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Plasma free 25-hydroxyvitamin D, vitamin D binding protein, and risk of breast cancer in the Nurses' Health Study II

Jun Wang,

Division of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts Amherst, 715 N. Pleasant Street, Amherst, MA 01003, USA

A. Heather Eliassen,

Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, USA. Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA

Donna Spiegelman,

Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA. Department of Biostatistics, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA

Walter C. Willett, and

Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, USA. Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA. Department of Nutrition, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA

Susan E. Hankinson

Division of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts Amherst, 715 N. Pleasant Street, Amherst, MA 01003, USA. Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115, USA. Department of Epidemiology, Harvard School of Public Health, 677 Huntington Avenue, Boston, MA 02115, USA

Jun Wang: junw@schoolph.umass.edu

Abstract

Purpose—Prior prospective studies, including our own, have evaluated total plasma 25-hydroxyvitamin D [25(OH)D] and breast cancer risk with inconsistent results. However, recent studies suggest that some vitamin D functions may be more relevant to the unbound (free) fraction of 25(OH)D. Vitamin D binding protein (DBP) influences the free 25(OH)D levels and thus possibly the biological activities of vitamin D.

Methods—We conducted a case–control study nested within the Nurses’ Health Study II to evaluate the association of plasma free 25(OH)D and DBP with breast cancer risk in predominantly premenopausal women. Plasma samples were assayed for 25(OH)D and DBP in 584 case–control pairs. Free 25(OH)D levels were calculated based on plasma levels of total 25(OH)D, DBP, and a constant value representing average albumin levels. Conditional logistic regression was used to estimate relative risks (RRs) and 95 % confidence intervals (CIs).

Results—We found no association between plasma calculated free 25(OH)D and risk of breast cancer overall (highest vs. lowest quartile RR 1.21, 95 % CI 0.83–1.77, trend test p value = 0.50). No association was observed for plasma DBP as well (highest vs. lowest quartile RR 0.95, 95 % CI 0.67–1.36, trend test p value = 0.96). Results were similar by tumor hormone receptor status. Neither the total nor the calculated free 25(OH)D and breast cancer association substantially varied by plasma DBP levels.

Conclusions—Our study does not support an important role of either calculated circulating free 25(OH)D or circulating DBP levels in breast cancer risk among predominantly premenopausal women.

Keywords

Free 25-hydroxyvitamin D; Vitamin D binding protein; Breast cancer; Epidemiology; Prospective

Introduction

Experimental studies indicate that vitamin D may have an important impact on both normal breast development and breast cancer [1]. Vitamin D, a steroid hormone, exerts most of its biological function through binding to the vitamin D receptor (VDR), which is expressed in normal mammary epithelial tissue. In animal models, knockout of VDR results in extensive ductal elongation and branching, as well as enhanced responsiveness to exogenous estrogen and progesterone [2]. Furthermore, substantial data suggest that vitamin D has anti-proliferative, pro-apoptotic, and anti-invasion properties in breast cancer models [1]. However, in prospective epidemiologic studies, the association between circulating 25-hydroxyvitamin D [25(OH)D], the main form of circulating vitamin D as well as the established biomarker of vitamin D status [3], and risk of breast cancer has been inconsistent [4–15]. One possible explanation for the conflicting results could be that current epidemiologic studies have only measured total circulating levels and have not differentiated the bound and unbound (free) fractions of vitamin D metabolites. According to the ‘free hormone’ hypothesis [16], unbound vitamin D metabolites are more likely to exert biological activity (i.e., entry through the cell membrane to bind to their intracellular receptors) than protein-bound vitamin D metabolites.

Approximately 85–90 % of circulating 25(OH)D and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], the biologically active form of vitamin D metabolite, are bound to vitamin D binding protein (DBP), and about 10–15 % are loosely bound to albumin, leaving <1 % as the free form [17, 18]. Consistent with the ‘free hormone’ hypothesis, several recent studies have shown that some functions of vitamin D may be more closely related to the free or bioavailable fraction of 25(OH)D than to total 25(OH)D levels. For instance, the free or

bioavailable fraction (defined as free plus albumin-bound portion) of circulating 25(OH)D was more strongly associated with bone mineral density than the total levels in healthy adults [19] and patients with kidney diseases [20].

