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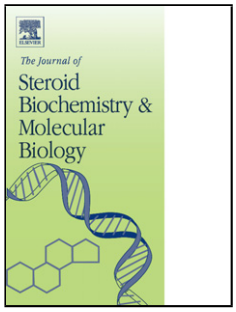

Title: Serum 25-Hydroxyvitamin D and breast cancer risk by pathological subtype (MCC-Spain)

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Serum 25-Hydroxyvitamin D and breast cancer risk by pathological subtype (MCC-Spain).

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Highlights

- High levels of serum Vitamin D seems to protect against breast cancer risk.
- The protective effect seems to be stronger for triple negative tumors
- No differences were observed by menopausal status, TNM staging or main risk factors
- Similar results were observed using cases sampled in the 1st month after diagnosis.

Abstract

Epidemiologic evidence on the association between vitamin D and breast cancer is still inconclusive. This study analyzes the association between serum 25-hydroxyvitamin D (25(OH)D) and breast cancer risk by pathologic subtype, stage at diagnosis and specific breast cancer risk factors. We conducted a population-based multicase-control study where 546 histologically-confirmed breast cancer cases and 558 population controls, frequently matched by geographic area, age and body mass index, were recruited in 12 Spanish provinces (MCC-Spain). Information was collected by a questionnaire and plasma 25(OH)D was measured by solid-phase extraction on-line coupled to liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS). Odds ratios and 95% confidence intervals were calculated using logistic and multinomial mixed regression models. We found a clear protective effect between 25(OH)D levels and breast cancer risk, with a significant dose-response trend (OR per 10 nmol/L= 0.88; 95%CI= 0.82-0.94). While no differences were observed between pre and postmenopausal women, stage at diagnosis, or across strata of the main breast cancer risk factors, the protection

was more pronounced for triple negative tumors (OR per 10 nmol/L= 0.64; p-heterogeneity=0.038). Similar results were observed when only cases sampled in the first month after diagnosis were considered. The protective effect of vitamin D on breast cancer risk may be subtype specific, being stronger for more aggressive tumors, which provides a new approach to prevent this disease.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; N, number of participants in each group; P25-P75, percentil 25 and percentil 75 of the mean 25(OH)D concentration.

Keywords: 25(OH)D; Vitamin D; Calcidiol; Breast neoplasm; Triple negative tumor; Stage at diagnosis

1. Introduction

Vitamin D is known as the "sunshine" vitamin, because sun exposure is by far the main source of this nutrient in humans. Vitamin D is the precursor to the steroid hormone calcitriol (1,25-dihydroxy-vitamin D), required to absorb and maintain calcium concentrations within the physiological range, and its deficiency causes rickets, osteomalacia and osteoporosis [1, 2]. Inside the body, vitamin D suffers a first hydroxylation in the liver and the serum concentration of the resulting 25-hydroxy-vitamin D (25(OH)D) is considered the main biomarker reflecting vitamin D status. A second hydroxylation in the kidney is required to transform 25(OH)D into calcitriol [1]. During the last decade, vitamin D has attracted a lot of attention due to its role in multiple signaling pathways involved in proliferation, apoptosis, differentiation, inflammation, invasion, angiogenesis and metastasis [3]. Calcitriol functions activating the vitamin D nuclear receptor (VDR) present in most cells in the body. In this way, calcitriol regulates as much as 3-5% of the human genome [1]. In fact, multiple laboratory studies support a role for vitamin D in retarding cancer development and progression [3].

There is no unanimous consensus on optimal levels of 25(OH)D. While the Institute of Medicine in the US established a cutoff of 50 nmol/L (20 ng/mL) [4], the US Endocrine Society considered that concentrations between 20-29 ng/mL indicate a relative insufficiency and set up a cutoff of 75 nmol/L (30 ng/mL) [5]. Using this definition, it has been estimated that Vitamin D insufficiency affects almost 50% of the population worldwide [6]. In Spain, despite favorable climatology, the levels are similar to, or even lower than, those described for Europe. These lower levels may be owing to more skin pigmentation, sunshine-avoiding

behavior, use of UV protection cream and air pollution with ozone and nitrogen dioxide, which reduce sun-induced vitamin D production [7, 8].

In 2008, a review by the International Agency for Research on Cancer concluded that observational studies regarding vitamin D and colorectal cancer give conclusive evidence of a protective effect, while studies linking vitamin D and breast cancer were more heterogeneous [9]. Since then, different meta-analyses have been published, generally reflecting an inverse relationship between 25(OH)D and breast cancer [10-13], more consistent in case-control than in cohort studies. Moreover, there is little information regarding the association of vitamin D status and breast cancer subtypes.

This paper examines the association between serum 25(OH)D and breast cancer risk, the dose-response shape of this relationship and possible differences in this association by menopausal status, pathologic subtype and stage at diagnosis in a subsample of untreated breast cancer cases and population-based controls from a large case-control study in Spain (MCC-Spain).

2. Materials and Methods

2.1. Study Population

MCC-Spain is a population-based multicase-control study conducted between 2008 and 2013 in 12 geographical areas in Spain, to identify environmental factors associated with malignant tumors with high incidence -breast cancer included- and/or presenting specific characteristics in our country (http://www.mccspain.org). The study design has been extensively described elsewhere [14]. Briefly, the study recruited more than 6000 patients 20-85 years old with histologically confirmed incident tumors (including 1738 breast cancer cases), and a single set of 4101 population controls (including 2038 women, 1910 suitable as breast cancer controls). Response rates were 69% for breast cancer cases and 54% among their controls [15]. Participants were interviewed by trained personnel using a computer-assisted program, gathering information on sociodemographic, life-style, reproductive history, hormonal factors, medications and personal and family medical history. Participants received a validated Food Frequency Questionnaire (FFQ) referred to the 12 months previous to diagnosis (cases) or recruitment (controls). This questionnaire was completed at home and mailed to recruiting centers (response rate of 89.9% for cases and 90.2 for controls). Blood samples were collected from 76% of participants. The study was approved by the Ethics Committees of the participating institutions and all participants signed an informed consent form.

