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# Plasma 25-hydroxyvitamin D and the risk of breast cancer in the European Prospective Investigation into Cancer and Nutrition: A nested case–control study

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**Key words:** vitamin D, breast cancer, 25-hydroxyvitamin D, prospective study, estrogen receptor status

**Abbreviations:** 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CI: confidence interval; DEQAS: Vitamin D External Quality Assurance Scheme; EPIC: European Prospective Investigation into Cancer and Nutrition; ER+: estrogen receptor positive; ER−: estrogen receptor negative; IARC: International Agency for Research on Cancer; ICD-O: International Classification of Diseases for Oncology; IRS: immunoreactive score; HRT: hormone replacement therapy; OR: odds ratio; PR+: progesterone receptor positive; PR−: progesterone receptor negative

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**Experimental evidence suggests that vitamin D might play a role in the development of breast cancer. Although the results of case–control studies indicate that circulating 25-hydroxyvitamin D [25(OH)D] is inversely associated with the risk of breast cancer, the results of prospective studies are inconsistent. A case–control study embedded in the European Prospective Investigation into Cancer and Nutrition (EPIC) was carried out comprising 1,391 incident breast cancer cases and 1,391 controls. Multivariable conditional logistic regression models did not reveal a significant overall association between season-standardized 25(OH)D levels and the risk of breast cancer ($OR_{Q4-Q1}$ [95% CI]: 1.07 [0.85–1.36], $p_{trend} = 0.67$). Moreover, 25(OH)D levels were not related to the risks of estrogen receptor positive tumors ($OR_{Q4-Q1}$ [95% CI]: 0.97 [0.67–1.38], $p_{trend} = 0.90$) and estrogen receptor negative tumors ($OR_{Q4-Q1}$ [95% CI]: 0.97 [0.66–1.42], $p_{trend} = 0.98$). In hormone replacement therapy (HRT) users, 25(OH)D was significantly inversely associated with incident breast cancer ($OR_{log2}$ [95% CI]: 0.62 [0.42–0.90], $p = 0.01$), whereas no significant association was found in HRT nonusers ($OR_{log2}$ [95% CI]: 1.14 [0.80–1.62], $p = 0.48$). Further, a nonsignificant inverse association was found in women with body mass indices (BMI) < 25 kg/m$^2$ ($OR_{log2}$ [95% CI]: 0.83 [0.67–1.03], $p = 0.09$), as opposed to a borderline significant positive association in women with BMI $\geq$ 25 kg/m$^2$ ($OR_{log2}$ [95% CI]: 1.30 [1.0–1.69], $p = 0.05$). Overall, prediagnostic levels of circulating 25(OH)D were not related to the risk of breast cancer in the EPIC study. This result is in line with findings in the majority of prospective studies and does not support a role of vitamin D in the development of breast cancer.**

**What's new?**

**Experimental studies have indicated that vitamin D may play a role in preventing tumor formation in the breast. However, in the present investigation, the largest prospective case-control study on circulating 25-hydroxyvitamin D (25(OH)D) and breast cancer risk conducted to date, pre-diagnostic levels of 25(OH)D were found to be unrelated to overall breast cancer risk. While the results support those of similar prospective studies, a significant inverse association was detected between 25(OH)D levels and incident breast cancer in women taking hormone replacement therapy, suggesting that background factors may influence risk associations.**

Experimental evidence from *in vitro* and animal studies suggests that vitamin D exerts proapoptotic, antiproliferative, prodifferentiating, antiangiogenetic, anti-invasive and anti-inflammatory effects on the breast epithelium.[1] Furthermore, there is evidence to indicate that vitamin D may block estrogen signaling by inhibiting estrogen synthesis and by downregulating estrogen receptor expression in breast cancer cells.[1] Currently, a potential role of vitamin D in preventing breast cancer is being discussed.[2,3]

With regard to vitamin D intake from food and supplements in relation to breast cancer risk, the epidemiological evidence is inconclusive.[2] A meta-analysis of six prospective studies from 2010 suggested a moderate inverse association between both overall and supplemental vitamin D intake and breast cancer risk,[4] whereas the majority of prospective studies published afterward did not support this finding.[5–10] Results of vitamin D trials with breast cancer as primary end point are not available so far. In the Women's Health Initiative, a trial in postmenopausal women with hip fracture as primary outcome, a combined vitamin D (10 μg/day) and calcium (1 mg/day) intervention was not related to breast cancer risk after 7 years of intervention.[11] Yet, breast cancer risk was significantly increased in women with a high total vitamin D intake (≥15 μg/day) at randomization. On the other hand, after exclusion of participants who reported personal vitamin D or calcium supplement use at randomization, the intake of interventional vitamin D/calcium supplements was associated with a significantly decreased breast cancer risk.[12]

Although the contribution of diet to the overall vitamin D supply is limited, exposure to sunlight, *i.e.*, UVB, is the most important determinant of vitamin D status.[13,14] Thus, rather

than focusing on vitamin D intake, the relationship between plasma 25(OH)D, which is the precursor of the active hormone 1,25$(OH)_2$ vitamin D and the most commonly used vitamin D status marker, and breast cancer risk has been assessed in several epidemiological studies. Case–control studies consistently revealed an inverse association of 25(OH)D levels and breast cancer risk,[15–17] even though lower circulating levels of 25(OH)D in cancer patients have been attributed to changes in lifestyle around the time of diagnosis and may therefore not reflect a causal role of vitamin D in breast cancer development.[15,18]

With respect to prospective nested case–control studies, the relationship between circulating 25(OH)D with breast cancer risk is less clear. No significant inverse association between 25(OH)D levels and breast cancer risk was found in a meta-analysis of four prospective studies in 2010,[15] whereas a meta-analysis in 2011 that included two additional prospective studies provided a significant inverse overall association.[16] However, 25(OH)D levels were significantly inversely associated with breast cancer risk in only one of the original studies included in the latter meta-analysis, the French E3N cohort, which is part of the multicenter European Prospective Investigation into Cancer and Nutrition (EPIC).[19] As no significant associations were observed in two further prospective studies that were published after both meta-analyses,[20,21] the evidence from prospective studies on a relationship between 25(OH)D levels and breast cancer risk remains inconclusive.