One of the major functions of DBP is the binding and transporting of vitamin D metabolites. Circulating DBP levels are approximately 20 times higher than the total circulating vitamin D levels [21]. Experimental studies show that DBP prolongs the half-life of serum 25(OH)D but can also inhibit the biological function of injected 1,25(OH)₂D₃ in mice [22]. In several recent nested case–control studies, circulating DBP levels modified the association between 25(OH)D and risk of several cancers, including bladder [23], pancreatic [24], and prostate [25], further suggesting a role of DBP in the etiology of cancer.

We previously found no association between plasma 25(OH)D and breast cancer risk in a study population of predominantly premenopausal women in the Nurses' Health Study II (NHS II) [12]. However, given the potentially important role of 'free' vitamin D in exerting its biological activities, in the current analysis, we examined the association between plasma calculated free 25(OH)D and breast cancer risk in the same study population. In light of the reported modifying role of DBP on 25(OH)D and risk of several cancers, we also assessed whether there was a direct association between circulating DBP and breast cancer risk and whether the 25(OH)D and breast cancer association varied by DBP levels.

Materials and methods

Study population

The NHSII was established in 1989, when 116,430 female registered nurses, aged 25–42 years, completed, and returned an initial questionnaire. The majority of the participants were white (~94 %) and approximately 2 % each for African American, Asian, and Hispanic. The cohort has been followed biennially by mailed questionnaires to update exposures and to ascertain newly diagnosed disease.

Between 1996 and 1999, 29,611 cohort members who were cancer-free and between 32 and 54 years old provided a blood sample in response to having been sent a blood collection kit and a short questionnaire. Details of this cohort and blood collection have been described previously [26]. Briefly, 18,521 premenopausal women who had not used exogenous hormones (i.e., postmenopausal hormonal therapy or oral contraceptives) or been pregnant in the past 6 months provided an initial blood sample drawn on the third to fifth day of the menstrual cycle (follicular blood collection) and a second sample drawn 7–9 days before the anticipated start of the next cycle (luteal blood collection). Participants were sent a blood collection kit and a short questionnaire. Follicular plasma was aliquoted by the participants and frozen in their home until the second blood collection. Participants shipped both follicular plasma and luteal whole blood samples via overnight courier with an ice pack, to the laboratory where luteal blood samples were processed and separated into plasma, red blood cell, and white blood cell components, and aliquoted into labeled cryotubes. Women who were not eligible to provide timed blood samples (e.g., perimenopausal or postmenopausal) or declined to provide timed samples gave a single blood sample. These samples were shipped and processed similarly to luteal samples. All samples have been

stored in liquid nitrogen freezers (less than -130°C) since collection. Characteristics of women who donated blood samples (e.g., age, BMI) were similar to the entire NHSII cohort [26]. The study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital and the Harvard School of Public Health.

Case identification and control selection

Details of the case ascertainment and control selection have been described elsewhere [12]. In brief, breast cancer cases were identified through the biennial questionnaires: Cases had no previously reported cancer diagnosis before blood collection and were diagnosed after blood collection but before June 1, 2007. A total of 610 cases who donated blood and participated in the previous study of plasma 25(OH)D were included [12], with mean time from blood drawn to diagnosis of 57 months (range 1–127 months). Cases were confirmed by either medical record review (95 %) or verbal confirmation by the nurse (5 %). The latter cases were included because of the >99 % concordance rate between medical record review and self-report. Although the previous study of plasma 25(OH)D included 2 controls per case, due to financial constraints, only one control was matched to each case in the current analysis ($n = 610$ controls) by age (± 2 years); race/ethnicity (Caucasian, African American, Hispanic, Asian, other); menopausal status at blood collection and diagnosis (pre-menopausal, postmenopausal, unknown/missing); month and year of blood drawn (± 2 months); luteal day (for timed samples; ± 1 day); time of day (± 2 h); and fasting status (<2, 2–4, 5–7, 8–11, >12 h).

Laboratory assays

Details of the plasma 25(OH)D measurements have been described previously [12]. Briefly, plasma 25(OH)D was assayed using a radioimmunoassay with radioiodinated tracers after acetonitrile extraction at Heartland Assays Inc. (Ames, IA, U.S.). Assays were run in two batches; the overall coefficients of variation (CVs) from blinded replicate quality control samples in each batch were 10.7 and 6.0 %. Plasma DBP was assayed in the laboratory of Dr. Nader Rifai (Children's Hospital, Boston, MA) using an enzyme-linked immunosorbent assay (ELISA) from R&D systems (Minneapolis, MN). All samples for DBP were assayed in a single batch, and the CV from blinded replicate quality control samples was 6.7 %. Case-control pairs were assayed together, and laboratory personnel were blinded to case, control, or quality control status. Of the 610 pairs, 26 pairs were not sent to the laboratory because of insufficient plasma remaining for DBP assays, leaving a total of 584 case-control sets in the final analysis.

