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Sascha Abbas, Jenny Chang-Claude* and Jakob Linseisen

Division of Cancer Epidemiology, German Cancer Research Center, Heidelberg, Germany

Laboratory and epidemiological data have linked vitamin D to breast cancer prevention. Beside dietary intake, endogenous production of vitamin D substantially contributes to a subject's vitamin D status. Most studies, however, have assessed dietary intake only. Although differential effects of vitamin D on premenopausal and postmenopausal breast cancer have been discussed, this is the first study to investigate the association of plasma 25-hydroxyvitamin D [25(OH)D], as indicator of the overall vitamin D status, with breast cancer risk with restriction to premenopausal women only. We used data of a population-based case-control study comprising 289 cases and 595 matched controls. Information on sociodemographic and breast cancer risk factors was collected by questionnaire and plasma 25(OH)D was measured by enzyme immunoassay. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using conditional logistic regression. We observed a significant inverse association between breast cancer risk and plasma 25(OH)D concentrations. Compared with the lowest category (<30 nmol/L), the ORs (95% CI) for the upper categories (30–45, 45–60, ≥60 nmol/L) were 0.68 (0.43–1.07), 0.59 (0.37–0.94) and 0.45 (0.29–0.70), respectively (p_{trend} = 0.0006). The association was shown to be nonlinear ($p_{nonlinearity}$ = 0.06) in fractional polynomial analysis with a stronger effect in women at low plasma 25(OH)D levels, providing some evidence of a threshold effect (at circa 50 nmol/L). The association was stronger in progesterone receptor negative tumors, with suggestive evidence of effect heterogeneity ($p_{heterogeneity}$ = 0.05, case-only model). Our findings support a protective effect of vitamin D for premenopausal breast cancer.

The anticarcinogenic potential of vitamin D in various cell types, including normal and malignant breast cells, by influencing the induction of cell differentiation, inhibition of cell growth and regulation of apoptosis is well-established.[1–6] Most of the epidemiologic studies regarding breast cancer risk have assessed the effects of vitamin D only for dietary intake yielding inconsistent results.[7–15] However, beside dietary intake, the main source of vitamin D is cutaneous production *via* sun exposure. Studies assessing the association of breast cancer risk and measures of sun exposure, as a proxy of endogenous vitamin D synthesis, have observed inverse associations.[6,17] However, an appropriate biomarker to measure vitamin D status in humans, accounting for both vitamin D from diet and endogenous production, is 25-hydroxyvitamin [25(OH)D]. 25(OH)D is converted to 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], the biologically active form of vitamin D, in the liver and other tissues including breast.[18–20]

Few studies have assessed the relationship between serum or plasma 25(OH)D and breast cancer risk.[21–25] Of 2 small hospital-based case-control studies in predominantly postmenopausal women, one study found a significantly inverse association[23] whereas the other study revealed no association between plasma 25(OH)D and breast cancer risk.[25] To date, 2 studies assessed the association of vitamin D metabolites with breast cancer risk with restriction to postmenopausal women only.[21,22] A study nested in the prostate, lung, colorectal and ovarian cancer screening trial reported no association between both higher plasma 25(OH)D and $1,25(OH)_2D$ concentration and breast cancer risk.[22] However, a strong statistically significant inverse association between postmenopausal breast cancer risk and serum 25(OH)D was found in a large case-control study.[21] A case-control study nested in the Nurses' Health Study (NHS) is the only study that so far reported results stratified by menopausal status.[24] The authors reported a nonsignificant decrease in breast cancer risk with both higher plasma 25(OH)D and $1,25(OH)_2D$ concentration for overall breast cancer risk. Stratification by menopausal status suggested an inverse association for postmenopausal women only, however power was low for their analysis in premenopausal women and risk estimates were not presented. As reviewed recently, prospective studies on dietary intake of vitamin D suggest a more pronounced effect for premenopausal breast cancer risk.[26] Furthermore, we recently reported a significant inverse association of dietary vitamin D with premenopausal breast cancer risk in the present study population.[13] To our knowledge, no study so far assessed the association of plasma 25(OH)D with restriction to premenopausal breast cancer only. Following our previous report on the association between postmenopausal breast cancer risk and serum 25(OH)D,[21] we here describe the results on plasma 25(OH)D and premenopausal breast cancer risk from another population-based case-control study conducted in broadly the same geographical region. We further assessed differential effects by receptor status of the tumor.

Material and methods

Participants in this population-based case-control study were recruited from 2 study regions in southern Germany (Freiburg and Rhein-Neckar-Odenwald) as previously described.[27] Cases were eligible if they were German speaking, diagnosed by the age of 50 years with an incident *in situ* or invasive breast cancer between January 1992 and December 1995. Cases were identified through frequent monitoring of hospital admissions, surgery schedules and pathology reports in 38 hospitals. Two controls per case matched by exact age and study region were selected from a random list of residents provided by the population registries. All study participants gave informed consent. The study was reviewed and approved by the ethics committee of the University of Heidelberg. Overall 706 of 1,020 eligible cases (69.2 percent) and 1,381 of 2,257 eligible controls (61.2 percent) participated, including 494 cases and 957 controls from the Rhein-Neckar-Odenwald study region.