Therefore, our first objective was to investigate the prospective relationship between 25(OH)D levels and breast cancer risk in a larger EPIC-wide prospective case–control study. Although experimental findings suggest that vitamin D may interfere with estrogen signaling and protect from estrogen receptor positive (ER+) tumors, there is a lack of evidence from prospective studies concerning this matter. Thus, our second objective was to assess the association between 25(OH)D and breast cancer risk by estrogen receptor status.

## Methods

### Study population

EPIC is a prospective cohort study that initially included 366,521 women and 153,457 men aged mostly between 35 and 70 years. Participants were enrolled at 23 centers in ten European countries (Denmark, France, Germany, Greece, Italy, Norway, Spain, Sweden, The Netherlands and the United Kingdom) between 1992 and 2000. Methods and design of the study have previously been described in detail.[22] In brief, anthropometric measurements and questionnaire data on diet, health status, medication use, reproductive and menstrual history, history of lifetime alcohol consumption and smoking, occupation, education and physical activity were collected at baseline. Also, blood samples were drawn from about 80% of the participants. Processed and aliquoted blood specimens were stored locally at the study centers and centrally at the International Agency for Research on Cancer (IARC) in Lyon, France. All participants gave written informed consent and the study was approved by the review board of the IARC and local ethics committees as necessary.

### Cases and controls

Incident cases of invasive breast cancer were ascertained by record linkage with regional or national cancer registries in most countries. In Germany, Greece and France, active follow-up procedures in combination with record linkage systems or health insurance data were used to identify breast cancer cases, followed by a systematic and detailed medical verification of self-reports by means of clinical and pathological records. Breast cancer incidence data were coded according to the International Classification of Diseases for Oncology (ICD-O-2/3). Only cases of primary epithelial breast cancer that occurred before center-specific closure dates between 2003 and 2006 and who had donated a blood sample at baseline were eligible for our study.

The case selection was motivated by three criteria: at first, all women with a breast cancer diagnosis before the age of 50 ($n = 591$) were selected to attain a sufficient number of premenopausal cases. As analyses by estrogen receptor status were a core objective of our study, all estrogen receptor negative (ER−) cases above the age of 50 ($n = 473$) were selected next. Subsequently, an equal number of ER+ cases above the age of 50 ($n = 473$) was chosen. The ER+ cases were characterized by the same distribution of country and year of diagnosis as the ER− cases, while apart from these criteria their selection was random. Cases and controls with a history of cancer (except nonmelanoma skin cancer) prior to the index date of breast cancer diagnosis were not considered. Because of a lack of remaining plasma samples, 141 cases and the same number of controls had to be excluded from the final selection. After further excluding three cases and two controls (and the corresponding matched subjects) with undetectable 25(OH)D values, a total of 2,782 participants (1,391 cases) remained for matched analyses.

As 25(OH)D in relation to breast cancer risk had been assessed before the start of our project in the Malmö Diet and Cancer Study, which is part of EPIC,[23] participants from Malmö were not included. In contrast, the case set for our study does include 160 incident cases from the French E3N cohort. These cases were also part of a parallel initiative based on a total of 636 incident cases from the E3N study that independently started at the same time as our study.[19]

An incidence density protocol was applied for matching. Accordingly, each control could be chosen more than once and also become a case at a later time point. Besides the factor "study center," the following baseline characteristics were used as matching criteria: age (±3 months), menopausal status (premenopausal, surgical postmenopausal, natural postmenopausal and perimenopausal/unknown), exogenous hormone use at blood donation, *i.e.*, the use of oral contraceptives or hormone replacement therapy (HRT) (yes, no or missing), time of the day of blood collection (±1 hr), fasting

status (<3, 3–6 and >6 hr) and phase of the menstrual cycle (early follicular, late follicular, periovulatory, midluteal and other luteal). Season of blood draw was not a matching factor, as the presented matching criteria were initially defined for a set of case–control studies on plasma antioxidants and circulating vitamin levels beyond 25(OH)D. Hence, season-standardized 25(OH)D values were calculated for our main analysis and subgroup analyses by season of blood draw were carried out (see below).

### Laboratory methods

The 25(OH)D levels were measured in the immunoassay laboratory of the Cancer Epidemiology Division at the German Cancer Research Center, Heidelberg (DKFZ) using citrate plasma samples that had been drawn at baseline. Samples of most participants were retrieved from the central liquid nitrogen storage at 2196C at the IARC in Lyon, France. Samples of Danish and Swedish participants were retrieved from local storage in nitrogen vapor at 2150 or from freezers at 270, respectively. Case and control sample sets were assayed within the same analytical batch, blinded to the lab personnel. Samples of Danish and Swedish participants were retrieved from local storage in nitrogen vapor at −150° or from freezers at −70°, respectively. Case and control sample sets were assayed within the same analytical batch, blinded to the lab personnel. For quantification of 25(OH)D in plasma samples an OCTEIA 25(OH)D enzyme immunoassay (IDS, Immunodiagnostic Systems, Boldon, UK) was used. Recovery of the assay determined by spiking a high with a low concentrated sample in different ratios ranged between 84 and 98% (at 21.4–50.9 nmol/L). Pearson's coefficient of correlation of 25(OH)D levels in 30 DEQAS (Vitamin D External Quality Assurance Scheme) samples determined with HPLC-MS/MS[24] and the OCTEIA enzyme immunoassay was 0.988. The overall intra-assay and interassay coefficients of variation from two DEQAS samples measured in each batch were 3.7 and 6.6% at 23.0 nmol/L, and 5.6 and 10.9% at 42.6 nmol/L, respectively. The lower limit of detection was 6.7 nmol/L, and the upper limit of detection was 175 nmol/L. Results of tumor receptor status assessments were provided by the study centers. The following standardized criteria were used to define a positive receptor status: ≥10% cells stained, any "plus-system" description, ≥20 fmol/mg, an Allred score of ≥3, an immunoreactive score (IRS) ≥ 2 or an H-score ≥ 10.[25]

### Statistical evaluation

Season-standardized 25(OH)D levels were obtained by adding the residuals from lowess regressions of raw 25(OH)D values on the calendar week of blood collection to the overall 25(OH)D mean.[26] Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to quartiles and continuous log2 values of season-standardized 25(OH)D values were calculated by conditional logistic regression analyses. First, a crude model was built accounting for matching variables only, *i.e.*, study center, age, menopausal status, exogenous hormone use, time of the day of blood collection, fasting status and phase of the menstrual cycle. Second, a multivariable model was built adjusting for potential confounders, *i.e.*, body mass index (BMI, <25, 25–29.9 and ≥30 kg/m$^2$), age at first menses (<7 or missing, 7–10, 11–13 and ≥13 years), number of full-term pregnancies (1, 2, ≥3 and nulliparous), age at first full-term pregnancy (≤20, 21–30 and >30 years), breastfeeding (yes or no), alcohol consumption (<1.5, 1.5–9.9, 10–19.9, 20–29.9 and ≥30 g/day), smoking status (never, former and current), education level (none or primary school completed, technical or professional school, secondary school and longer education including university degree) and physical activity according to the Cambridge Physical Activity Index (inactive, moderately inactive, moderately active and active). A category for missing values was created for all adjustment variables. In addition to the analyses based on season-standardized 25(OH)D levels, the described models were calculated using quartiles, *a priori* defined categories (<25, 25–49.9, 50–74.9 and ≥75 nmol/L) and log2 continuous values of crude 25(OH)D levels adjusting for season.