Covariate data

Demographic and breast cancer risk factor information was obtained from the biennial NHS II questionnaires, and a questionnaire completed at the time of the blood drawn. Age at menarche, weight at age 18, and height were queried in the baseline questionnaire in 1989. Family history of breast cancer was ascertained in 1989 and 1997. Parity, age at first birth, diagnosis of benign breast disease, alcohol consumption, menopausal status, weight, and oral contraceptive use were assessed biennially. Weight and alcohol consumption were also

asked at the time of blood collection. For covariates with multiple assessments, information from the questionnaire completed closest to the blood collection was used.

Statistical analysis

We first assessed statistical outliers using the generalized extreme studentized deviate many-outlier detection approach [27]. Five participants with low 25(OH)D values (< 10.7 nmol/L) were identified; however, these low values were likely to be true because these participants had very low vitamin D dietary intake (i.e., 50–100 IU/day) and donated blood during the winter, and thus, these values were retained in the analysis. Only one participant who had an outlier value at the low end of DBP (837.1 nmol/L) was identified. However, results were materially unchanged by excluding this outlier; thus, we kept this participant in the analyses.

Plasma-free 25(OH)D was calculated by the following equation [18]:

$$\text{Free 25(OH)D} = \frac{\text{Total 25(OH)D}}{1 + ([6 \times 10^5] \times [5.8 \times 10^{-6}]) + ([7 \times 10^8] \times \text{DBP})}$$

where 6×10^5 and 7×10^8 represent the affinity constants of 25(OH)D to albumin and DBP, respectively, and all concentrations are expressed in mol/L. Calculated circulating free 25(OH)D using this equation and measured free 25(OH)D using centrifugal ultrafiltration were reported to be highly correlated ($r = 0.9$) [18]. Plasma albumin was not measured in our study; instead, a constant (5.8×10^{-6} mol/L) was used in the analysis based on previous studies [28] given that only a small proportion (10–15 %) of circulating 25(OH)D binds to albumin and the inter-individual variation of albumin is small [28]. Thus, the quantitative impact of albumin on circulating free 25(OH)D levels is unsubstantial. For instance, for a given free 25(OH)D value of 18.96 pmol/L calculated with albumin as 5.8×10^{-6} mol/L, this value remained the same when using albumin levels of either 4.6×10^{-6} mol/L or 7.3×10^{-6} mol/L, levels representing the normal lower and upper range of circulating albumin. Of note, the ratio of total 25(OH)D to DBP has previously been used as an index of free 25(OH)D [29]. Without measuring circulating albumin levels, the molar ratio and calculated free 25(OH)D levels are perfectly correlated and only differ by the albumin constant.

For the primary analysis, conditional logistic regression was used to estimate relative risks (RRs) and 95 % confidence intervals (CIs). Quartile cutoff points of total 25(OH)D and calculated free 25(OH)D were defined in two ways: The first set was determined based on the distribution among all controls; the second set was based on season-specific cutoff points based on blood collection dates among the controls: February to April, May to July, August to October, and November to January. Quartile cutoff points of DBP, which does not vary by season, were determined based on the distribution among all controls. Multivariate models were adjusted for body mass index (BMI) at age 18 and at blood collection, age at menarche, parity and age at first birth, history of benign breast disease, family history of breast cancer, and alcohol consumption (see Tables 2, 3, 4 footnote for exposure categories). Trend tests were conducted by modeling quartile medians and calculating the Wald statistic. The dose–response relationship was assessed using restricted cubic spline models [30, 31]. Tests of interaction between plasma total, free 25(OH)D or DBP and a potential modifying

variable were performed using the likelihood ratio test by comparing models with and without a cross-product term of the quartile medians of each vitamin D variable and the modifying variable. In stratified analyses, unconditional logistic regression adjusted for the matching factors and the covariates given above was used to maximize statistical power. Results from the multivariable unconditional and conditional logistic regression were very similar. In stratified analyses by tumor subtype (i.e., ER-/PR-), we collapsed several categories of alcohol consumption due to small numbers of cases or controls in some categories.

