostate cancer (41); it was suggested that the anticarcinogenic properties of 1,25(OH)2D may be less effective because its production by the kidney might be reduced in case of high calcium intake. In contrast, low dietary calcium might transiently reduce the calcium serum concentration through PTH feedback control, and would enhance the conversion rate of 1,25(OH)2D from 25(OH)D in order to increase the efficacy of intestinal calcium absorption.

Our data suggested that the benefit of a high 25(OH)D3 serum concentration is restricted to women with normal BMI (<25 kg/m^2), a result which might be due to a lack of power in our study, and a low prevalence of overweight and obese women. In our study, lower 25(OH)D3 serum concentrations have been found in overweight individuals, likely due to greater uptake of vitamin D into adipocytes rather than to less sun exposure or less effective vitamin D synthesis (42). However, BMI was not a confounder in the association between vitamin D and breast cancer risk, unlike findings from the Women's Health Initiative, in which a lower risk of breast cancer associated with high baseline 25(OH)D serum concentrations disappeared after adjustment for BMI (23). Nevertheless, the decreased bioavailability of vitamin D in tissues could explain the increased risk of cancer.

Strengths and limitations

One strength of our results lies in the fact that we took into account both geographic localization and date of blood collection as matching criteria, so that a latitude effect or a potential seasonal effect was unlikely to have influenced our results. Three ecological studies (43-45) showed a significant inverse association between UVB exposure and risk of breast cancer, while another found no association (46). Although we did not record the sunbathing habits of women in our study, we captured sunlight exposure by specifically assessing the 25(OH)D3 serum concentration of both 25(OH)D2 and 25(OH)D3 instead (as is done in most studies), which reflects endogenous and exogenous vitamin D sources. Moreover, no statistically significant correlation was found between dietary vitamin D and serum vitamin D concentrations in our study and added to evidence that assessment of vitamin D serum concentration is a key observation to account for misclassification of exposure in studies examining dietary sources only.

Although we adjusted for a large number of major parameters implicated either in vitamin D status or breast cancer incidence, our study had some limitations. We cannot exclude the possibility that the associations we observed resulted from a confounding bias. Cases and controls were from a selected population of highly educated women willing to participate in both the dietary survey and blood collection. Although this population was not representative of the general population, it is not clear how selection could have affected our results. However, although information on menopausal status was accurate, premenopausal women did not provide information on the menstrual phase cycle on the date of blood collection; how this might have modified the association between the 25(OH)D3 serum concentration and breast cancer risk, specifically when adjusting for both serum estradiol and progesterone in this population, is unknown. Nonetheless, results were similar whether or not the model was adjusted for the two hormonal biomarkers, which suggests only a weak effect of this missing information on our findings. Another limitation is that we did not have relevant data on doses of calcium and vitamin D intake at blood collection, which could therefore affect our associations. In particular, among women taking vitamin D supplements, we lacked information as to whether the supplement was vitamin D2 or D3. Because we measured only 25(OH)D3 serum concentrations, some misclassification of vitamin D concentrations might have occurred in women taking vitamin D_2. However, our findings were similar when excluding vitamin D supplement users, thus suggesting that this potential bias was of minor importance. It may also be questioned whether it is appropriate to use a single determination of 25(OH)D because vitamin D status results from a combination of various lifestyle characteristics which could change during the study (sunbathing habits, exact sun exposure at the time of blood collection, weather; ref. 47). We assessed 25(OH)D3 and other biomarker concentrations during an average of 4 years prior to breast cancer diagnosis. Nonetheless, it remains unclear as to which period is optimal for measuring the vitamin D serum concentration, although blood collection several years prior to breast cancer diagnosis is preferred (48). Interestingly, results from our sensitivity analyses indicated that the associations were stronger when excluding breast cancer cases diagnosed during the year following blood collection. In future studies, multiple measurements at different periods before diagnosis may provide more accurate indicators for analysis.

Conclusions

Our findings support the conclusion that the maintenance of adequate vitamin D levels should be encouraged, especially in populations with low 25(OH)D serum concentrations, as in our study. To maintain a 25(OH)D serum concentration of >30 ng/mL, assuming a baseline of 10 ng/mL in sedentary women with very little sun exposure (49), an intake of 2,000 IU/d is necessary; this corresponds to the U.S. National Academy of Sciences' upper limit (50). However, recommendations from food agencies concerning vitamin D intakes are between 200 and 400 IU/d, which is why scientists advocate raising these recommendations (30, 51). Alternatively, 12 minutes of sun exposure per day, on a clear day, with 50% of the skin area exposed and if climate and season allow, are equivalent to an approximate oral intake of 3,000 IU of vitamin D_3 (52); however, these recommendations are not adapted to latitudes above 35 degrees, where there is minimal, if any, previtamin D_3 production in the skin during winter (52). These arguments support the aggressive supplementation and fortification of foods such as milk, dairy products, or orange juice in European countries, to be encouraged by food and health agencies. Further randomized intervention trials with different doses of vitamin D supplementation are also required to confirm its benefits on breast cancer risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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