Information on risk factors and sociodemographic data was obtained by a self-administered questionnaire. Information was truncated at the reference date, which was date of diagnosis for

Abbrevations: BMI, body mass index; CI, confidence interval; ER, estrogen receptor; NHS, Nurses' Health Study; OR, odds ratio; PR, progesterone receptor; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D.

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***Correspondence to:** Department of Cancer Epidemiology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. Fax: 49-6221-422203. E-mail: j.chang-claude@dkfz.de

cases and date of study questionnaire completion for controls. Women were defined as postmenopausal if they reported a natural menopause 6 months before reference date or a bilateral oophorectomy. The menopausal status of women with previous hysterectomy not accompanied by bilateral oophorectomy was classified as unknown. Information on hormone receptor status was obtained from pathology reports from the respective hospitals.

Among the 1,451 participants from Rhein-Neckar-Odenwald, plasma samples were available for 1,131 women (77.9 percent). With restriction to premenopausal women, 289 cases (including 18 *in situ* cases) and 595 controls were included in the present analysis.

We measured 25(OH)D in plasma with the 25-hydroxyvitamin D enzyme immunoassay (IDS, Immunodiagnostic Systems Limited, Boldon, UK). Samples were stored in aliquots at −80°C until measurement and analyzed in a single batch in October 2007. The coefficient of variation was 3.1% for intra-assay and 3.3% for interassay determination. Recovery ranged from 96 to 105% as previously reported.[21] We measured 88 random samples (10.0%) in duplicate with an average absolute deviation from the mean of 1.7%.

Because of the skewed distribution of plasma 25(OH)D, geometric means adjusted for time of blood collection and body mass index (BMI) are calculated and compared among cases and controls using analysis of variance. We assessed the association of plasma 25(OH)D with risk of premenopausal breast cancer by means of conditional logistic regression with stratification by age (continuous, in years). Odds ratio (OR) estimates and 95% confidence intervals (CIs) were calculated assessing 25(OH)D concentration both as continuous (per 10 nmol/L increment) and as categorical variable divided into 4 categories (<30, 30–45, 45–60, ≥60 nmol/L). Variables were entered as covariates in the logistic regression model if (*i*) including the covariate considerably changed the OR for the main variables of interest (>5% change in the ORs), (*ii*) if the covariate is a known or potential breast cancer risk factor or, (*iii*) if the covariate was likely to be associated with the main variable of interest (biological plausibility). OR estimates were both adjusted for time of blood collection in four categories, January–March, April–June, July–September, October–December only and with additional adjustment for first-degree family history of breast cancer (yes/no), number of births (0, 1–2, ≥3), duration of breast feeding (continuous, in months), age at menarche (<13, 13–14, ≥15), BMI (continuous, in kg/m^2) and alcohol consumption (0, 1–18, ≥18 g ethanol/day). Age at first birth, smoking and education did not affect the estimates considerably and were therefore not included in the final model.

Linear trend test was performed using the median values in each category as an ordinal variable. We further assessed dose-response-relation and departure from linearity of the log OR function for predictive 25(OH)D concentrations by using fractional polynomials.[28] The continuous 25(OH)D variable was entered into the multivariate logistic regression model *via* a set of defined transformations [x^{-2}, x^{-1}, $x^{-0.5}$, $x^{0.5}$, x^2, x^3 and log(x)], allowing a maximum of 2 terms (including the untransformed variable) in the model. The function that best fitted the data was selected by the −2 log likelihood of the respective model. Test for nonlinearity was performed using the Wald statistics of the transformed term (best model selected by the −2 log likelihood) including both the transformed and the linear term in the model. Effect modification was evaluated by including a cross-product term of the continuous 25(OH)D variable and potential interaction variables and testing for multiplicative interaction with the likelihood ratio test. Analysis stratified by receptor status of the tumor was performed by polytomous logistic regression adjusting for the same covariates as in the overall model with additional adjustment on age. Effect heterogeneity of 25(OH)D status by estrogen receptor (ER) or progesterone receptor (PR) status of the tumor was assessed by means of a case-only analysis. ER or PR status was used as the dependent variable (outcome) and 25(OH)D (continuously) as independent variable in the logistic regression model. Differences in cases and controls were assessed with χ^2-tests for categorical variables. All tests were two-sided with a significance level of $p \leq 0.05$. Statistical analyses were performed with the software SAS 9.1 (SAS Institute, Cary, NC).