To test whether the association between calculated free 25(OH)D or DBP and breast cancer risk differed by estrogen receptor (ER) or progesterone receptor (PR) status of the tumor, we used polytomous unconditional logistic regression [32]. Because there were few cases with ER+/PR- or ER-/PR+, tests of disease heterogeneity were performed primarily for ER+/PR+ versus ER-/PR-. A likelihood ratio test comparing a model with a common slope for calculated free 25(OH)D or DBP with a model with separate slopes for each case group was used. All *p* values were based on two-sided tests and considered statistically significant if <0.05 . All analyses were performed using SAS software version 9.2 (SAS Institute, Cary, NC, USA).

Results

Cases were more likely to have a family history of breast cancer and a history of benign breast disease than controls (Table 1). BMI at age 18 and at blood collection was slightly higher in controls than in cases. Plasma levels of calculated free 25(OH)D and DBP were similar in cases and controls.

Calculated free 25(OH)D was highly correlated with total 25(OH)D (Spearman's correlation coefficient [*r*] among controls = 0.76, $p < 0.0001$) and inversely correlated with DBP ($r = -0.51$ among controls, $p < 0.0001$). In addition, among controls, free 25(OH)D was inversely correlated with BMI at blood drawn ($r = -0.29$, $p < 0.0001$) while positively correlated with alcohol consumption ($r = 0.19$, $p < 0.0001$). The high correlation between total and calculated free 25(OH)D remained after controlling for age, BMI at blood collection, and alcohol consumption ($r = 0.73$, $p < 0.0001$). DBP was not correlated with age, BMI, smoking, or alcohol consumption.

As expected, plasma total 25(OH)D was not associated with risk of breast cancer overall in this analysis (the highest versus lowest quartile RR 1.18, 95 % CI 0.80–1.76; trend test *p* value = 0.49), and the estimates were very similar to the results in our prior report (top vs. bottom quartile RR 1.20, 95 % CI 0.88–1.63; trend test *p* value = 0.32) [12]. Calculated free 25(OH)D was not significantly associated with breast cancer overall (the highest vs. lowest quartile RR 1.21, 95 % CI 0.83–1.77; trend test *p* value = 0.50, Table 2). Further analyses by tumor subtypes did not show significant differences. The effect estimates were similar in invasive cases (the highest vs. lowest quartile RR 1.15, 95 % CI 0.77–1.72; trend test *p* value = 0.69). When stratified by tumor hormone receptor status, no significant heterogeneity was found (heterogeneity test *p* value for ER+/PR+ vs. ER-/PR- = 0.12). There was no significant linear or nonlinear association detected when using restricted cubic

spline models. Calculated free 25(OH)D levels were significantly higher in the months of May to October than in the months of November to April ($p < 0.0001$, data not shown). When stratifying by season of blood drawn (i.e., May–July, August–October, November–January, February–April), we found an overall null association in each of the strata. A non-significant positive association was observed for months with more light (i.e., May to July, the highest vs. lowest RR 1.26, 95 % CI 0.62–2.56, trend test p value = 0.42) as well as for darker months (i.e., November to January, the highest vs. lowest RR 1.43, 95 % CI 0.66–3.11; trend test p value = 0.42). We further performed analyses using season-specific cutoff points for calculated free 25(OH)D, and the results remained very similar to those without using season-specific cutoffs (data not shown).

Plasma DBP was not associated with breast cancer overall (the highest vs. lowest quartile RR 0.95, 95 % CI 0.67–1.36; trend test p value = 0.96, Table 3) or invasive cases (the highest vs. lowest quartile RR 1.18 95 % CI 0.82–1.71; trend test p value = 0.25) in our study population. Further, no significant heterogeneity was observed by hormone receptor subtype (heterogeneity test p value for ER+/PR+ vs. ER-/PR- = 0.78). No significant linear or nonlinear association was detected for DBP as well using the restricted cubic spline models.

When we stratified by the median level of plasma DBP, we did not observe any significant variation in the associations of either total or calculated free 25(OH)D with breast cancer risk (interaction test p value = 0.75 and 0.82, respectively; Table 4). We also tested whether BMI at blood collection (<25 vs. ≥ 25 kg/m²) modified the association between calculated free 25(OH)D or DBP and breast cancer, and observed a significant interaction for free 25(OH)D (interaction test p value <0.0001) but not for DBP (interaction test p value = 0.75). There was an increasing risk associated with increased calculated free 25(OH)D levels among women with BMI ≥ 25 kg/m² (trend test p value = 0.01), with a more than doubled risk observed in the highest versus lowest quartile (OR = 2.11, 95 % CI 1.21–3.70). No significant association was found among women with BMI <25 kg/m².