Because of our study objective and design, subgroup analyses by ER status and age at diagnosis (≤50 *vs.* >50 years) were carried out. Further, we separately analyzed the associations between 25(OH)D levels and breast cancer risk by progesterone receptor (PR) status and combined ER/PR status. Moreover, we carried out *a priori*-defined analyses stratified by menopausal status at blood donation, HRT use at blood donation, season and country. The analysis stratified by menopausal status was conducted, as one previous study had shown an interaction between 25(OH)D levels and menopausal status,[19] while most prospective studies consisted of predominantly postmenopausal women. HRT use was considered for stratified analyses, because HRT has been proposed to interfere with vitamin D signaling in relation to breast cancer risk.[27] Given the seasonal variation in 25(OH)D levels, we carried out subgroup analyses in subjects who donated blood in summer (June to November) and winter (December to May). Finally, because of the multicenter set-up of our study, we stratified our analyses by study center.

A meta-analytic approach to test for heterogeneity across strata was applied calculating Cochran's $Q$ and the $I^2$ statistic.[28] Sensitivity analyses were carried out excluding breast cancer cases that had occurred within the first 2 years after baseline. Tests for linear trends were conducted by using the median 25(OH)D values of each quartile as quantitative score. All statistical tests were two-sided and $p$-values <0.05 were considered to be statistically significant. SAS 9.2 (SAS Institute, Cary, NC) was used for all analyses.

## Results

Overall, 1,391 breast cancer cases and 1,391 controls were selected for our study. Both groups had a mean age of 50.7 (±8.8) years at baseline. The mean time interval between

**Table 1.** Characteristics of 1,391 breast cancer cases and 1,391 matched controls

| | Cases, *N* (%) | Controls, *N* (%) | $p^1$ | | Cases, *N* (%) | Controls, *N* (%) | $p^1$ |
|---|---|---|---|---|---|---|---|
| **Estrogen receptor status** | | | | **Age at first full-term pregnancy** | | | |
| Negative | 547 (39.3) | | | ≤20 years | 139 (10.0) | 191 (13.7) | 0.01 |
| Positive | 643 (46.2) | | | >20–30 years | 838 (60.2) | 831 (59.8) | |
| Unknown | 201 (14.5) | | | >30 years | 134 (9.6) | 99 (7.1) | |
| **Progesterone receptor status** | | | | Missing | 101 (7.3) | 104 (7.5) | |
| Negative | 472 (33.9) | | | Nulliparous | 179 (12.9) | 166 (11.9) | |
| Positive | 462 (33.2) | | | **Number of full-term pregnancies** | | | |
| Unknown | 457 (32.9) | | | ≤20 years | 233 (16.7) | 190 (13.7) | 0.10 |
| **Age at diagnosis** | | | | >20–30 years | 528 (38.0) | 531 (38.2) | |
| ≤50 years | 543 (39.0) | | | >30 years | 309 (22.2) | 349 (25.1) | |
| >50 years | 848 (61.0) | | | Missing | 142 (10.2) | 155 (11.1) | |
| **Age at blood collection** | | | | Nulliparous | 179 (12.9) | 166 (11.9) | |
| ≤45 | 461 (33.1) | 461 (33.1) | 0.99 | **Age at first menstrual period** | | | |
| 46–58 | 640 (46.0) | 637 (45.8) | | 7–10 years | 50 (3.6) | 48 (3.5) | 0.91 |
| >58 | 290 (20.9) | 293 (21.1) | | 11–13 years | 778 (55.9) | 774 (55.6) | |
| **Season-standardized Vitamin D quartiles** | | | | >13 years | 479 (34.4) | 492 (35.4) | |
| ≤39.3 nmol/L | 342 (24.6) | 346 (24.9) | 0.72 | >7 years or missing | 84 (6.0) | 77 (5.5) | |
| >39.3–50.9 nmol/L | 357 (25.7) | 350 (25.1) | | **Ever breastfed** | | | |
| >50.9–63.0 nmol/L | 324 (23.3) | 346 (24.9) | | No | 319 (22.9) | 310 (22.3) | 0.81 |
| >63.0 nmol/L | 368 (26.4) | 349 (25.1) | | Yes | 877 (63.1) | 875 (62.9) | |
| **Season of blood draw** | | | | Missing | 195 (14.0) | 206 (14.8) | |
| June to November | 640 (46.0) | 678 (48.7) | 0.15 | **Smoking status** | | | |
| December to May | 751 (54.0) | 713 (51.3) | | Never | 730 (52.5) | 731 (52.5) | 0.91 |
| **Menopausal status** | | | | Former | 338 (24.3) | 325 (23.3) | |
| Premenopausal | 538 (38.7) | 538 (38.7) | 1.0 | Smoker | 296 (21.3) | 309 (22.3) | |
| Perimenop./unknown | 221 (15.9) | 221 (15.9) | | Missing | 27 (1.9) | 26 (1.9) | |
| Postmenopausal | 632 (45.4) | 632 (45.4) | | **BMI categories** | | | |
| **Exogenous hormone use at blood donation** | | | | >25 kg/m$^2$ | 801 (57.6) | 815 (58.6) | 0.59 |
| No | 914 (65.7) | 914 (65.7) | 1.0 | ≥25 kg/m$^2$ | 590 (42.4) | 576 (41.4) | |
| Yes | 380 (27.3) | 380 (27.3) | | **Alcohol consumption at recruitment** | | | |
| Missing | 97 (7.0) | 97 (7.0) | | >1.5 g/day | 441 (31.7) | 465 (33.4) | 0.25 |
| **HRT use at blood donation**[2] | | | | 1.5–9.9 g/day | 492 (35.4) | 498 (35.8) | |
| No | 340 (53.8) | 346 (54.8) | 0.91 | 10–19.9 g/day | 223 (16.0) | 231 (16.6) | |
| Yes | 272 (43.0) | 268 (42.4) | | 20–29.9 g/day | 102 (7.3) | 96 (6.9) | |
| Missing | 20 (3.2) | 18 (2.8) | | ≥30 g/day | 133 (9.6) | 101 (7.3) | |
| **Highest school level** | | | | **Physical activity** | | | |
| Primary school | 342 (24.6) | 360 (25.9) | 0.72 | Inactive | 260 (18.7) | 245 (17.6) | 0.75 |
| Technical/prof. school | 341 (24.5) | 352 (25.3) | | Moderately inactive | 432 (31.1) | 457 (32.9) | |
| Secondary school | 352 (25.3) | 328 (23.6) | | Moderately active | 281 (20.2) | 289 (20.8) | |
| Longer education | 313 (22.5) | 315 (22.6) | | Active | 252 (18.1) | 234 (16.8) | |
| Missing | 43 (3.1) | 36 (2.6) | | Missing | 166 (11.9) | 166 (11.9) | |