TABLE I – SOCIODEMOGRAPHIC CHARACTERISTICS AND RISK FACTORS FOR PREMENOPAUSAL BREAST CANCER IN THE STUDY POPULATION

Characteristics	Cases (*N* = 289)		Controls (*N* = 595)		p^2
	N^1	%	N^1	%	
Age at diagnosis/ recruitment (years)					0.78
30–34	21	7.3	53	8.9	
35–39	47	16.2	115	19.3	
40–44	97	33.6	193	32.4	
45–49	100	34.6	186	31.3	
50	14	4.8	26	4.4	
BMI (kg/m^2)					0.66
<18.5	8	2.8	15	2.5	
18.5–29.9	247	85.7	523	87.9	
≥30	33	11.5	57	9.6	
Educational level					0.7
Low	32	11.1	71	11.9	
Middle	184	63.7	361	60.7	
High	73	25.2	163	27.4	
First degree family					<0.01
History of breast cancer					
No	251	86.9	572	96.1	
Yes	38	13.1	23	3.9	
Number of births					0.01
0	63	21.8	140	23.5	
1–2	206	71.3	373	62.7	
≥3	20	6.9	82	13.8	
Age at menarche (years)					0.65
≤12	103	35.8	230	38.7	
13–14	140	48.6	282	47.4	
≥15	45	15.6	83	13.9	
Duration of breast feeding (months)					0.54
0	133	46.0	270	45.4	
1–3	65	22.5	112	18.8	
4–6	38	13.1	78	13.1	
7–12	34	11.8	84	14.1	
≥13	19	6.6	51	8.6	
Alcohol consumption (g/day)					0.02
0	60	20.8	95	16.0	
1–8	191	66.1	446	74.9	
≥19	38	13.1	54	9.1	
Physical activity (MET; hr/week)[3]					0.32
<83.15	80	40.0	167	34.4	
83.15–132.42	67	33.5	167	34.4	
≥132.43	53	26.5	151	31.2	
Hormonal receptor status of the tumor[4]					
Estrogen receptor					
Positive	128	59.0			
Negative	89	41.0			
Progesterone receptor					
Positive	117	54.9			
Negative	96	45.1			
Time of blood collection					0.36
January–March	80	27.7	136	22.9	
April–June	71	24.6	152	25.6	
July–September	69	23.9	167	28.1	
October–December	69	23.9	140	23.5	

[1]Numbers do not always add up to total numbers due to missing values.–[2]χ^2-test.–[3]MET, metabolic equivalents, data available for 200 cases and 485 controls.–[4]Data on estrogen receptor and progesterone receptor status were available for 217 and 213 cases, respectively.

Results

Characteristics of the 289 cases and 595 controls are shown in Table I. Mean age was 42.1 and 41.6 years for cases and controls, respectively. Statistically significant differences between cases

TABLE II – ODDS RATIOS FOR PREMENOPAUSAL BREAST CANCER BY PLASMA 25-HYDROXYVITAMIN D CONCENTRATION

Plasma 25-hydroxyvitamin D	Cases		Controls		Crude model[1] OR (95% CI)	Adjusted model[2] OR (95% CI)
	N	%	*N*	%		
Categorized (nmol/L)						
<30	66	22.9	87	14.6	1	1
30–45	72	24.9	131	22.0	0.70 (0.45–1.09)	0.68 (0.43–1.07)
45–60	68	23.5	140	23.5	0.66 (0.42–1.04)	0.59 (0.37–0.94)
≥60	83	28.7	237	39.9	0.48 (0.31–0.74)	0.45 (0.29–0.70)
p_{trend}					0.001	0.0006
Continuous per 10 nmol/L increment	289		595		0.91 (0.85–0.96)	0.90 (0.84–0.96)

CI = confidence interval, OR = odds ratio.
[1]Conditional logistic regression stratified by age and adjusted for time of blood collection.–[2]Conditional logistic regression stratified by age and adjusted for time of blood collection, number of births, first-degree family history, age at menarche, duration of breast-feeding, BMI, alcohol consumption.

and controls were observed with respect to family history of breast cancer in at least one first-degree relative, number of births and alcohol consumption.

Geometric means of plasma 25(OH)D adjusted for time of blood collection and BMI were 45.4 nmol/L and 51.3 nmol/L in cases and controls, respectively ($p < 0.0001$). Median (25th–75th percentile) serum 25(OH)D was 46.7 (31.7–63.7) 52.8 (36.4–71.5), in cases and controls, respectively. We observed a statistically significant inverse association between plasma concentration of 25(OH)D and risk of premenopausal breast cancer. Compared with the lowest category (<30 nmol/L), the ORs [OR (95% CI)] for higher plasma concentrations of 30–45, 45–60, and ≥60 nmol/L were 0.68 (0.43–1.07), 0.59 (0.37–0.94) and 0.45 (0.29–0.70), respectively ($p_{trend} = 0.0006$) (Table II). We found a significantly reduced risk of breast cancer with an OR of 0.90 (0.84–0.96) per 10 nmol/L increment when considering 25(OH)D as a continuous variable. Using fractional polynomial analysis, a nonlinear association between the plasma 25(OH)D concentration and the log OR with borderline statistical significance was observed ($p_{nonlinearity} = 0.06$). The function that best fitted our data was $OR(x) = \exp[28.6 \times (1/(x + 1))]$ (−2 log likelihood = 946.7 as compared to 950.0 for the linear model). For graphical illustration we used the median of the lowest category from the categorical analysis, *i.e.* 25.2 nmol/L 25(OH)D in the controls, as reference (Fig. 1). The graph indicates a more pronounced inverse association in the lower concentration ranges of plasma 25(OH)D, while the risk function flattens with increasing 25(OH)D concentrations. The function fitted the categorical risk estimates fairly well. For comparison between the linear and nonlinear model, the linear risk function is displayed as a dotted line.