For this study, we selected those breast cancer cases who had donated a blood sample before starting chemotherapy, radiotherapy or hormonotherapy (546 cases). Based on pathology records, the cases were classified into three groups, using a simplified version of the St Gallen international consensus [16]: 1) estrogen receptor positive (ER+) and/or progesterone receptor positive (PR+) without overexpression of the human epidermal growth factor receptor 2 (HER2), 2) HER2 positive tumors (HER2+), and 3) triple negative (ER-, PR- and HER2-) tumors. Regarding stage at diagnosis, we considered stage I, stage II and stages III & IV. The last two were included in a single category, due to the small number of women showing metastasis at diagnosis (16 women).

Cases and controls were frequency matched on geographic area, age (5-year groups) and body mass index (BMI) (5-unit groups) (n=558).

2.2. Biochemical Analyses

Details of the method for determination of 25(OH)D have been described elsewhere [17]. In brief, an on-line arrangement of automatic solid-phase extraction–liquid chromatography–tandem mass spectrometry (SPE–LC–MS/MS) was used, in which 200 µL of filtered serum spiked with the deuterated standards of the analytes was introduced for cleanup–chromatographic separation as required–tandem mass spectrometry detection. Quantitation was carried out using calibration models with the ratio between the area of the chromatographic peak from each analyte and that of the corresponding deuterated standard.

2.3. Statistical Methods

For descriptive purposes, characteristics of cases and controls were summarized as percentages and mean values, and compared using chi-square and *t*-tests. Differences in the concentration of 25(OH)D according to these characteristics were checked out in the control population (*t*-test and ANOVA tests). The association between 25(OH)D and breast cancer was studied using mixed logistic regression models, considering the geographical area as a random effect term (included in all multivariate analyses). Four logistic models with increasing degrees of adjustment were fitted: Model 1 only adjusted for the matching variables: age and BMI. Model 2 adds menopausal status and the day when the sample was extracted. This variable was included using natural splines, to take into account the non-linear variation of vitamin D levels throughout the year. Model 3a, further adjusted for educational level, ethnicity, age at first full term delivery (with a category of nulliparous), family history of breast cancer, personal history (previous breast biopsies, hypercholesterolemia and hormone replacement therapy (HRT) use), skin color, and physical activity in the last 5 years (MET-

h/week). Finally, a sensitivity analysis (model 3b) was fitted further adjusting for total energy intake, calcium and alcohol intake using cases and controls who completed the FFQ. All these models were fitted with 25(OH)D concentration (nmol/L) categorized into 5 levels, according to the quintiles in the control group, and as a continuous variable. The shape of the dose-response relationship was investigated using natural splines with 5 nodes at percentiles 5, 27.5, 50, 72.5 and 95.

Differences in the effect of 25(OH)D according to menopausal status and breast cancer subtype were explored considering both a 3-category variable, based on controls' tertiles, and the continuous variable. For menopausal status, differences in pre- and postmenopausal women were tested including in the final model the corresponding interaction term. For breast cancer subtypes, we fitted multinomial logistic regression models adjusting for the same factors. Heterogeneity of effects was tested comparing the coefficients (linear effect) obtained for the three subtypes (Wald test).

In order to know to what extent the duration and/or extension of the disease may have affected our results, multinomial models were also used to quantify the effect of 25(OH)D according to: 1) time from diagnosis to sample extraction in cases (<30 days, 30-60 days, >60 days or unknown) and 2) stage at diagnosis (stage I, stage II, stages III and IV).

Finally, possible effect modifications were contrasted by including interaction terms of 25(OH)D level (continuous) with each of the covariates in the final model. All analyses were performed using STATA/MP 14.2 software.

3. Results

We initially selected 558 breast cancer cases and 558 controls, but 12 cases where excluded because they had initiated chemotherapy before blood extraction. Thus, the final sample cohort included 546 cases and 558 controls. Table 1 describes both groups. Their mean age was 56 years, around 2/3 were postmenopausal and 97% were Caucasian. Cases had more relatives with breast cancer and higher prevalence of previous biopsies, while tended to be less educated and have darker skin color than controls. Cases had a lower concentration of 25(OH)D, even though the percentage of samples collected in summer and fall (seasons where Vit D concentrations are higher) was greater in this group (Table 1).

Table 2 shows the distribution of 25(OH)D levels according to socioeconomic, reproductive and life style characteristics in the control group. Apart from seasonal and geographical variations, the concentration of 25(OH)D decreased with age and BMI. It was lower in non-Caucasians and higher in women with hypercholesterolemia and in those who had used HRT.

The association of 25(OH)D levels with breast cancer risk is shown in Table 3. Model 1 included, apart from the geographical area, the other matching variables: age and BMI. Model 2 added menopausal status and day of sample extraction. Model 3a added to Model 2 the following confounders: educational level, ethnicity, age at first full term delivery, family history of breast cancer, previous breast biopsies, physical activity, hypercholesterolemia, HRT use and skin color. Finally, Model 3b further adjusted for the following dietary factors: total energy intake, calcium intake and alcohol intake to test their possible role as confounders. In all models, a clear protective effect is seen, with ORs decreasing with increasing concentrations of 25(OH)D, with a dose-response trend highly significant (OR per 10 nmol/L= 0.88; 95%CI= 0.82-0.94). The protective effect, though, seems to level-off at the fourth quintile. According to the final model (model 3a), women in the two highest quintiles had a reduction of risk over 50% (OR_{Q4vsQ1}= 0.40; 95%CI= 0.26-0.61; OR_{Q5vsQ1}= 0.46; 95%CI= 0.30-0.70).

This apparent level-off is explained by the shape of the dose-response curve (Figure 1), that showed a clear and statistically significant departure from linearity (*p* value<0.001). Breast cancer risk clearly declined for concentrations between 30 and 70 nmol/L, and the risk seems to increase afterwards, though it should be noted that only 13% of controls and 10% of cases had concentrations over 70 nmol/L, and around 3% had levels greater than 90 nmol/L. For this reason, in subsequent subgroup analyses, we used tertiles of 25(OH)D and dose-response tests assumed a linear trend.