[1]Chi-squared test.
[2]Postmenopausal women only.

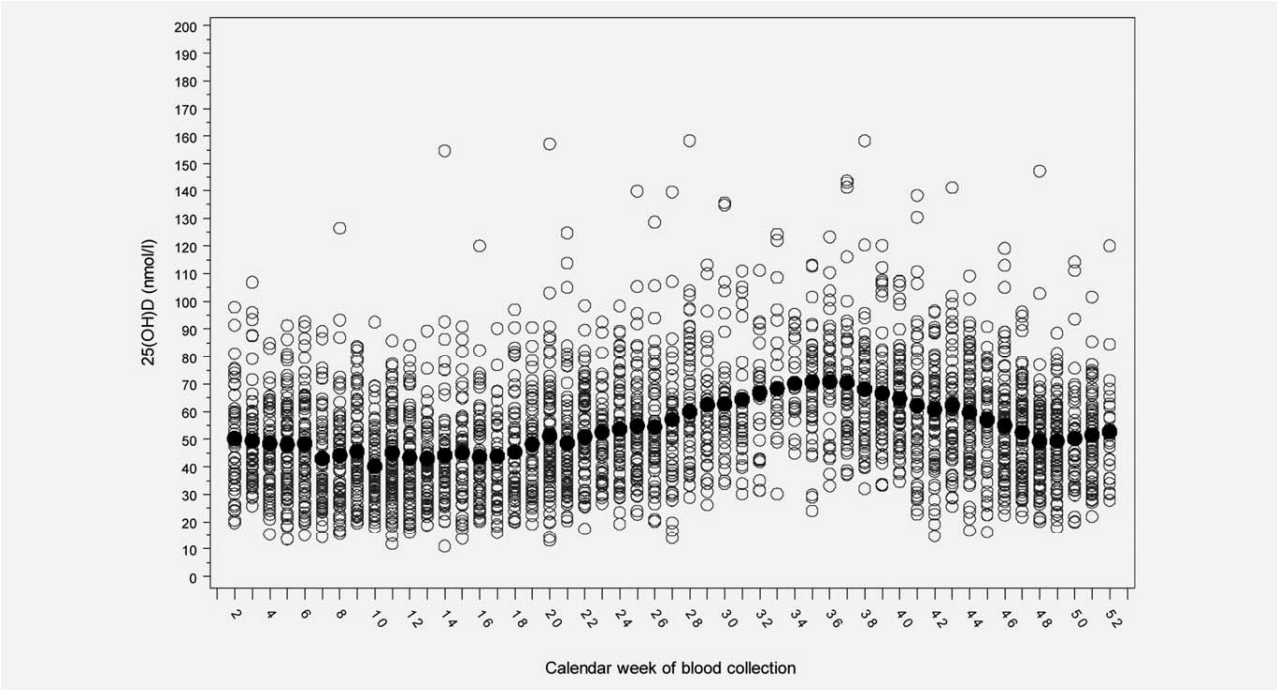


**Figure 1.** Seasonal variation of 25(OH)D values. The gray circles represent individual 25(OH)D values. The black circles represent the predicted mean 25(OH)D values for each calendar week after smoothing by locally weighted polynomial regression.

recruitment and diagnosis was 4.1 (±2.8) years, and the mean age at diagnosis was 54.8 (±9.1) years. Average plasma 25(OH)D concentrations were 53.1 (±21.5) nmol/L in cases and 52.8 (±20.0) nmol/L in controls ($p = 0.69$). Further descriptive characteristics of cases and controls are shown in Table 1. We observed no statistically significant differences between cases and controls concerning most of the presented variables, even though the proportion of women younger than 20 years at the time of first full-term pregnancy was higher among controls. Lowess regression models revealed varying plasma 25(OH)D levels by calendar week of blood collection, with higher values between weeks 20 and 46, *i.e.*, in the time from June to November (Fig. 1). After season standardization, mean 25(OH)D levels were 53.2 (±19.5) nmol/L in cases and 52.7 (±18.2) nmol/L in controls ($p = 0.49$).

ORs of breast cancer according to 25(OH)D plasma concentrations are presented in Table 2. The matched conditional logistic regression analysis did not show a significant overall association between 25(OH)D levels and the risk of breast cancer, neither using 25(OH)D quartiles ($OR_{Q4-Q1}$ [95% CI]: 1.07 [0.86–1.34], $p_{trend} = 0.65$) nor using log2-transformed 25(OH)D levels on a continuous scale ($OR_{log2}$ [95% CI]: 1.02 [0.87–1.19], $p = 0.81$). Adjustment for potential confounders did not change this association ($OR_{Q4-Q1}$ [95% CI]: 1.07 [0.85–1.36], $p_{trend} = 0.67$; $OR_{log2}$ [95% CI]: 1.01 [0.86–1.19], $p = 0.86$). Furthermore, 25(OH)D levels were not related to the risk of either ER− or ER+ tumors separately (Table 2).