There was no significant interaction between plasma 25(OH)D and first-degree family history of breast cancer, age at menarche, duration of breast feeding, number of births, alcohol intake or BMI. We further examined the association of plasma 25(OH)D with breast cancer risk by receptor status of the tumor. The association was somehow stronger in estrogen receptor negative (ER−) ($N = 89$) than in ER+ tumors ($N = 128$), but heterogeneity was not significant ($p_{heterogeneity} = 0.27$ for the continuous variable in a case-only model) (Table III). Heterogeneity reached statistical significance for the PR status of the tumor. The association was stronger in PR− ($N = 96$) [OR (95% CI) = 0.29 (0.15–0.56)] than in PR+ tumors ($N = 117$) [OR (95% CI) = 0.73 (0.39–1.37)] comparing the highest (≥60 nmol/L) with the lowest (<30nmol/L) plasma 25(OH)D category ($p_{heterogeneity} = 0.05$) (Table III). However, there were few cases for this analysis ($N = 43$, 22, 42 and 22 in the highest category for ER+, ER−, PR+, and PR− tumors, respectively).

Median difference between time of diagnosis and time of blood collection in cases was 189 days (25th–75th percentile = 25–471). To evaluate potential effects of diagnosis or cancer therapy on circulating 25(OH)D concentration we analyzed the data stratified by time of blood collection since diagnosis (<6 months or ≥6 months). Comparing the highest with the lowest 25(OH)D category, the inverse association remained significant with ORs (95% CI) of 0.45 (0.29–0.70) for all cases and 0.33 (0.18–0.60) and 0.57 (0.32–0.99) when including cases with time of blood collection <6 months and ≥6 months since diagnosis, respectively. However, the test for trend was no longer significant for the latter group ($p_{trend} = 0.0006$, 0.0001 and 0.15, respectively).

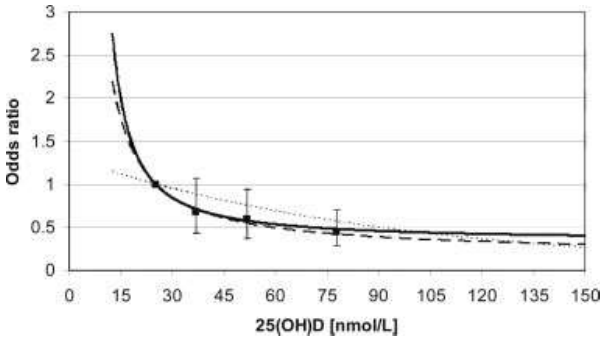

FIGURE 1 – Odds ratios for premenopausal breast cancer by 25(OH)D concentrations using fractional polynomial as dose-response analysis. The resulting function, odds ratio $(x) = \exp[28.6 \times (1/(x + 1))]$, is displayed, setting the median of the lowest category from the categorical analysis as reference (25.2 nmol/L 25(OH)D) (solid line). The solid squares and respective bars represent the odds ratios and 95% CIs of the categorical analysis (30–45, 45–60, ≥60 nmol/L). For graphical illustration categorical odds ratio estimates were displayed at the median value in the controls of each category, at 25.2 (reference), 36.8, 51.9, 77.8 nmol/L, respectively. For comparison between the linear and nonlinear model the linear risk function is displayed as a dotted line. The previously published risk function odds ratio $(x) = \exp[10.4 \times (x + 1)^{-0.5}]$ for postmenopausal breast cancer in another case-control study is displayed as a dashed line (Abbas et al., 2008, see discussion).

Discussion

There is ample evidence from cellular and animal studies linking 25(OH)D to breast cancer, *e.g.* the known anticarcinogenic effects of vitamin D with regard to apoptosis, cell differentiation and proliferation and growth inhibition of human mammary epithelial cells by both $1,25(OH)_2D$ and 25(OH)D. In addition, the presence and expression of the vitamin D receptor and CYP27B1—the enzyme that converts 25(OH)D to active $1,25(OH)_2D$—as well as the uptake of the vitamin D binding protein-25(OH)D complex in mammary cells provide further support for possible anticarcinogenic effects in breast tissue.[29,30] Confirming our a priori hypothesis, we observed in this population-based case-control study a significantly inverse association between

TABLE III – ODDS RATIOS[1] FOR PREMENOPAUSAL BREAST CANCER BY PLASMA 25-HYDROXYVITAMIN D ACCORDING TO ESTROGEN RECEPTOR AND PROGESTERONE RECEPTOR STATUS OF THE TUMOR

Plasma 25-hydroxyvitamin D	Co	ER-positive tumors		ER-negative tumors		PR-positive tumors		PR-negative tumors	
			OR (95% CI)		OR (95% CI)		OR (95% CI)		OR (95% CI)
Mean (nmol/L)[2]	51.3	46.3		43.7		47.2		42.8	
Categorized (nmol/L)	N_{Co}	N_{Ca}		N_{Ca}		N_{Ca}		N_{Ca}	
<30	87	27	1	21	1	21	1	27	1
30–45	131	28	0.66 (0.36–1.21)	24	0.76 (0.39–1.49)	28	0.85 (0.44–1.61)	22	0.52 (0.27–1.01)
45–60	140	30	0.64 (0.35–1.17)	22	0.62 (0.31–1.25)	26	0.72 (0.37–1.39)	25	0.52 (0.27–1.01)
≥60	237	43	0.56 (0.31–1.00)	22	0.40 (0.20–0.81)	42	0.73 (0.39–1.37)	22	0.29 (0.15-0.56)
p_{trend}			0.10		0.009		0.39		0.0007
Continuous per 10 nmol/L increment	595	128	0.93 (0.86–1.01)	89	0.86 (0.77–0.96)	117	0.95 (0.87–1.03)	96	0.84 (0.76–0.94)