Table 4 presents the results in pre and postmenopausal women (top) and according to breast cancer subtypes (medium rows) and stage at diagnosis (bottom). No clear differences were observed between pre and postmenopausal women (*p*-interaction=0.597). Regarding breast cancer subtypes, ER+/PR+ tumors and

HER2+ tumors presented similar effects (OR per 10 nmol/L= 0.89 and 0.88 respectively), but 25(OH)D seemed to be particularly protective against triple negative tumors (OR per 10 nmol/L= 0.64; p-heterogeneity=0.038), though this result is based on a reduced number of cases (36 breast cancer cases and 558 controls. Regarding stage at diagnosis, only 5 cases had metastasis at diagnosis, so stages III and IV were combined in a single category. No differences in the effect of 25(OH)D were observed according to breast cancer stage (*p*-heterogeneity=0.706).

Figure 2 plots the linear effect of 25(OH)D serum concentration on breast cancer risk (per 10 nmol/L) in subgroup analyses by categories of the following variables: age, education, menarche, age at first child, family history of breast cancer, previous biopsies, BMI, tobacco, physical activity, skin color, hypercholesterolemia, HRT use and season. No differences were seen across strata, and all interaction *p*-values were greater than 0.30.

Finally, we tested whether the mean 25(OH)D levels in cases differed according to the time elapsed between diagnosis and blood sampling. No differences were observed in 25(OH)D levels in cases sampled in the first 15 days compared to those sampled in week 3rd and 4th after diagnosis (45.89 nmol/L and 45.96 nmol/L, *p*-value=0.977). However, 25(OHD) level seemed to decrease after the first month of diagnosis (25(OH)D mean=41.75 nmol/L in women sampled in the second month; *p*-value=0.067; and 25(OH)D mean=40.95 nmol/L in women sampled afterwards; *p*-value=0.109). Owing to these differences, a sensitivity analysis was conducted considering only breast cancer cases sampled in the first month (298 cases). The overall effect per 10 nmol/L was slightly attenuated, though no different from that obtained using the whole sample (OR=0.92; 95%CI=0.85-0.99; p-value=0.034). The corresponding OR for the second and third tertiles of 25(OH)D were 0.63 (95%CI=0.44-0.92) and 0.49 (95%CI=0.32-0.73), respectively. The heterogeneity of effects according to breast cancer subtype was confirmed (*p*-heterogeneity=0.012). The OR per 10 nmol/L of 25(OH)D was 0.92 for ER+|PR+&HER2- (95%CI=0.84-1.01), 1.00 for HER2+ tumors (95%CI= 0.85-1.17) and 0.53 for triple negative tumors (95%CI=0.36-0.78). These results are based on 205 ER+|PR cases, 43 HER2+ cases and 21 triple negative tumors (see Supplemental Material, Table S1).

4. Discussion

To our knowledge, this is the first study providing information on the association of 25(OH)D levels with breast cancer risk by pathologic subtype in Spain. Our results show a consistent protective effect with increase 25(OH)D serum levels on breast cancer risk This effect is similar in pre and postmenopausal women, but, interestingly enough, it seems to be stronger for triple negative tumors. Even though the non-linear shape of the dose-response might suggest an increased risk in women at the upper extreme of the 25(OH)D range, very few women had concentrations over 90 nmol/L.

Mean serum 25(OH) concentration in our study was similar to that reported in other European countries [18], similar to that detected in small studies carried out in different Spanish regions [8] and slightly lower than the concentration detected in larger Spanish studies, where the average levels fluctuated between 56 and 62 nmol/L [19-21]. The prevalence of vitamin D deficiency (<50nmol/L) in our study (55% in controls and 69% in BC cases) was higher than that reported among pre and post Mexican women (36%) [22], higher than the reported at European level (40%) [18], and also higher than that reported in other Spanish studies [19-21, 23], but much lower than that detected by Almirall et al. in 2010 [24] (80%) and Aguado et al. in 2000 [25] (87%) among postmenopausal Spanish women.

Some meta-analyses have investigated the association between serum 25(OH)D levels and breast cancer risk reporting controversial results. Among those focused solely on prospective studies, two detected an inverse association only in postmenopausal women [26, 27], while the most recent reported a weak and nonsignificant inverse association [12]. Other meta-analyses that separated analysis for case-control and prospective studies found that the inverse association was restricted to case-control studies [11, 13]. However, Chen et al, in a meta-analysis of 11 nested case-control and retrospective studies and 10 case-control studies, suggested that higher blood vitamin D levels were associated with a significantly reduced risk of breast cancer [10].

The non-linear dose-response association detected in our study has been previously described by Bauer et al. [26]. In this study, 25(OH)D levels at or above 27 ng/mL (67.5 nmol/L) threshold were associated with a 12% lower risk of postmenopausal breast cancer per 5ng/mL increase in 25(OH)D. However, no further reductions in risk were observed above 35 ng/mL (87.5 nmol/L). In our study, these cut-off points were left-shifted (30

and 70 mmol/L, respectively), although it should be noted that serum 25(OH)D levels in our women were lower than those reported in this meta-analysis.

In consonance with previous studies [28-31], women with triple negative breast cancer presented the lowest mean 25(OH)D serum concentration and therefore, high levels of vitamin D seemed to be particularly protective against this pathological subtype. Two previous epidemiological studies also found an inverse association between serum 25(OH)D concentrations and triple negative breast cancer risk [32, 33]. Approximately two-thirds of these tumors express VDR [34], and it has been demonstrated that ligand bound VDR inhibits the proliferation of triple negative breast cancer cell lines, inhibits the triple negative breast cancer stem-like cells, induces differentiation and attenuates metastatic potential [34-36]. Moreover, interesting studies revealed that calcitriol can stabilize DNA repair protein 53BP1 levels in tumor cells, contributing to reduce proliferation of breast cancer with the poorest prognosis [37, 38], and can also induce de novo E-cadherin expression by promoter demethylation in triple-negative breast cancer cells [39].

On the other hand, since calcitriol can suppress the expression of aromatase, reducing estrogen synthesis via direct and indirect pathways [36], most studies have found an inverse association mainly in postmenopausal women [26, 27]. Although we did not detect statistically significant differences between pre and postmenopausal women in our study, a stronger protective effect was observed in postmenopausal women.