Discussion

To our knowledge, this is the first investigation of the association between circulating free 25(OH)D, DBP, and risk of breast cancer. We observed no association between plasma calculated free 25(OH)D and risk of breast cancer overall or according to tumor invasiveness or hormone receptor status. Similarly, we did not find an association between plasma DBP and risk of breast cancer overall or by tumor subtype. Our data also suggest no significant interaction between circulating DBP and either total or calculated free 25(OH)D on risk of breast cancer overall. The significant interaction between calculated free 25(OH)D and BMI was expected, given a similar interaction was observed for total 25(OH)D in our previous study [12] and the high correlation between total and calculated free 25(OH)D ($r = 0.76$ among controls).

Although an overall null association was found for plasma total 25(OH)D and breast cancer in this and in our previous study [12], both of which consisted of predominantly premenopausal women, an earlier study reported a suggestive inverse association in

predominantly postmenopausal women in the NHS (RR for highest vs. lowest quintile = 0.73, 95 % CI 0.49–1.07; trend test p value = 0.06) [4]. Consistent with this apparent differential effect of plasma total 25(OH)D on breast cancer by menopausal status, a recent dose–response meta-analysis also suggested a possible nonlinear inverse association in postmenopausal but not premenopausal women [33]. However, the mechanisms underlying a potential difference by menopausal status are not known.

Although no studies to date have evaluated the effect of circulating free 25(OH)D on risk of breast cancer, several prospective studies have assessed the association between the molar ratio of 25(OH)D to DBP, a proxy for free circulating 25(OH)D [29], and risk of cancers of different origins. Given that we did not measure albumin levels in our study, the calculated 25(OH)D based on a constant value representing average albumin levels is statistically identical to the molar ratio. In case–control studies nested within the Alpha-Tocopherol Beta-Carotene (ATBC) Cancer Prevention study cohort, the molar ratio of 25(OH)D to DBP was positively associated with risk of pancreatic cancer (RR for the highest vs. the lowest quartile = 1.86, 95 % CI 0.97–3.56; trend test p value = 0.04) [24] and prostate cancer (RR for the highest vs. the lowest quintile = 1.36, 95 % CI 0.97–1.91; trend test p value = 0.04) [25] and suggestively associated with renal cell carcinoma (RR for the highest vs. lowest quartile = 1.61, 95 % CI 0.95–2.73; trend test p value = 0.09) [34]. However, in another case–control study nested within the ATBC study cohort, a non-significant inverse association was reported for bladder cancer [23], although results from a nested case–control study within the prostate, lung, colorectal, and ovarian (PLCO) cancer screening trial showed no association for bladder cancer [35]. In contrast to the positive association with prostate cancer found in the ATBC study, no association was observed in a nested case–control study in the Physicians' Health Study [36].

Several nested case–control studies including those mentioned above have examined the effects of circulating DBP on risk of several types of cancer. The findings were generally null for both bladder [23, 35] and prostate cancers [25, 36, 37], while inverse associations were reported for pancreatic cancer (RR for the highest vs. the lowest quintile = 0.67, 95 % CI 0.37–1.19; trend test p value = 0.02) [24] and renal cell carcinoma (RR for the top vs. bottom quartile = 0.17, 95 % CI 0.08–0.33; trend test p value <0.0001) [34]. Despite the lack of a consistent overall association of DBP on risk of cancer at different sites, several analyses have suggested that the 25(OH)D–cancer association may vary by level of DBP [23–25]. The inverse 25(OH)D–bladder cancer [23] and the positive 25(OH)D–pancreatic cancer [24] associations were stronger among those with lower circulating DBP levels (below the median), suggesting that the 'free' fraction of 25(OH)D may be particularly relevant to the etiology of these cancers. However, in contrast, the positive 25(OH)D prostate cancer association was stronger among those with higher circulating DBP levels [25].