Ca, cases; Co, controls; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; OR, odds ratio.
[1]Polytomous logistic regression adjusted for age, time of blood collection, number of births, first-degree family history, age at menarche, duration of breast-feeding, BMI, alcohol consumption.–[2]Geometric mean adjusted for time of blood collection and BMI. $p_{heterogeneity} = 0.27$ and 0.05 for estrogen and progesterone receptor status in a case-only model, respectively.

plasma 25(OH)D and premenopausal breast cancer risk. The result fits well with the recent findings in a case-control study on postmenopausal breast cancer recruited in broadly the same geographical region.[21] A consistent effect of vitamin D on premenopausal and postmenopausal breast cancer was found by dose-response analysis for 25(OH)D concentrations using fractional polynomials, which showed very similar functions for premenopausal and postmenopausal breast cancer risk (Fig. 1). Vitamin D exerts its anticarcinogenic effects by different mechanisms which involve the regulation of a variety of genes acting on growth regulation. These genes include the proto-oncogenes c-fos and c-myc, cyclin-dependent kinase inhibitors p21 and p27, as well as different genes involved in apoptosis, *e.g.* the bcl-2 family, insulin-like growth factor I/II, and tumor necrosis factor-alpha.[2,31,32] As these general mechanisms of cellular growth equally apply to premenopausal and postmenopausal women, a protective effect of vitamin D on both premenopausal and postmenopausal breast cancer is likely.

To our knowledge, this is the first study that assesses plasma 25(OH)D and breast cancer risk in premenopausal women only. So far the relationship between vitamin D metabolites and breast cancer has been assessed in studies including postmenopausal or predominantly postmenopausal women without adequate statistical power to address differences by menopausal status.[21–25,33] The largest cohort to date nested in the prostate, lung, colorectal and breast cancer screening trial assessing postmenopausal breast cancer and vitamin D metabolites reported no association.[22] In the NHS stratification by menopausal status did not suggest an inverse association in premenopausal women but power was low in their analyses and neither subgroup specific risk estimates nor *p*-values of test for statistical interaction were presented.[24] Prospective studies on dietary intake tend to indicate a more pronounced effect in premenopausal women as reviewed recently.[26] The NHS reported a significant association between dietary vitamin D and premenopausal but not postmenopausal breast cancer risk.[7] Similar observations of an inverse association for total vitamin D intake in premenopausal but not postmenopausal women was also recently reported by the Women's Health Study.[15] One proposed explanation lies in the interaction of vitamin D and insulin-like growth factors, thus the potential of vitamin D to suppress IGF-stimulated cell growth.[34,35] Because of the decline of IGF levels with age, the effect of vitamin D on IGF-related tumor development may be more pronounced in premenopausal compared to postmenopausal women. Furthermore, vitamin D metabolism is impaired with aging, including a declined cutaneous synthesis as well as reduced renal production of $1,25(OH)_2D$.[36] Thus higher amounts of vitamin D in older people may be necessary to achieve the same beneficial effects in postmenopausal than in premenopausal women. However, these hypotheses could not be confirmed in our analyses in premenopausal and postmenopausal[21] women.

As reported also for postmenopausal breast cancer,[21] the analysis using fractional polynomials indicate a more pronounced inverse association in the lower concentration ranges of 25(OH)D. These findings contribute to further understanding the dose-response relationship of vitamin D and breast cancer risk. In addition, our data may provide additional information in explaining diverging study results, since the range of 25(OH)D levels vary widely among studies. Median plasma 25(OH)D concentrations were 52.8 nmol/L in our control population as compared to much higher levels ranging from 67 to 80 nmol/L in the UK or US.[22–24] Differences in 25(OH)D concentrations may in part be explained by seasonal differences in 25(OH)D levels that may be more or less pronounced in different countries. As expected, we observed much higher levels of 25(OH)D in cases and controls in summer (April–September) as compared to winter (October–March) (Median 25(OH)D in nmol/L = 48.5 and 42.2 for cases and 59.5 and 49.3 for controls, in summer and winter respectively). However, stratifying the main logistic regression model in samples collected in summer and winter time did not result in considerable changes of the risk estimates. Comparing the highest with the lowest 25(OH)D category, the ORs (95% CI) were 0.40 (0.20–0.82), 0.44 (0.23–0.84) and 0.45 (0.29–0.70), for summer, winter and all samples respectively. Moreover, the collected blood samples were more or less equally distributed over the quarters of the year. We therefore, conclude that potential seasonal effects did not influence our results.