The major limitation of the present analysis is the possibility of reverse causation, a particular concern in case-control studies. Since vitamin D levels were assessed after diagnosis, it may be possible that the progression of the disease or changes in patients' lifestyle would have adversely affected 25(OH)D concentrations. In an attempt to minimize this bias a sensitivity analysis was also performed considering only breast cancer cases sampled in the first month after diagnosis (time in which the serum 25(OH)D concentrations were not altered in our participants), and results of this sub analysis were very similar to those obtained using the whole sample. This one-month time window is in agreement with the serum half-life of 25(OH)D, estimated approximately in 3 weeks [40]. Although prospective studies with serum 25(OH)D samples collected prior to diagnosis are preferred, optimal timing of vitamin D assessment is uncertain. There is previous evidence that follow-up periods after serum sampling should not be too long for breast cancer since it develops rapidly, concluding

that case–control studies of breast cancer incidence provide reliable results and should be used rather than nested case-control studies with samples taken many years before diagnosis [41, 42]. Another relevant issue is that the pre-existing local inflammatory lesions involved in the onset of the disease could have contributed to reducing serum 25(OH)D levels, mainly in the case of triple negative breast tumors [43]. However, there is previous evidence that vitamin D inhibits inflammation, suggesting the reverse, that is, reduced vitamin D levels might increase inflammation [3]. Another relevant issue in case-control studies is the potential of selection bias. This study attempted to recruit all cases with a first diagnosis of breast cancer in the selected health areas, ensuring that very few incident cases were missed, and general practitioner lists were used to select controls. On the other hand, despite having adjusted for the most established risk factors, residual confounding cannot be ruled out. However, those characteristics with a geographical distribution have been at least partly accounted for through the random effect province term included in our statistical analyses. Finally, we were limited by the small sample size when evaluating the association by stage at diagnosis and by pathologic breast cancer subtype, mainly in the case of triple negative tumors whose frequency is very low in our context [44]. Despite these limitations, to date, this is the largest epidemiological study conducted in Spain that analyzes the association between serum 25(OH)D levels and breast cancer risk by pathological subtype or stage at diagnosis. On the other hand, histologically confirmed cases and population controls were recruited in 12 Spanish regions located throughout the Spanish geography, which allowed us to have a broad representation of the lifestyle and dietary habits that coexist in Spain. An important strength of our study is the use of BMI as a matching factor, since BMI affects both vitamin D serum levels and breast cancer risk in postmenopausal women. Finally, the LC-MS/MS method can be considered the gold standard for 25(OH)D determination, demonstrating better performance than other automated methods [45].

5. Conclusions

Our results confirm an inverse association between 25(OH)D serum levels and breast cancer risk, which was more pronounced in triple negative tumors. Public health and clinical strategies aimed at improving vitamin D levels would be desirable, taking into account the high proportion of women with inadequate concentrations of 25(OH)D.

CONFLICT OF INTEREST

The authors declare they have no actual or potential competing financial interests

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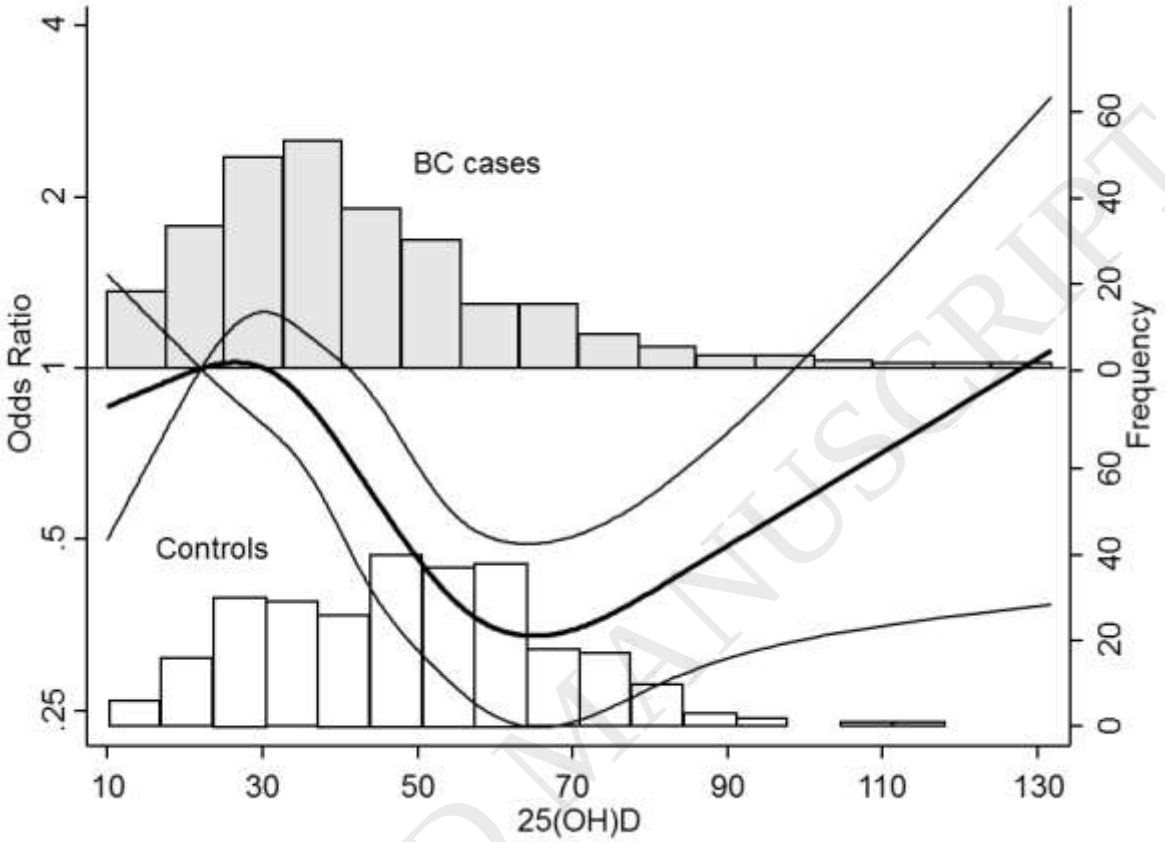
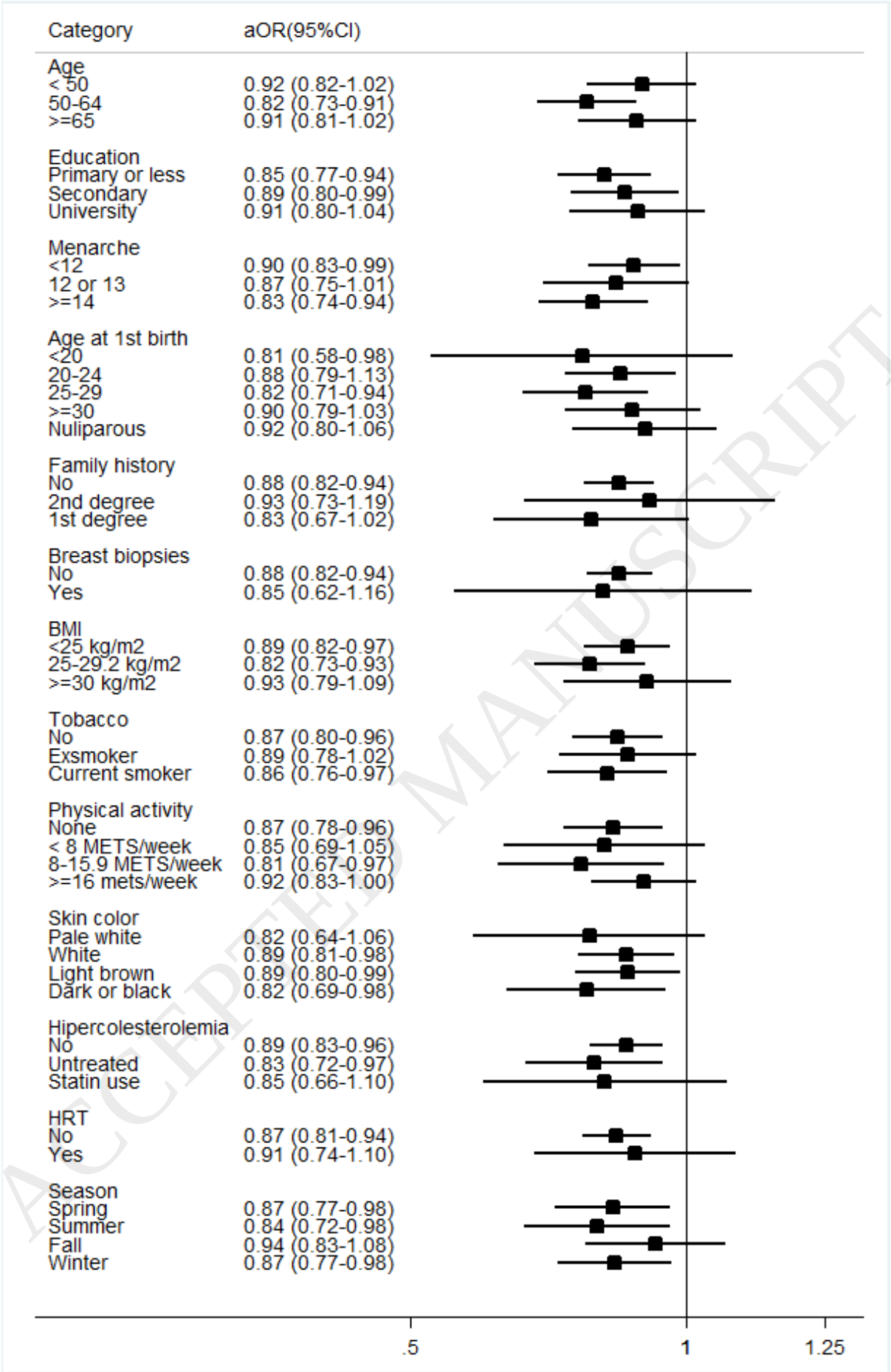
Figure 1. Dose-response relationship between 25-hydroxyvitamin D levels and breast cancer risk.