Overall, the current literature on the association between circulating 'free' or the proxy for 'free' 25(OH)D, DBP, and risk of cancer is limited and consists largely of results from the ATBC study. Differences in either the study population (e.g., ATBC participants were male smokers while ~10 % of our female participants smoked) or in the etiology of the cancers examined might partly explain the differing results. Of note, the correlation between

circulating total and calculated free 25(OH) in our study and one of the nested case-control studies in the ATBC trial [24] were very similar ($r = 0.72$ in ATBC and $r = 0.76$ in ours). Despite the relatively high correlation, total 25(OH)D levels only accounted for 52–58 % (r^2) of the variation in free levels based on these two studies. Future studies with larger sample sizes are needed to provide further insights of the relationship between the free portion and the total of 25(OH)D.

In addition, contrary to the ‘free hormone hypothesis,’ recent experimental data have shown the existence of cellular uptake pathways for the DBP-bound vitamin D complex by the plasma membrane receptor megalin. Through this mechanism, carrier-bound vitamin D metabolites also may enter into the cell and be internalized via megalin-mediated endocytosis [38], thus potentially contributing to the activation of the VDR pathway [39]. Megalin is expressed in human mammary epithelial cell lines as well as some breast cancer cell lines [39]. Megalin-mediated cellular uptake of the DBP-bound vitamin D complex has been shown in several epithelial tissues, including the mammary gland [39, 40], but has not yet been reported in pancreas, bladder, or prostate tissues. Further epidemiologic studies of these associations as well as laboratory studies to further determine the presence and role of megalin are needed.

The major strengths in our study include the prospective collection of blood samples, relatively large study population, and low technical assay error (CV ranges 6–10 %). One limitation is that plasma 25(OH)D and DBP were measured at one point in time, although we have shown that a single measurement of 25(OH)D reliably reflects levels over at least a 3-year period in the NHS (intraclass correlation coefficient = 0.72) [41]. Circulating DBP levels maintain stable and do not vary substantially by season [29]. Another limitation is that we did not measure individual albumin levels; instead, a fixed value has been assigned to individuals. However, as mentioned above, it is unlikely to change the results given the negligible impact of circulating albumin levels on free 25(OH)D levels.

In conclusion, findings from our prospective study indicate no substantial impact of plasma calculated unbound 25(OH)D on risk of breast cancer. Further, we found no evidence of an important role of DBP in the vitamin D and breast cancer association in predominately premenopausal women.

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Abbreviations

| | |
|----------------|---------------------------|
| 25(OH)D | 25-Hydroxyvitamin D |
| DBP | Vitamin D binding protein |
| VDR | Vitamin D receptor |

| | |
|------------------------------------------|--------------------------------------|
| 1,25(OH)₂D₃ | 1,25-Dihydroxyvitamin D ₃ |
| NHS | Nurses' Health Study |
| CV | Coefficient of variation |
| ER | Estrogen receptor |
| PR | Progesterone receptor |
| BMI | Body mass index |
| RR | Relative risk |
| CI | Confidence interval |

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Table 1Characteristics of breast cancer cases and controls in the NHS II^a

| | Cases (<i>n</i> = 584) | Controls (<i>n</i> = 584) |
|-------------------------------------------------------------|-------------------------|----------------------------|
| Age at blood collection (year) | 45.1 (4.5) | 45.0 (4.4) |
| Age at menarche, 13 years (%) | 45 | 46 |
| BMI at age 18 (kg/m ²) | 20.7 (2.7) | 21.1 (3.0) |
| BMI at blood collection (kg/m ²) | 25.0 (5.0) | 26.0 (6.1) |
| Smoking, current (%) | 10 | 7 |
| Alcohol consumption, 1 drinks/week (%) | 39 | 34 |
| Physical activity, MET-h/week | 17.9 (25.0) | 18.9 (25.6) |
| Ever used oral contraceptive (%) | 85 | 88 |
| Duration of past oral contraceptive use, month ^b | 55.4 (46.3) | 51.9 (44.2) |
| Nulliparous (%) | 22 | 20 |
| Parity ^c | 2.2 (0.9) | 2.4 (1.0) |
| Age at first birth ^c | 26.5 (4.6) | 26.0 (4.6) |
| Pre-menopausal at blood collection (%) | 76 | 74 |
| Family history of breast cancer (%) | 17 | 10 |
| History of benign breast disease (%) | 23 | 16 |
| Plasma 25(OH)D ^d (nmol/L) | 62.6 (47.0, 78.1) | 61.4 (45.6, 76.4) |
| Calculated plasma-free 25(OH)D ^d (pmol/L) | 19.5 (14.1, 26.3) | 19.2 (13.3, 25.5) |
| Plasma DBP ^d (nmol/L) | 4,642 (3,641, 5,671) | 4,626 (3,760, 5,588) |