Our findings of a significant inverse association in PR− and ER− tumors only confirm the recent observation for dietary intake of vitamin D in the same premenopausal study population.[13] However, effect heterogeneity reached statistical significance only for PR status of the tumor. Data from the NHS also suggests an inverse association in ER− tumors only.[24] However, we did not find any evidence for effect modification in postmenopausal breast cancer by ER or PR status of the tumor.[21] Anticarcinogenic effects of vitamin D could be mediated *via* the estrogen pathway by downregulation of the ER and therefore disruption of estrogen mediated mitogenic signals.[37–39] Yet responsiveness to the growth inhibitory effects of vitamin D in ER− tumor cells have also been reported.[40,41] The observations on effect modification by receptor status of the tumor could have been a chance finding since the number of cases was very small, and test for heterogeneity in a case-only model was borderline significant only for PR status of the tumor.

Our study results raise the concern of bias due to the retrospective study design. A cancer diagnosis may change plasma 25(OH)D levels due to less sun exposure or less dietary vitamin D ingestion or as a consequence of cancer treatment. Therefore, reverse causality, *i.e.* the lower 25(OH)D levels were a consequence of the cancer diagnosis and not *vice versa*, cannot be excluded in our study. However, short-term changes in diet or behavioural habits are less likely to influence plasma concentration, because the half-life of 25(OH)D is rather long (about 3 weeks)[42] and plasma levels of 25(OH)D have been reported to be

fairly consistent over time.[43] Although prospective studies with measurement in blood samples collected prior to diagnosis are the preferred study design, it is, however, still unclear at what time in life 25(OH)D should be measured to best predict breast cancer risk.[26] Effects of vitamin D and analogues have been reported in early initiation and promotion phases of cancer development[44,45] as well as in late phases when used as a chemotherapeutic agent.[46] To overcome these problems, repeated measurements at different time points before diagnosis would be most valuable.

The effects of chemotherapy or hormonal therapy on 25(OH)D concentration are not profoundly studied. However, a notable change in 25(OH)D concentration after chemotherapeutic treatment was not observed in 2 small studies.[47,48] Chemotherapy is commonly given within the first 6 months after diagnosis. We therefore stratified our analysis by time of blood collection since time of diagnosis using 6 months as cutpoint. The association was weaker in cases whose blood samples were collected $\geq$6 months since diagnosis than that for those collected $<$6 months since diagnosis, suggesting a potential overestimation of the protective effect in the main analysis. However, the risk estimate for the highest category of plasma 25(OH)D remained significant in both groups.

Selection bias is of further concern in the this study. However, comparison between the study group with information on 25(OH)D and the original study population provided only minor differences with respect to sociodemographic variables and breast cancer risk factors. In addition, 25(OH)D status in the control group was fairly comparable to data from the representative German National Health Survey for women in this age range.[49] Another concern in our study lies in the validity of the 25(OH)D measurement since various publications reported variation within different assays and laboratories.[50–52] However, the IDS enzyme immunoassay used in our study gave comparable results to the gold standard, high performance liquid chromatography, in the international Vitamin D Quality Assessment Scheme (DEQAS).[53] Moreover, the low intra- and interassay variation as well as the low deviation from the mean between 2 duplicates give assurance on the validity of the assay.

A major strength of our study is the population-based study design and the ability to account for potential breast cancer risk factors and confounders. Furthermore, 25(OH)D status was assessed in detail using a categorical, a continuous and a fractional polynomial approach to assess dose-response relationships.

In summary, high concentrations of plasma 25(OH)D were associated with a significantly reduced premenopausal breast cancer risk. In a dose-response analysis, the association was found to be nonlinear. There was a stronger inverse association at low plasma 25(OH)D concentration ranges and a flattening of the risk function with higher 25(OH)D concentrations. The findings of a stronger inverse association in PR− tumors deserve further investigation. Our results suggest a protective effect of vitamin D as measured by plasma 25(OH)D for premenopausal breast cancer.