Abbreviations: BC. breast cancer; 25(OH)D, 25-hydroxyvitamin D.
Adjusted for age, body mass index, menopausal status, day of sample extraction, educational level, ethnicity, age at first full term delivery, family history of breast cancer, previous breast biopsies, hypercholesterolemia, hormone replacement therapy use, skin color, and physical activity in the last 5 years. Geographical area introduced as a random effect term.

Figure 2. Breast cancer risk for every 10 nmol/L increase in serum 25-hydroxyvitamin D concentration according to women characteristics.



Abbreviations: OR(95%CI), odds ratio an 95% confidence interval; BMI, body mass index; HRT, hormone replacement therapy.

Adjusted for age, body mass index, menopausal status, day of sample extraction, educational level, ethnicity, age at first full term delivery, family history of breast cancer, previous breast biopsies, hypercholesterolemia, hormone replacement therapy use, skin color and physical activity in the last 5 years. Geographical area introduced as a random effect term.

Table 1. Descriptive characteristics of breast cancer cases and controls.

	Controls (n=558)	Cases (n=546)	*p*-value[a]
Geographical region [n (%)]			0.996
Madrid	115 (20.6)	113 (20.7)	
Barcelona	50 (9.0)	55 (10.1)	
Navarra	54 (9.7)	46 (8.4)	
Gipuzkoa	132 (23.7)	122 (22.3)	
Leon	89 (16.0)	88 (16.1)	
Asturias	40 (7.2)	44 (8.1)	
Huelva	4 (0.7)	3 (0.6)	
Cantabria	42 (7.5)	44 (8.1)	
Valencia	9 (1.6)	8 (1.5)	
Girona	23 (4.1)	23 (4.2)	
Age (y) (mean±SD)	56 ±12.0	56 ±11.7	0.541
Ethnicity [n (%)]			0.745
Caucasian	543 (97.3)	533 (97.6)	
Other	15 (2.7)	13 (2.4)	
Educational level [n (%)]			0.076
Less than primary school	66 (11.8)	76 (13.9)	
Primary school completed	173 (31.0)	195 (35.7)	
Secondary school	192 (34.4)	180 (33.0)	
University graduate	127 (22.8)	95 (17.4)	
Body mass index (Kg/m^2) (mean±SD)			
Premenopausal women	23.7±3.4	23.6±3.5	0.829
Postmenopausal women	26.3±4.1	26.6±4.5	0.480
Age at menarche (y) [n (%)]			0.740
<12	115 (20.6)	109 (20.0)	
12-13	260 (46.6)	267 (48.9)	
>=14	183 (32.8)	170 (31.1)	
Menopausal status [n (%)]			0.347
Postmenopausal	200 (35.8)	181 (33.2)	
Premenopausal	358 (64.2)	365 (66.8)	
Age at first birth (y) [n (%)]			0.889
<20	21 /4.8)	24 (5.5)	
20-24	126 (28.5)	130 (20.8)	
25-29	179 (40.5)	168 (38.5)	
>=30	114 (25.8)	109 (25.0)	
Unknown	2 (0.5)	5 (1.2)	
Number of children [n (%)]			0.608
None	116 (20.8)	110 (20.1)	
1-2	318 (57.0)	325 (59.5)	
3-4	100 (17.9)	95 (17.4)	
>4	24 (4.3)	16 (2.9)	
Family history of breast cancer [n (%)]			<0.001
No	483 (86.6)	412 (75.5)	
Second degree only	32 (5.7)	53 (9.7)	
1 First degree	40 (7.2)	73 (13.4)	
> 1 First degree	3 (0.5)	8 (1.5)	
Previous biopsies [n (%)]			<0.001
No	547 (98.0)	511 (93.6)	