^a Mean (standard deviation) for continuous variables and % for categorical variables^b Duration of oral contraceptive use among ever users^c Among parous women^d Median (25, 75 %) values were given

Table 2

Plasma calculated free 25(OH)D and risk of breast cancer overall and by tumor subtypes in the NHS II

| Cut-off points (pmol/L) | Quartile 1 < 13.3 | Quartile 2 13.3 to < 19.2 | Quartile 3 19.2 to < 25.5 | Quartile 4 | 25.5 | Trend test <i>p</i> value |
|---------------------------|---------------------|---------------------------|---------------------------|------------------|------|---------------------------|
| All cases | | | | | | |
| Cases/controls | 124/145 | 162/147 | 139/146 | 159/146 | | |
| Simple ^a | 1.00 ^{Ref} | 1.29 (0.93–1.79) | 1.15 (0.81–1.63) | 1.30 (0.92–1.85) | | 0.26 |
| Multivariate ^b | 1.00 ^{Ref} | 1.24 (0.87–1.76) | 1.09 (0.75–1.58) | 1.21 (0.83–1.77) | | 0.50 |
| Invasive | | | | | | |
| Cases/controls | 89/145 | 107/147 | 98/146 | 104/146 | | |
| Multivariate ^c | 1.00 ^{Ref} | 1.25 (0.85–1.82) | 1.14 (0.77–1.69) | 1.15 (0.77–1.72) | | 0.69 |
| ER+/PR+ | | | | | | |
| Cases/controls | 54/145 | 65/147 | 71/146 | 70/146 | | |
| Multivariate ^c | 1.00 ^{Ref} | 1.27 (0.81–2.00) | 1.41 (0.89–2.23) | 1.31 (0.82–2.09) | | 0.32 |
| ER-/PR- | | | | | | |
| Cases/controls | 17/145 | 23/147 | 10/146 | 16/146 | | |
| Multivariate ^d | 1.00 ^{Ref} | 1.26 (0.62–2.57) | 0.49 (0.20–1.16) | 0.85 (0.37–1.93) | | 0.35 |

^aUnivariate conditional logistic regression model^bMultivariate conditional logistic regression model adjusted for BMI at age 18 (<20, 20 to <23, 23 kg/m²), BMI at blood drawn (continuous), age at menarche (<12, 12, 13, >13 years), parity and age at first birth (nulliparous, 1–2 children and <25 years, 1–2 children and 25–29 years, 1–2 children and 30 years, 3 children and <25 years, 3 children and 25 years), family history of breast cancer, history of benign breast disease, and alcohol consumption at blood collection (none, 1–3 drinks/month, 1 drink/week, 2–4 drinks/week, >4 drinks/week)^cMultivariate unconditional logistic regression models adjusted for the same covariates in b and matching factors: age at blood collection (continuous), race (white, other), date of blood collection (February–April, May–July, August–October, November–January), time of blood collection (1–4 a.m., 5–6 a.m., 7 a.m. to 12 p.m., treated as continuous), fasting status (>8 h, 8 h), luteal day (<8, 8, untimed collection), and menopausal status at blood collection (premenopausal, postmenopausal, unknown)^dMultivariate unconditional logistic regression model adjusted for the same covariates and matching variables in c except that alcohol consumption was adjusted using three categories (none, 1–3 drinks/month, 1 drink/week)*Ref* referent

Table 3

Plasma DBP and risk of breast cancer overall and by tumor subtype in the NHS II

| Cutoff points (nmol/L) | Quartile 1 < 3,760 | Quartile 2 3,760 to < 4,626 | Quartile 3 4,626 to < 5,588 | Quartile 4 5,588 | Trend test <i>p</i> value |
|---------------------------|---------------------|-----------------------------|-----------------------------|------------------|---------------------------|
| All cases | | | | | |
| Cases/controls | 156/146 | 133/146 | 139/146 | 156/146 | |
| Simple ^a | 1.00 ^{Ref} | 0.85 (0.61–1.19) | 0.88 (0.63–1.24) | 0.99 (0.71–1.38) | 0.94 |
| Multivariate ^b | 1.00 ^{Ref} | 0.83 (0.58–1.18) | 0.89 (0.62–1.28) | 0.95 (0.67–1.36) | 0.96 |
| Invasive | | | | | |
| Cases/controls | 95/146 | 87/146 | 100/146 | 116/146 | |
| Multivariate ^c | 1.00 ^{Ref} | 0.86 (0.59–1.27) | 1.05 (0.72–1.53) | 1.18 (0.82–1.71) | 0.25 |
| ER+/PR+ | | | | | |
| Cases/controls | 63/146 | 65/146 | 64/146 | 68/146 | |
| Multivariate ^c | 1.00 ^{Ref} | 0.94 (0.61–1.45) | 1.02 (0.66–1.57) | 1.05 (0.68–1.61) | 0.76 |
| ER-/PR- | | | | | |
| Cases/controls | 14/146 | 14/146 | 17/146 | 21/146 | |
| Multivariate ^d | 1.00 ^{Ref} | 0.92 (0.41–2.05) | 1.13 (0.52–2.44) | 1.45 (0.69–3.04) | 0.25 |