No significant associations between 25(OH)D levels were observed in subgroup analyses within strata defined by age at diagnosis, season at blood donation or menopausal status at blood donation (Fig. 2). Subgroup analyses by PR status and combined ER/PR status did not show significant associations, either (data not shown). By contrast, we did observe differential associations between 25(OH)D values and breast cancer risk depending on the participants' BMI (Fig. 2). There was a nonsignificant inverse association between 25(OH)D levels and breast cancer risk in women with BMI <25 kg/m$^2$ ($OR_{log2}$ [95% CI]: 0.83 [0.67–1.03], $p = 0.09$), as opposed to a borderline significant positive association in overweight and obese women ($OR_{log2}$ [95% CI]: 1.30 [1.00–1.69], $p = 0.054$). Within strata of BMI, there were no significant interactions between 25(OH)D levels and menopausal status (data not shown).

Although 25(OH)D levels were significantly inversely associated with breast cancer risk in postmenopausal women who used HRT at blood donation ($OR_{log2}$ [95% CI]: 0.62 [0.42–0.90], $p = 0.01$) in the fully adjusted model, there was no significant association in HRT nonusers ($OR_{log2}$ [95% CI]: 1.14 [0.80–1.62], $p = 0.48$) (Fig. 2). We detected no significant associations when separately analyzing the data of postmenopausal HRT users and nonusers, or premenopausal and postmenopausal women by ER status (data not shown). Also, analyses stratified by overall exogenous hormone use at blood donation (oral contraceptives and HRT) in all women, by oral contraceptive use at blood donation in premenopausal women and by ever use of oral contraceptives in all women

Table 2. Odds ratios (95% CIs) of breast cancer according to season-standardized quartiles of plasma 25(OH)D

| | | | Crude model[1] | | Adjusted model[2] | |
|---|---|---|---|---|---|---|
| 25(OHD) | Cases (%) | Controls (%) | OR (95% CI) | $p_{trend}$ | OR (95% CI) | $p_{trend}$ |
| **Overall** | | | | | | |
| ≤39.3 nmol/L | 342 (24.6) | 346 (24.9) | 1.0 (reference) | | 1.0 (reference) | |
| >39.3–50.9 nmol/L | 357 (25.7) | 350 (25.1) | 1.03 (0.83–1.27) | | 1.03 (0.83–1.29) | |
| >50.9–63.0 nmol/L | 324 (23.3) | 346 (24.9) | 0.94 (0.75–1.18) | | 0.94 (0.74–1.19) | |
| >63.0 nmol/L | 368 (26.4) | 349 (25.1) | 1.07 (0.86–1.34) | 0.65 | 1.07 (0.85–1.36) | 0.67 |
| Log2 (continuous) | | | 1.02 (0.87–1.19) | 0.81 | 1.01 (0.86–1.19) | 0.86 |
| **ER positive** | | | | | | |
| ≤39.3 nmol/L | 153 (23.8) | 162 (25.2) | 1.0 (reference) | | 1.0 (reference) | |
| >39.3–50.9 nmol/L | 154 (24.0) | 154 (23.9) | 1.07 (0.77–1.48) | | 0.97 (0.68–1.37) | |
| >50.9–63.0 nmol/L | 169 (26.2) | 160 (24.9) | 1.14 (0.82–1.59) | | 1.04 (0.73–1.50) | |
| >63.0 nmol/L | 167 (26.0) | 167 (26.0) | 1.08 (0.77–1.50) | 0.66 | 0.97 (0.67–1.38) | 0.90 |
| Log2 (continuous) | | | 1.06 (0.84–1.34) | 0.61 | 0.99 (0.77–1.28) | 0.95 |
| **ER negative** | | | | | | |
| ≤39.3 nmol/L | 141 (25.8) | 119 (21.7) | 1.0 (reference) | | 1.0 (reference) | |
| >39.3–50.9 nmol/L | 138 (25.2) | 151 (27.6) | 0.76 (0.54–1.07) | | 0.74 (0.52–1.06) | |
| >50.9–63.0 nmol/L | 111 (20.3) | 141 (25.8) | 0.64 (0.44–0.93) | | 0.62 (0.42–0.91) | |
| >63.0 nmol/L | 157 (28.7) | 136 (24.9) | 0.97 (0.68–1.38) | 0.95 | 0.97 (0.66–1.42) | 0.98 |
| Log2 (continuous) | | | 0.92 (0.71–1.18) | 0.51 | 0.90 (0.68–1.18) | 0.43 |

Results from conditional logistic regression analyses including 1,391 cases and 1,391 matched controls. $p_{trend}$ calculated treating quartile medians as quantitative score.
[1]Matched for study center, age at blood donation, menopausal status, exogenous hormone use at blood donation, time of the day of blood collection, fasting status and phase of the menstrual cycle.
[2]Adjusted for BMI, age at first period, age at first full-term pregnancy, number of full-term pregnancies, breastfeeding, alcohol consumption, smoking status, education level and physical activity.
Abbreviations: OR: odds ratio; CI: confidence interval.

did not reveal significant associations between 25(OH)D levels and breast cancer risk (data not shown).

Finally, there was significant heterogeneity in the estimates obtained from country-specific fully adjusted analyses ($I^2$ = 51, $p_{heterogeneity}$ >0.01). Although no significant associations between 25(OH)D and breast cancer risk were observed in most countries, higher 25(OH)D levels were significantly related to a higher risk in Dutch participants ($OR_{log2}$ [95% CI]: 2.46 [1.14–5.32], $p$ = 0.02) and to a lower risk in French participants ($OR_{log2}$ [95% CI]: 0.47 [0.25–0.88], $p$ = 0.02) (Fig. 3). To investigate if the detected by-country heterogeneity was due to differences in the prevalences of overweight and obesity and HRT use, respectively, we recalculated country-specific, fully adjusted conditional logistic regression models subsequently adding interaction terms of continuous log2 25(OH)D values with BMI and HRT use categories. Heterogeneity between country-specific ORs was reduced adjusting for the interaction between 25(OH)D levels and BMI categories ($I^2$ = 37%, $p_{heterogeneity}$ = 0.13). By additionally including an interaction term of 25(OH)D levels and HRT use categories into the models heterogeneity across countries further decreased ($I^2$ = 11%, $p_{heterogeneity}$ = 0.34). Notably, there was also no significant between-country heterogeneity when using conditional logistic regression analyses based on matching factors without adjustments for additional covariates ($I^2$ = 24%, $p_{heterogeneity}$ = 0.23) (Fig. 3). The association between 25(OH)D and breast cancer risk obtained by the crude model was significantly positive in women from the Netherlands ($OR_{log2}$ [95% CI]: 1.79 [1.02–3.15], $p$ = 0.04), even though less strong than in the fully adjusted model. By contrast, the crude model revealed a nonsignificant association in participants from France ($OR_{log2}$ [95% CI]: 0.89 [0.57–1.39], $p$ = 0.60) (Fig. 3).