Yes	11 (2.0)	35 (6.4)	
Tobacco consumption [n (%)]			0.813
No	298 (53.4)	296 (54.2)	
Exsmoker	116 (20.8)	118 (21.6)	
Current smoker	144 (25.8)	132 (24.2)	
Physical activity (MET-h/week) [n (%)]			0.201
No	214 (38.4)	237 (43.4)	
< 8	76 (13.6)	56 (10.3)	
8-15.9	79 (14.2)	71 (13.0)	
>=16	189 (33.9)	182 (33.3)	
Skin color [n (%)]			0.094
Pale white	42 (7.5)	31 (5.7)	
White	240 /43.0)	239 (43.8)	
Light brown	189 (33.9)	208 (38.1)	
Dark brown	83 /14,9)	68 (12.5)	
Black	4 (0.2)	0 (0.0)	
Eye color [n (%)]			0.707
Dark brown	312 (55.9)	296 (54.2)	
Light brown or green	169 (30.3)	178 (32.6)	
Blue or gray	76 (13.6)	71 (13.0)	
Unknown	1 (0.2)	1 (0.2)	
Hipercolesterolemia [n (%)]			0.344
No	399 (71.5)	411 (75.3)	
Yes, not treated	116 (20.8)	101 (18.5)	
Treated with statins	43 (7.7)	34 (6.2)	
Use of corticoids [n (%)]			0.171
No	399 (71.5)	483 (88.5)	
Yes	58 (10.4)	45 (8.2)	
Unknown	32 (5.7)	18 (3.3)	
Hormone replacement therapy use [n (%)]			0.892
Never	497 (89.1)	490 (89.7)	
<= 5 years	43 (7.7)	28 (5.1)	
> 5 years	11 (2.0)	10 (1.8)	
Unknown	18 (3.2)	18 (3.3)	
Alcohol consumption (g/day) [n (%)]			0.018
No	195 (34.9)	179 (32.8)	
< 15	256 (45.9)	235 (43.0)	
15-29.9	38 (6.8)	40 (7.3)	
>=30	15 (2.7)	36 (6.6)	
Unknown [b]	54 (9.7)	56 (10.3)	
Total energy intake (kcals) (mean±SD) [b]	1766.9±552.0	1826.6±516.9	0.068
Total calcium intake (g/day) (mean±SD) [b]	922.7±303.3	930.5±328.5	0.698
Total vitamin D intake (µg/day) (mean±SD) [b]	2.5±1.2	2.6±1.4	0.045
Vitamin D (nmol/L) (mean±SD)			
25-hydroxyvitamin D	48.2±19.9	43.6±22.3	<0.001
Season [n (%)]			0.010
Spring	217 (38.9)	174 (31.9)	
Summer	74 (13.3)	106 (19.4)	
Fall	109 (19.5)	120 (22.0)	
Winter	158 (28.3)	146 (26.7)	

[a] *p*-values were computed with the chi-square test and *t*-test.

[b] Participants who did not answer the food frequency questionnaire (54 controls and 56 cases) do not have information.

Table 2 shows the distribution of 25(OH)D levels according to socioeconomic, reproductive and life style characteristics in the control group. Apart from seasonal and geographical variations, the

concentration of 25(OH)D decreased with age and BMI. It was lower in non-Caucasians and higher in women with hypercholesterolemia and in those who had used HRT.

Table 2. Variables influencing 25-hydroxyvitamin D levels. Mean levels according to baseline characteristics in the control group.

		25(OH)D[a]		
Variable	N	Mean	P25-P75	*p*-value[b]
Geographical region				0.047
Madrid	115	52.48	40.90- 64.50	
Barcelona	50	46.02	28.80- 57.20	
Navarra	54	45.10	28.20- 61.00	
Gipuzkoa	132	49.51	36.80- 59.75	
Leon	89	43.27	31.30- 52.90	
Asturias	40	49.24	34.25- 61.85	
Huelva	4	57.05	41.80- 72.30	
Cantabria	42	46.41	32.50- 57.60	
Valencia	9	54.88	44.70- 69.10	
Girona	23	43.31	27.10- 54.10	
Age (y)				0.025
<40	35	53.86	31.10- 65.40	
40-49	140	44.57	29.80- 55.35	
50-59	164	49.43	36.70- 59.75	
60-69	138	50.02	35.00- 63.70	
>=70	81	45.20	29.60- 58.90	
Ethnicity				<0.001
Caucasian	543	48.60	34.40- 61.40	
Other	15	26.89	20.70- 30.40	
Educational level				0.882
Less than primary school	66	46.45	36.00- 58.60	
Primary school completed	173	48.00	33.30- 60.00	
Secondary school	192	47.99	31.30- 61.90	
University graduate	127	48.90	34.50- 62.00	
Body mass index (Kg/m^2)				0.024
<25	295	49.39	34.00- 62.70	
25-29.9	187	48.13	33.30- 59.70	
>=30	76	42.44	27.15- 53.65	
Age at menarche (y)				0.212
<12	115	45.35	29.30- 57.30	
12-13	260	48.17	32.85- 61.00	
>=14	183	49.49	34.40- 62.00	
Menopausal status				0.093
Postmenopausal	358	49.08	36.50- 61.90	
Premenopausal	200	46.13	29.80- 57.45	
Age at first birth (y)				0.708
<20	21	44.07	25.80- 56.90	
20-24	126	48.74	34.80- 60.60	
25-29	179	47.70	31.90- 60.00	
>=30	114	49.05	35.20- 61.90	
Number of children				0.324
None	116	47.52	31.25- 62.15	
1-2	318	48.56	34.00- 61.50	
3-4	100	48.58	36.35- 60.70	
>4	24	40.90	27.30- 54.40	
Family history of breast cancer				0.742
No	483	48.26	32.80- 61.20	