^aUnivariate conditional logistic regression model

^bMultivariate conditional logistic regression model adjusted for BMI at age 18 (<20, 20 to <23, 23 kg/m²), BMI at blood drawn (continuous), age at menarche (<12, 12, 13, >13 years), parity and age at first birth (nulliparous, 1–2 children and <25 years, 1–2 children and 25–29 years, 1–2 children and 30 years, 3 children and <25 years, 3 children and 25 years), family history of breast cancer, history of benign breast disease, and alcohol consumption at blood collection (none, 1–3 drinks/month, 1 drink/week, 2–4 drinks/week, >4 drinks/week)

^cMultivariate unconditional logistic regression models adjusted for the same covariates in b and matching factors: age at blood collection (continuous), race (white, other), date of blood collection (February–April, May–July, August–October, November–January), time of blood collection (1–4 a.m., 5–6 a.m., 7 a.m.–12 p.m., treated as continuous), fasting status (>8 h, 8 h), luteal day (<8, 8, untimed collection), and menopausal status at blood collection (premenopausal, postmenopausal, unknown)

^dMultivariate unconditional logistic regression model adjusted for the same covariates and matching variables in c except alcohol consumption was adjusted using three categories (none, 1–3 drinks/month, 1 drink/week)

Ref referent

Table 4

Plasma total and calculated free 25(OH)D and risk of breast cancer by plasma DBP levels in the NHS II

| | Quartile 1 | Quartile 2 | Quartile 3 | Quartile 4 | Trend test <i>p</i> value |
|---------------------------|---------------------|------------------|------------------|------------------|---------------------------|
| Total 25(OH)D | | | | | |
| DBP <median | | | | | |
| Case/control | 74/78 | 72/75 | 77/80 | 67/61 | |
| Multivariate ^a | 1.00 ^{Ref} | 0.91 (0.56–1.48) | 0.90 (0.55–1.48) | 1.09 (0.64–1.84) | 0.79 |
| DBP median | | | | | |
| Case/control | 55/68 | 77/70 | 66/68 | 96/84 | |
| Multivariate ^a | 1.00 ^{Ref} | 1.54 (0.91–2.60) | 1.25 (0.72–2.17) | 1.39 (0.82–2.37) | 0.39 |
| Calculated free 25(OH)D | | | | | |
| DBP <median | | | | | |
| Case/control | 34/41 | 50/52 | 72/78 | 134/123 | |
| Multivariate ^a | 1.00 ^{Ref} | 1.16 (0.62–2.19) | 1.08 (0.59–1.98) | 1.23 (0.69–2.19) | 0.51 |
| DBP median | | | | | |
| Case/control | 90/104 | 112/95 | 67/68 | 25/23 | |
| Multivariate ^a | 1.00 ^{Ref} | 1.45 (0.94–2.24) | 1.20 (0.73–1.97) | 1.17 (0.57–2.42) | 0.63 |

^aMultivariate unconditional logistic regression models adjusted for BMI at age 18 (<20, 20 to <23, 23 kg/m²), BMI at blood drawn (continuous), age at menarche (<12, 12, 13, >13 years), parity and age at first birth (nulliparous, 1–2 children and <25 years, 1–2 children and 25–29 years, 1–2 children and >29 years, 3 children and <25 years, 3 children and 25 years), family history of breast cancer, history of benign breast disease, and alcohol consumption at blood collection (none, 1–3 drinks/month, 1 drink/week, 2–4 drinks/week, >4 drinks/week), and matching factors listed in Table 3

p value for test of interaction: total 25(OH)D *p* = 0.75; calculated free 25(OH)D *p* = 0.82

Ref referent