Results from regression models including predefined 25(OH)D cut points (<25, 25–49.99, 50–74.99 and ≥75 nmol/L), quartiles of raw 25(OH)D values or continuous 25(OH)D values with adjustment for season instead of season-standardized 25(OH)D levels did not substantially differ from the reported results (data not shown). Also, sensitivity analyses excluding cases that occurred within the first 2 years after baseline did not considerably change our primary results (data not shown). Lastly, a separate analysis of cases diagnosed within 2 years after blood donation ($n$ = 368) did not reveal a significant association between 25(OH)D levels and breast cancer risk (data not shown).

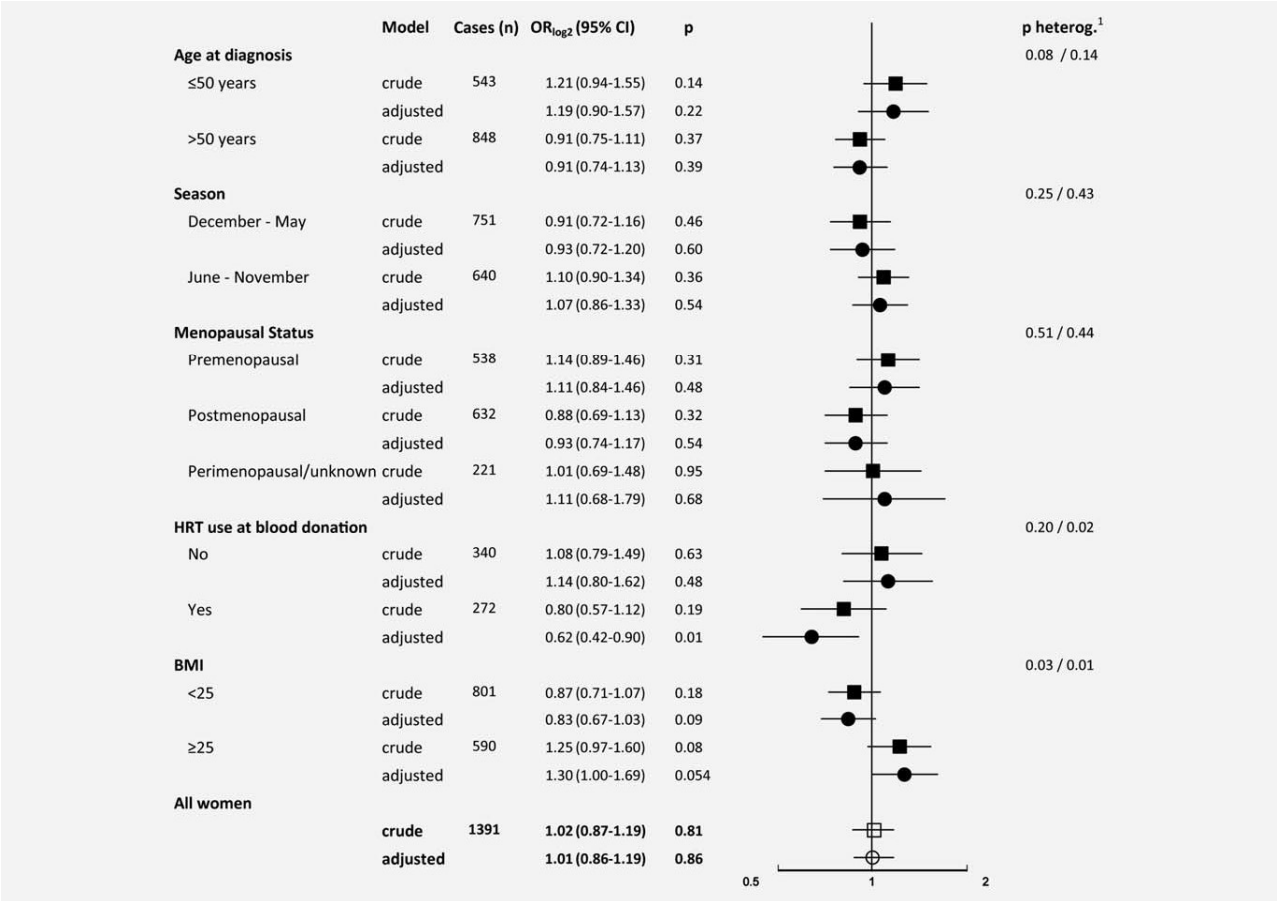


**Figure 2.** Odds ratios (95% CIs) of breast cancer according to season-standardized 25(OH)D levels on the log2 scale, stratified analyses. OR: odds ratio; CI: confidence interval; [1]*p* for heterogeneity for the crude/adjusted model; results for menopausal status and all women from conditional logistic regression analyses matched for study center, age at blood donation, menopausal status, exogenous hormone use at blood donation, time of the day of blood collection, fasting status and phase of menstrual cycle (crude model) and adjusted for BMI, age at first period, age at first full-term pregnancy, number of full-term pregnancies, breastfeeding, alcohol consumption, smoking status, education level and physical activity (adjusted model). Results on HRT use, season and BMI from unconditional logistic regression analyses adjusted for the matching factors (crude model) and additionally adjusted for BMI, age at first period, age at first full-term pregnancy, number of full-term pregnancies, breastfeeding, alcohol consumption, smoking status, education level and physical activity (adjusted model).

## Discussion

In our study, we did not observe significant associations between 25(OH)D plasma levels and the risk of breast cancer, neither overall nor in most stratified analyses. Nonetheless, we observed heterogeneity in the relation between 25(OH)D levels and breast cancer risk across strata of postmenopausal HRT use and BMI within the EPIC cohort, demonstrating that different prevalences of specific background factors may underlie varying associations between 25(OH)D and breast cancer risk in different populations. Consistent with this idea, it appeared that between-country heterogeneity was smaller and no longer statistically significant, when adjusting for interaction terms of 25(OH)D levels with BMI and HRT use. In turn, heterogeneity among countries only occurred in fully adjusted models, which further supports the notion that certain background factors affect the relationship between 25(OH)D and breast cancer risk.