Second degree only	32	44.40	33.85- 56.30	
1 First degree	40	48.28	30.50- 61.50	
> 1 First degree	3	44.33	17.60- 63.70	
Previous biopsies				0.137
No	547	47.84	32.50- 60.80	
Yes	11	56.84	50.60- 73.80	
Tobacco consumption				0.155
No	298	47.79	32.50- 60.60	
Exsmoker	116	50.91	35.25- 63.55	
Current smoker	144	46.18	32.00- 57.00	
Physical activity (MET-h/week)				0.492
No	214	46.36	31.30- 58.60	
< 8	76	49.08	34.60- 61.40	
8-15.9	79	48.98	37.70- 61.10	
>=16	189	49.06	31.90- 62.50	
Skin color				0.087
Pale white	42	52.18	41.20- 61.50	
White	240	46.41	31.65- 58.50	
Light brown	189	48.61	34.00- 62.70	
Dark brown	83	50.13	31.50- 62.50	
Black	4	29.30	15.40- 43.20	
Eye color				0.493
Dark brown	312	47.13	31.60- 59.75	
Light brown or green	169	49.33	35.00- 61.90	
Blue or gray	76	48.58	33.85- 61.20	
Hipercolesterolemia				0.058
No	399	46.79	31.60- 58.70	
Yes, not treated	116	51.66	37.55- 63.70	
Treated with statins	43	49.55	36.80- 60.90	
Use of corticoids				0.582
No	468	48.04	32.50- 61.05	
Yes	58	49.57	34.50- 63.10	
Hormone replacement therapy use				0.042
Never	497	47.19	31.70- 59.80	
<= 5 years	32	52.27	39.15- 62.25	
> 5 years	11	60.11	46.80- 70.30	
Alcohol consumption (g/day)				0.407
No	195	47.63	33.20- 58.10	
< 15	256	48.61	33.45- 61.20	
15-29.9	38	50.84	38.90- 62.70	
>=30	15	41.10	18.70- 54.50	
Energy intake (kcals)				0.263
<1385	126	48.11	30.40- 60.90	
1385-1700	126	46.33	34.60- 56.60	
1701-2070	126	51.00	37.60- 62.60	
>2070	126	47.25	31.70- 61.00	
Calcium intake (g/day)				0.080
<720	126	44.72	30.60- 57.10	
720-895	126	47.55	31.80- 60.90	
896-1126	126	49.91	36.50- 60.20	
>1126	126	50.51	33.30- 64.70	
Vitamin D intake (µg/day)				0.650
< 1.531	126	48.91	31.80- 62.70	
1.531-2.420	126	47.10	31.60- 59.70	
2.421-3.135	126	47.06	34.00- 58.90	
> 3.135	126	49.63	35.80- 61.30	
Season				<0.001
Spring	217	44.48	30.00- 56.50	
Summer	74	54.76	40.60- 67.30	

Fall	109	51.42	37.40- 64.20
Winter	158	47.38	31.90- 61.50

[a] 25(OH)D levels in nmol/L
[b] *p*-values were computed with *t*-test.

Table 3. Association between 25-hydroxyvitamin D levels and breast cancer risk.

	Controls (n=558)	Cases (n=546)	MODEL 1 [a] OR(95%CI)	*p*-value	MODEL 2 [b] OR(95%CI)	*p*-value	MODEL 3a [c] OR(95%CI)	*p*-value	MODEL 3b [d] OR(95%CI)	*p*-value
25(OH)D (nmol/L)										
<=29.98	111	153	1.00		1.00		1.00		1.00	
]29.98-41.74]	112	149	0.97(0.68, 1.37)	0.768	0.90(0.63, 1.28)	0.562	0.96(0.66, 1.39)	0.817	0.96(0.64, 1.42)	0.823
]41.74-52.70]	113	97	0.63(0.43, 0.90)	0.009	0.56(0.38, 0.81)	0.003	0.55(0.37, 0.82)	0.004	0.61(0.40, 0.93)	0.022
]52.70-63.70]	112	68	0.44(0.30, 0.65)	<0.001	0.39(0.26, 0.58)	<0.001	0.40(0.26, 0.61)	<0.001	0.41(0.26, 0.64)	<0.001
>63.70	110	79	0.52(0.36, 0.77)	0.001	0.43(0.29, 0.64)	<0.001	0.46(0.30, 0.70)	<0.001	0.50(0.32, 0.79)	0.003
Trend per 10 nmol/L			*0.90(0.85, 0.96)*	*0.001*	*0.88(0.83, 0.94)*	*<0.001*	*0.88(0.82, 0.94)*	*<0.001*	*0.89(0.83, 0.95)*	*0.001*

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; OR(95%CI), odds ratio an 95% confidence interval.
[a] Adjusted for age and body mass index. Geographical area introduced as a random effect term.
[b] Additionally adjusted for menopausal status and day of sample extraction.
[c] Additionally adjusted for educational level, ethnicity, age at first full term delivery, family history of breast cancer, previous breast biopsies, hypercholesterolemia, hormone replacement therapy use, skin color, and physical activity in the last 5 years.
[d] Additionally adjusted for total energy intake, calcium intake and alcohol intake (54 controls and 56 cases without information on diet are excluded).

Table 4. Association between 25-hydroxyvitamin D levels and breast cancer risk by menopausal status, tumor subtype and stage at diagnosis.