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References

1. Bouillon R, Eelen G, Verlinden L, Mathieu C, Carmeliet G, Verstuyf A. Vitamin D and cancer. J Steroid Biochem Mol Biol 2006;102:156–162.
2. Colston KW, Hansen CM. Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer. Endocrine Relat Cancer 2002;9:45–59.
3. Christakos S, Dhawan P, Liu Y, Peng X, Porta A. New insights into the mechanisms of vitamin D action. J Cell Biochem 2003;88:695–705.
4. Eisman JA, MacIntyre I, Martin TJ, Frampton RJ, King RJ. Normal and malignant breast tissue is a target organ for 1,25-(0H)2 vitamin D3. Clin Endocrinol (Oxford) 1980;13:267–272.
5. Saez S, Falette N, Guillot C, Meggouh F, Lefebvre MF, Crepin M. William L. McGuire Memorial Symposium. 1,25(OH)2D3 modulation of mammary tumor cell growth *in vitro* and *in vivo*. Breast Cancer Res Treat 1993;27:69–81.
6. Trump DL, Hershberger PA, Bernardi RJ, Ahmed S, Muindi J, Fakih M, Yu WD, Johnson CS. Anti-tumor activity of calcitriol: pre-clinical and clinical studies. J Steroid Biochem Mol Biol 2004;89–90:519–26.
7. Shin MH, Holmes MD, Hankinson SE, Wu K, Colditz GA, Willett WC. Intake of dairy products, calcium, and vitamin d and risk of breast cancer. J Natl Cancer Inst 2002;94:1301–11.
8. McCullough ML, Rodriguez C, Diver WR, Feigelson HS, Stevens VL, Thun MJ, Calle EE. Dairy, calcium, and vitamin D intake and postmenopausal breast cancer risk in the Cancer Prevention Study II Nutrition Cohort. Cancer Epidemiol Biomarkers Prev 2005;14:2898–904.
9. Witte JS, Ursin G, Siemiatycki J, Thompson WD, Paganini-Hill A, Haile RW. Diet and premenopausal bilateral breast cancer: a case-control study. Breast Cancer Res Treat 1997;42:243–51.
10. Potischman N, Swanson CA, Coates RJ, Gammon MD, Brogan DR, Curtin J, Brinton LA. Intake of food groups and associated micronutrients in relation to risk of early-stage breast cancer. Int J Cancer 1999;82:315–21.
11. Levi F, Pasche C, Lucchini F, La Vecchia C. Dietary intake of selected micronutrients and breast-cancer risk. Int J Cancer 2001;91:260–3.
12. Simard A, Vobecky J, Vobecky JS. Vitamin D deficiency and cancer of the breast: an unprovocative ecological hypothesis. Can J Public Health 1991;82:300–3.
13. Abbas S, Linseisen J, Chang-Claude J. Dietary vitamin D and calcium intake and premenopausal breast cancer risk in a german case-control study. Nutr Cancer 2007;59:54–61.
14. Robien K, Cutler GJ, Lazovich D. Vitamin D intake and breast cancer risk in postmenopausal women: the Iowa Women's Health Study. Cancer Causes Control 2007;18:775–82.
15. Lin J, Manson JE, Lee IM, Cook NR, Buring JE, Zhang SM. Intakes of calcium and vitamin D and breast cancer risk in women. Arch Int Med 2007;167:1050–9.
16. John EM, Schwartz GG, Dreon DM, Koo J. Vitamin D and breast cancer risk: the NHANES I Epidemiologic follow-up study, 1971–1975 to 1992. National Health and Nutrition Examination Survey. Cancer Epidemiol Biomarkers Prev 1999;8:399–406.
17. John EM, Schwartz GG, Koo J, Wang W, Ingles SA. Sun Exposure, Vitamin D Receptor Gene Polymorphisms, and Breast Cancer Risk in a Multiethnic Population. Am J Epidemiol 2007;166:1409–19.
18. Friedrich M, Rafi L, Mitschele T, Tilgen W, Schmidt W, Reichrath J. Analysis of the vitamin D system in cervical carcinomas, breast cancer and ovarian cancer. Recent Results Cancer Res 2003;164:239–46.
19. Welsh JE, Wietzke JA, Zinser GM, Byrne B, Smith K, Narvaez CJ. Vitamin D-3 receptor as a target for breast cancer prevention. J Nutr 2003;133:2425S–33S.
20. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, Hewison M. Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. J Clin Endocrinol Metab 2001;86:888–94.
21. Abbas S, Linseisen J, Slanger T, Kropp S, Mutschelknauss EJ, Flesch-Janys D, Chang-Claude J. Serum 25-hydroxyvitamin D and risk of post-menopausal breast cancer–results of a large case-control study. Carcinogenesis 2008;29:93–9.
22. Freedman DM, Chang SC, Falk RT, Purdue MP, Huang WY, McCarty CA, Hollis BW, Graubard BI, Berg CD, Ziegler RG. Serum levels of vitamin D metabolites and breast cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. Cancer Epidemiol Biomarkers Prev 2008;17:889–94.
23. Lowe LC, Guy M, Mansi JL, Peckitt C, Bliss J, Wilson RG, Colston KW. Plasma 25-hydroxy vitamin D concentrations, vitamin D receptor genotype and breast cancer risk in a UK Caucasian population. Eur J Cancer 2005;41:1164–9.
24. Bertone-Johnson ER, Chen WY, Holick MF, Hollis BW, Colditz GA, Willett WC, Hankinson SE. Plasma 25-hydroxyvitamin d and 1,25-dihydroxyvitamin d and risk of breast cancer. Cancer Epidemiol Biomarkers Prev 2005;14:1991–7.
25. Janowsky EC, Lester GE, Weinberg CR, Millikan RC, Schildkraut JM, Garrett PA, Hulka BS. Association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk. Public Health Nutr 1999;2:283–91.