To date, only one prospective study, the E3N study, has shown a significantly decreased risk of breast cancer in participants with higher 25(OH)D levels.[19] One further prospective study showed a borderline significant inverse association between 25(OH)D levels and breast cancer risk.[29] The results of these studies contributed to the result of the most recent meta-analysis[16] that revealed an inverse overall risk estimate from six prospective studies.[11,19,23,29–31] However, the clear null results of our study and two other recent studies[20,21] suggest that the prospective overall relationship of 25(OH)D levels and breast cancer risk is weak in magnitude, if present at all.

We observed significant heterogeneity in the association between 25(OH)D and breast cancer risk across strata of BMI. Contrary to a nonsignificant inverse relationship in women with BMI $< 25$ kg/m$^2$, 25(OH)D values were

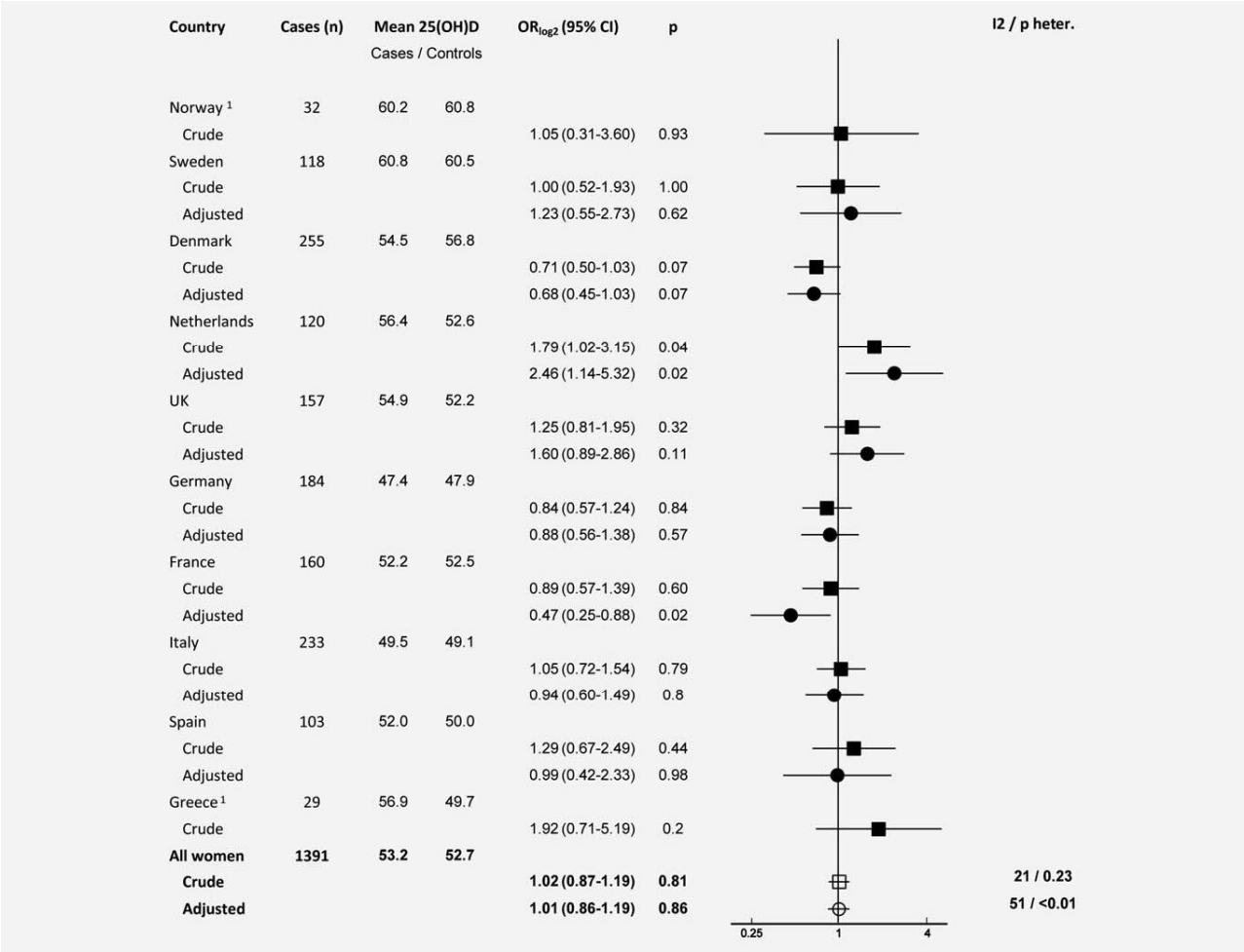


**Figure 3.** Country-specific odds ratios (95% CIs) of breast cancer according to season-standardized 25(OH)D levels on the log2 scale. OR: odds ratio; CI: confidence interval; *p* heter.: *p* for heterogeneity. Results from conditional logistic regression matched for study center, age at blood donation, menopausal status, exogenous hormone use at blood donation, time of the day of blood collection, fasting status and phase of menstrual cycle (crude model) with additional adjustment for BMI, age at first period, age at first full-term pregnancy, number of full-term pregnancies, breastfeeding, alcohol consumption, smoking status, education level and physical activity (adjusted model). [1]Results from unadjusted models only are reported for Greece and Norway, as multiple adjustment was restricted because of the low case numbers from these countries.

positively associated with breast cancer risk in overweight and obese women at borderline significance. A higher BMI has been found to be related to lower circulating 25(OH)D.[32] However, this does not explain an increased breast cancer risk among overweight and obese women with higher 25(OH)D levels, or an inverse association between 25(OH)D and breast cancer risk restricted to normal weight women, as observed in our analyses. One previous prospective study has also shown an increased breast cancer risk at higher 25(OH)D levels in overweight and obese participants, whereas no association was found in participants with BMI $< 25$ kg/m$^2$.[21] Although this result is obviously consistent with ours, underlying mechanisms remain to be identified. As no effect modification by BMI was observed in several other prospective studies[23,29–31,33] and case–control studies,[27,34] the differential association depending on BMI observed in our study may also be due to chance and requires further exploration.