MENOPAUSAL STATUS		**OVERALL**			**PREMENOPAUSAL**				**POSTMENOPAUSAL**				
25(OH)D nmol/L	Controls	Cases	OR (95% CI)[a]	*p*-value	Controls	Cases	OR (95% CI)[a]	*p*-value	Controls	Cases	OR (95% CI)[a]	*p*-value	*p*-int
Mean (95% CI)	48.3 (45.9, 50.7)	43.2 (40.8, 45.6)			47.2 (43.6, 50.8)	42.2 (38.5, 46.0)			48.9 (46.2-51.5)	43.5 (40.9-46.1)			
<=37.65	184	260	1.00		80	83	1.00		104	177	1.00		
]37.65-55.2]	187	158	0.56 (0.41, 0.76)	<0.001	61	53	0.79 (0.46, 1.34)	0.374	126	105	0.44 (0.30, 0.65)	<0.001	
>55.2	187	128	0.44 (0.31, 0.61)	<0.001	59	45	0.54 (0.30, 0.98)	0.043	128	83	0.36 (0.24, 0.54)	<0.001	
trend per 10 nmol/L			*0.88 (0.82, 0.94)*	*<0.001*			*0.85 (0.75, 0.97)*	*0.012*			*0.88 (0.80, 0.95)*	*0.001*	*0.597*

TUMOR SUBTYPE[b]		**ER+/PR+ & HER2-**			**HER2+**			**TRIPLE NEGATIVE**			
25(OH)D nmol/L	Controls	Cases	OR (95% CI)[a]	*p*-value	Cases	OR (95% CI)[a]	*p*-value	Cases	OR (95% CI)[a]	*p*-value	*p*-het
Mean (95% CI)	48.3 (45.9, 50.7)	43.5 (40.9, 46.2)			43.2 (38.6, 47.9)			35.4 (28.6, 42.2)			
<=37.65	184	176	1.00		38	1.00		21	1.00		
]37.65-55.2]	187	104	0.57 (0.40, 0.80)	0.001	27	0.70 (0.39, 1.26)	0.231	11	0.46 (0.19, 1.08)	0.073	
>55.2	187	85	0.43 (0.29, 0.63)	<0.001	19	0.49 (0.25, 0.97)	0.040	4	0.16 (0.05, 0.51)	0.002	
trend per 10 nmol/L			*0.89 (0.82, 0.96)*	*0.002*		*0.88 (0.77, 1.01)*	*0.071*		*0.64 (0.49, 0.82)*	*<0.001*	*0.038*

STAGE AT DIAGNOSIS[c]		**Stage I**			**Stage II**			**Stage III-IV**			
25(OH)D nmol/L	Controls	Cases	OR (95% CI)[a]	*p*-value	Cases	OR (95% CI)[a]	*p*-value	Cases	OR (95% CI)[a]	*p*-value	*p*-het
Mean (95% CI)	48.3 (45.9, 50.7)	43.0 (39.8, 46.2)			42.5 (39.2, 45.8)			40.8 (35.2, 46.3)			
<=37.65	184	96	1.00		91	1.00		34	1.00		
]37.65-55.2]	187	62	0.55 (0.36, 0.84)	0.006	53	0.55 (0.36, 0.84)	0.006	10	0.31 (0.14, 0.68)	0.003	
>55.2	187	58	0.43 (0.28, 0.68)	<0.001	40	0.37 (0.23, 0.59)	<0.001	12	0.31 (0.14, 0.89)	0.004	
trend per 10 nmol/L			*0.86 (0.78, 0.95)*	*0.002*		*0.85 (0.77, 0.93)*	*0.001*		*0.79 (0.66, 0.95)*	*0.011*	*0.706*

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; OR(95%CI), odds ratio an 95% confidence interval; p-int, p-value for interaction; p-het, p-value for heterogeneity; ER+/PR+ & HER2-, estrogen receptor positive and/or progesterone receptor positive tumors with human epidermal growth factor receptor 2 negative; HER2+, human epidermal growth factor receptor 2 positive tumors.

[a] Adjusted for age, body mass index, menopausal status, day of sample extraction, educational level, ethnicity, age at first full term delivery, family history of breast cancer, previous breast biopsies, hypercholesterolemia, hormone replacement therapy use, skin color, and physical activity in the last 5 years. Geographical area introduced as a random effect term.

[b] Sixty-one breast cancer cases could not be classified.

[c] Ninety breast cancer cases could not be classified.

Supplementary Material

Table S1. Association between 25-hydroxyvitamin D levels and breast cancer risk considering only cases sampled in the first month after diagnosis.

MENOPAUSAL STATUS	**OVERALL**				**PREMENOPAUSAL**				
25(OH)D nmol/L	Controls	Cases	OR (95% CI)[a]	*p*-value	Controls	Cases	OR (95% CI)[a]	*p*-value	Controls
<=37.65	184	130	1.00		80	45	1.00		104
]37.65-55.2]	187	89	0.63 (0.44-0.92)	0.015	61	28	0.69 (0.36-1.31)	0.255	126
>55.2	187	79	0.49 (0.32-0.73)	<0.001	59	31	0.60 (0.30-1.22)	0.156	128
trend per 10 nmol/L			*0.92 (0.85-0.99)*	*0.034*			*0.89 (0.77-1.03)*	*0.117*	

TUMOR SUBTYPE[b]	**ER+/PR+ & HER2-**				**HER2+**			
25(OH)D nmol/L	Controls	Cases	OR (95% CI)[a]	*p*-value	Cases	OR (95% CI)[a]	*p*-value	Cas
<=37.65	184	88	1.00		17	1.00		1
]37.65-55.2]	187	63	0.68 (0.45-1.04)	0.073	12	0.70 (0.31-1.62)	0.407	5
>55.2	187	54	0.50 (0.31-0.79)	0.003	14	0.69 (0.29-1.65)	0.401	1
trend per 10 nmol/L			*0.92 (0.84-1.01)*	*0.081*		*1.00 (0.85-1.17)*	*0.959*	

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; OR(95%CI), odds ratio an 95% confidence interval; p-int, p-value for interaction; p-het, p-value for heterogeneity; ER+/PR+ & HER2-, estrogen receptor positive and/or progesterone receptor positive tumors with human epidermal growth factor receptor 2 negative; HER2+, human epidermal growth factor receptor 2 positive tumors.

[a] Adjusted for age, body mass index, menopausal status, day of sample extraction, educational level, ethnicity, age at first full term delivery, family history of breast cancer, previous breast biopsies, hypercholesterolemia, hormone replacement therapy use, skin color, and physical activity in the last 5 years. Geographical area introduced as a random effect term.

[b] Twenty nine breast cancer cases could not be classified.