26. Bertone-Johnson ER. Prospective studies of dietary vitamin D and breast cancer: more questions raised than answered. Nutr Rev 2007; 65:459–66.
27. Chang-Claude J, Eby N, Kiechle M, Bastert G, Becher H. Breastfeeding and breast cancer risk by age 50 among women in Germany. Cancer Causes Control 2000;11:687–95.
28. Royston P, Ambler G, Sauerbrei W. The use of fractional polynomials to model continuous risk variables in epidemiology. Int J Epidemiol 1999;28:964–74.
29. Rowling MJ, Kemmis CM, Taffany DA, Welsh J. Megalin-mediated endocytosis of vitamin D binding protein correlates with 25-hydroxycholecalciferol actions in human mammary cells. J Nutr 2006;136: 2754–9.
30. Kemmis CM, Salvador SM, Smith KM, Welsh J. Human mammary epithelial cells express CYP27B1 and are growth inhibited by 25-hydroxyvitamin D-3, the major circulating form of vitamin D-3. J Nutr 2006;136:887–92.
31. Hansen CM, Binderup L, Hamberg KJ, Carlberg C Vitamin D and cancer: effects of 1,25(OH)2D3 and its analogs on growth control and tumorigenesis. Front Biosci 2001;6:D820–D48.
32. Swami S, Raghavachari N, Muller UR, Bao YP, Feldman D. Vitamin D growth inhibition of breast cancer cells: gene expression patterns assessed by cDNA microarray. Breast Cancer Res Treat 2003;80:49–62.
33. Hiatt RA, Krieger N, Lobaugh B, Drezner MK, Vogelman JH, Orentreich N. Prediagnostic serum vitamin D and breast cancer. J Natl Cancer Inst 1998;90:461–63.
34. Diorio C, Berube S, Byrne C, Masse B, Hebert-Croteau N, Yaffe M, Cote G, Pollak M, Brisson J. Influence of insulin-like growth factors on the strength of the relation of vitamin D and calcium intakes to mammographic breast density. Cancer Res 2006;66:588–97.
35. Xie SP, James SY, Colston KW. Vitamin D derivatives inhibit the mitogenic effects of IGF-I on MCF-7 human breast cancer cells. J Endocrinol 1997;154:495–504.
36. Bell NH. Vitamin D metabolism, aging, and bone loss. J Clin Endocrinol Metab 1995;80:1051.
37. James SY, Mackay AG, Binderup L, Colston KW. Effects of a new synthetic vitamin D analogue. EB1089, on the oestrogen-responsive growth of human breast cancer cells. J Endocrinol 1994;141:555–63.
38. Swami S, Krishnan AV, Feldman D. 1 alpha,25-Dihydroxyvitamin D3 down-regulates estrogen receptor abundance and suppresses estrogen actions in MCF-7 human breast cancer cells. Clin Cancer Res 2000;6:3371–9.
39. Peng X, Jhaveri P, Hussain-Hakimjee EA, Mehta RG. Overexpression of ER and VDR is not sufficient to make ER-negative MDA-MB231 breast cancer cells responsive to 1alpha-hydroxyvitamin D5. Carcinogenesis 2007;28:1000–7.
40. Flanagan L, Packman K, Juba B, O'Neill S, Tenniswood M, Welsh J. Efficacy of Vitamin D compounds to modulate estrogen receptor negative breast cancer growth and invasion. J Steroid Biochem Mol Biol 2003;84:181–192.
41. Elstner E, Linker-Israeli M, Said J, Umiel T, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP. 20-epi-vitamin D3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. Cancer Res 1995;55:2822–30.
42. Holick MF. Vitamin D: biosynthesis, metabolism, and mode of action. In: DeGroot LJ, Cahill GF Jr, Martini L, Nelson DH, Odell WD, Potts JT, Jr, Steinberger E, Winegrad AI, eds. Endocrinology. New York: Grune and Stratton, 1989. 902–26.
43. Feskanich D, Ma J, Fuchs CS, Kirkner GJ, Hankinson SE, Hollis BW, Giovannucci EL. Plasma vitamin D metabolites and risk of colorectal cancer in women. Cancer Epidemiol Biomarkers Prev 2004;13:1502–8.
44. Mehta R, Hawthorne M, Uselding L, Albinescu D, Moriarty R, Christov K, Mehta R. Prevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats by 1alpha-hydroxyvitamin D(5). J Natl Cancer Inst 2000;92:1836–40.
45. Mehta RG, Moriarty RM, Mehta RR, Penmasta R, Lazzaro G, Constantinou A, Guo L. Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1alpha-hydroxyvitamin D5. J Natl Cancer Inst 1997;89:212–8.
46. VanWeelden K, Flanagan L, Binderup L, Tenniswood M, Welsh J. Apoptotic regression of MCF-7 xenografts in nude mice treated with the vitamin D3 analog. EB1089. Endocrinology 1998;139:2102–10.
47. Kailajarvi ME, Salminen EK, Paija OM, Virtanent AM, Leino AE, Irjala KA. Serum bone markers in breast cancer patients during 5-fluorouracil, epirubicin and cyclophosphamide (FEC) therapy. Anticancer Res 2004;24:1271–4.
48. Gao Y, Shimizu M, Yamada S, Ozaki Y, Aso T. The effects of chemotherapy including cisplatin on vitamin D metabolism. Endocrinol J 1993;40:737–42.
49. Hintzpeter B, Mensink GB, Thierfelder W, Muller MJ, Scheidt-Nave C. Vitamin D status and health correlates among German adults. Eur J Clin Nutr 2007 May 30; Epub ahead of print.
50. Binkley N, Krueger D, Cowgill CS, Plum L, Lake E, Hansen KE, DeLuca HF, Drezner MK. Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. J Clin Endocrinol Metab 2004;89:3152–7.
51. Hollis BW. Editorial: The determination of circulating 25-hydroxyvitamin D: no easy task. J Clin Endocrinol Metab 2004;89:3149–51.
52. Zerwekh JE. The measurement of vitamin D: analytical aspects. Ann Clin Biochem 2004;41:272–81.
53. Carter GD, Carter R, Jones J, Berry J. How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. Clin Chem 2004;50:2195–7.