Heterogeneity in the association between circulating 25(OH)D and breast cancer risk was also detected across strata of HRT use at blood donation. Plasma 25(OH)D levels were significantly inversely associated with breast cancer risk in HRT users, whereas no significant association in HRT nonusers was observed. The significant inverse association in HRT users might indicate that vitamin D is protective at high levels of sex hormones, possibly because of a vitamin D-induced inhibition of sex hormone-related growth signaling or estrogen receptor expression in breast cancer cells. However, further stratifying our analyses in current HRT users by hormone receptor status did not reveal heterogeneity in the association between 25(OH)D levels and breast cancer risk. Alternatively, HRT use has been reported to increase blood levels of the vitamin D-

binding protein.[35] As the vitamin D-binding protein has the functions of maintaining 25(OH)D levels and transporting 25(OH)D to target organs,[36] one may speculate that HRT users with high 25(OH)D levels benefit from an improved supply of the breast tissue with vitamin D. On the other hand, the bioavailability of 25(OH)D may rather depend on its free circulating fraction.[36,37] According to the "free hormone hypothesis," presumably higher levels of free 25(OH)D among HRT users with comparably high total 25(OH)D values may therefore be another explanation for our finding.

Admittedly, there is a lack of evidence for a differential association between 25(OH)D and breast cancer risk according to HRT use. Three previous studies that assessed interactions between circulating 25(OH)D and HRT use included larger numbers of postmenopausal women. Although one case–control study involving 1,394 postmenopausal women revealed a stronger inverse association between 25(OH)D levels and breast cancer risk in HRT nonusers, when compared to HRT users,[27] no significant effect modifications by HRT use were detected in two prospective studies in 1,005[30] and 1,080 postmenopausal women,[33] respectively. Two further, but smaller prospective studies also showed no significant 25(OH)D–HRT interactions.[29,31] Given the lack of evidence from previous studies, we cannot exclude that our subgroup finding was due to chance.

Interestingly, the E3N study, the only prospective study that has shown a significant inverse association between 25(OH)D levels and breast cancer risk to date, is characterized by the highest proportion of postmenopausal HRT users and the lowest proportion of overweight and obese women among the cohorts of the EPIC project. In our nested case–control study, the prevalence of HRT use at the time of blood collection among the participants from the E3N subpopulation was 69.2% compared to 36.5% among the rest of the postmenopausal population, and the prevalence of overweight and obesity was 24.1% compared to 44.2% among the rest of the participants. Accordingly, when excluding the E3N cohort members from our EPIC-wide analyses, the associations between 25(OH)D levels and breast cancer risk in postmenopausal HRT users ($OR_{log2}$ [95% CI]: 0.72 [0.45–1.14], $p = 0.16$) and in overweight and obese women ($OR_{log2}$ [95% CI]: 1.28 [0.98–1.68], $p = 0.08$) were attenuated.

Our results regarding hormone receptor positive and negative breast cancer subtypes are in agreement with those from most previous studies. One prospective study revealed that 25(OH)D levels might be inversely associated with the risk of ER−/PR− tumors in particular,[29] and two case–control studies suggested stronger inverse associations between circulating 25(OH)D and the risk of ER−[34,38] or PR− tumors,[34] when compared to ER+ and PR+ tumors, respectively. However, the numbers of ER− and PR− breast cancer cases were rather low in each of these studies. Also, basic research findings imply an impact of vitamin D on hormone receptor positive rather than hormone receptor negative tumors.[39] Our analyses including 643 ER+, 462 PR+ and 383 ER+/PR+ cases showed no association between 25(OH)D and breast cancer risk according to ER or PR status, independent of the participants' menopausal status. Thus, our findings as well as recent findings from other prospective[21,31] and case–control studies[27,40,41] do not indicate a role of vitamin D in breast cancer development related to hormone receptor status.

Unlike in our study, heterogeneity in the association between 25(OH)D levels and breast cancer risk by menopausal status was observed in one prospective[19] and one case–control study.[40] As several other prospective studies[21,23,29,31] and one case–control study[33] did not show differential associations, the evidence for a 25(OH)D–menopausal status interaction related to breast cancer is weak. Results of two prospective studies further pointed at possible modifications of the relationship between 25(OH)D levels and breast cancer risk by age,[19,29] whereas our study and other prospective studies did not show heterogeneity depending on age.[21,30,31] Finally, there was no indication for heterogeneity across seasons of blood draw with respect to 25(OH)D and breast cancer risk in our study and other studies.[21,23,30,31]

Contradictory findings from case–control and prospective studies on vitamin D and breast cancer risk have been attributed to possible lifestyle changes of cancer patients affecting 25(OH)D levels.[15,18] The average follow-up time of 4.1 years between blood donation and diagnosis in our study makes it unlikely that 25(OH)D levels were influenced by lifestyle modifications due to cancer. Moreover, it has been suggested that the time interval between blood donation and breast cancer diagnosis affects the association between 25(OH)D levels and breast cancer risk and that 25(OH)D levels might decrease not earlier than 3 years before breast cancer detection.[42] However, excluding breast cancer cases that occurred within the first 2 years after baseline did not substantially affect the results of our analyses. Also, a separate analysis of cases diagnosed within 2 years after baseline showed no relationship between 25(OH)D and breast cancer risk. Even though there is evidence from several case–control studies[15,16] and some prognostic studies among breast cancer patients,[43–45] that lower 25(OH)D levels could be a consequence of manifest cancer and be caused by an increased vitamin D turnover related to tumor growth, our results imply that vitamin D may not play a major role in breast cancer development.

Our study was the largest prospective case–control study on the association of 25(OH)D and breast cancer so far and we were able to conduct well-powered analyses stratified by menopausal status, hormone receptor status, BMI and other factors. Also, we could adjust for a range of potential confounders. Still, residual confounding cannot be ruled out. A limitation of our study, which was common in all comparable studies too, was that vitamin D status assessment was only available at a single time point. Although repeated measurements are desirable, several cohort studies have shown that 25(OH)D levels remain relatively stable over time.[46–48] Lastly, recent study results suggest that levels of the vitamin D-binding protein may affect associations between 25(OH)D and cancer risk.[49,50] Thus, another limitation of our study is that circulating levels of the vitamin D-binding protein were not assayed.

In summary, we did not find a prospective association between 25(OH)D levels and the risk of breast cancer. This result was independent of ER and PR status, menopausal status, age or time between blood donation and breast cancer diagnosis. Overall, our results are in line with the results from the majority of other prospective studies on this topic and do not point to a potential role of vitamin D in primary breast cancer prevention. Nevertheless, future meta-analyses will facilitate the identification of background factors that may affect associations between 25(OH)D levels and breast cancer risk such as HRT use and BMI.

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