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Circulating vitamin D and breast cancer risk: an international pooling project of 17 cohorts

A full list of authors and affiliations appears at the end of the article.

Abstract

Laboratory and animal research support a protective role for vitamin D in breast carcinogenesis, but epidemiologic studies have been inconclusive. To examine comprehensively the relationship of circulating 25-hydroxyvitamin D [25(OH)D] to subsequent breast cancer incidence, we harmonized and pooled participant-level data from 10 U.S. and 7 European prospective cohorts. Included were 10,484 invasive breast cancer cases and 12,953 matched controls. Median age (interdecile range) was 57 (42–68) years at blood collection and 63 (49–75) years at breast cancer diagnosis. Prediagnostic circulating 25(OH)D was either newly measured using a widely accepted immunoassay and laboratory or, if previously measured by the cohort, calibrated to this assay to permit using a common metric. Study-specific relative risks (RRs) for season-standardized 25(OH)D concentrations were estimated by conditional logistic regression and combined by random-effects models. Circulating 25(OH)D increased from a median of 22.6 nmol/L in consortium-wide decile 1 to 93.2 nmol/L in decile 10. Breast cancer risk in each decile was not statistically significantly different from risk in decile 5 in models adjusted for breast cancer risk factors, and no trend was apparent (P -trend = 0.64). Compared to women with sufficient 25(OH)D based on Institute of Medicine guidelines (50–<62.5 nmol/L), RRs were not statistically significantly different at either low concentrations (<20 nmol/L, 3% of controls) or high concentrations (100–<125 nmol/L, 3%; 125 nmol/L, 0.7%). RR per 25 nmol/L increase in 25(OH)D was 0.99 (95% confidence interval [CI]: 0.95–1.03). Associations remained null across subgroups, including those defined by body mass index, physical activity, latitude, and season of blood collection. Although none of the associations by tumor characteristics reached statistical significance, suggestive inverse associations were seen for distant and triple negative tumors. Circulating 25(OH)D, comparably measured in 17 international cohorts and season-standardized,

Corresponding author: Regina G. Ziegler, regina.ziegler@nih.gov.

^aContributed equally as first authors.

^bDeceased.

^cContributed equally as senior authors.

Author contributions

The Circulating Biomarkers and Breast and Colorectal Cancer Consortium was conceived by WCW, SSW, and RGZ and directed by SSW and RGZ. KV, AMM, AZJ, MW, MHG, SSY, SJW, MLM, AHE, NRC, TH, SSW, and RGZ designed the breast cancer study and developed the methodology. Data were provided by investigators from each participating cohort and harmonized by KV, AMM, AZJ, SSY, TH, TKM, SSW, and RGZ. The vitamin D assays were conducted in the laboratory of RLH. Epidemiologic and statistical analyses were conducted by MW, MHG, SSY, and TH with KV, AMM, AZJ, SSW, and RGZ providing input and interpretation. RGZ wrote the initial draft of the manuscript, with KV, AMM, AZJ, and SSW providing extensive input and review on multiple versions of the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

Competing interests

No authors have reported conflicts of interest related to the information presented in this manuscript.

Ethics approval and consent to participate

Each participating cohort and the BBC3 coordinating center received approval of this study from its institutional ethics review board.

was not related to subsequent incidence of invasive breast cancer over a broad range in vitamin D status.

Keywords

25-hydroxyvitamin D; biomarker; breast cancer; calibration; pooled analysis; prospective cohort study; vitamin D

Introduction

Breast cancer is the most common cancer among women, both in the U.S.(1) and worldwide. (2) The 2.3 million cases of breast cancer diagnosed globally in 2020 accounted for one in four of female cancer cases.(2) Breast cancer, already the leading cause of cancer mortality among women worldwide, recently became the leading cause among U.S. women.(2)

Compelling evidence from laboratory and animal research suggests that vitamin D can inhibit cancer initiation and progression by regulating multiple pathways, including differentiation, proliferation, invasion, apoptosis, angiogenesis, inflammation, and immunity. (3, 4) Many of these experimental models used mammary tissue or mammary tumors(3–5) and are thus directly relevant to human breast cancer. Vitamin D receptors and CYP27B1 and CYP24A1, the enzymes that synthesize and metabolize $1\alpha,25$ -dihydroxyvitamin D, the active form of vitamin D, have been identified in normal human breast tissue and in breast cancers.(5) In addition, vitamin D can modulate the influence of endogenous estrogen, an accepted breast cancer promoter, by decreasing aromatase expression and estrogen production in breast adipose tissue and downregulating estrogen receptor (ER)-alpha expression.(4) Circulating concentrations of 25(OH)D, the accepted measure of vitamin D status, can be increased with minimal risk by dietary supplements, fortified foods, oily fish consumption, and modest sunlight exposure. Therefore, it has been proposed that vitamin D offers a simple, effective strategy for breast cancer prevention.

However, evidence from epidemiologic research, both observational studies and clinical trials, for a role of vitamin D in breast cancer prevention has been inconclusive. Of the 12 cohort studies of prediagnostic circulating 25(OH)D and risk of breast cancer completed at the time we designed our pooling project, two had reported statistically significant inverse associations;(6, 7) five had reported statistically non-significant inverse associations; (8–12) two had reported statistically non-significant positive associations;(13, 14) and three had reported essentially null associations.(11, 15, 16) Importantly, these studies used different assays and laboratories to measure vitamin D, and vitamin D concentrations differ noticeably when different assay methods are employed.(17, 18) Therefore, these studies, and meta-analyses combining them,(19–21) were unable to explore the vitamin D-breast cancer relationship with a common metric.

In the Women's Health Initiative (WHI), which included the largest randomized, placebo-controlled trial of supplemental vitamin D completed to date, 400 IU of vitamin D₃ and 1000 mg of calcium daily did not lower breast cancer incidence.(22, 23) However, the low

vitamin D dose, limited adherence, and participants continuing their personal vitamin D supplements during the trial complicated interpretation of results.(22, 24)

In 2011, the Institute for Medicine (IOM; now the National Academy of Medicine) concluded that the evidence of vitamin D benefits for cancer prevention was too inconsistent and insufficient to inform the nutritional requirements for vitamin D, and more research was needed.(3, 25) Thus, IOM public health recommendations for vitamin D are based on bone health. In 2018, the World Cancer Research Fund/American Institute for Cancer Research reported that evidence about vitamin D and breast cancer risk was too limited to reach a conclusion.(26)

We have explored how circulating 25(OH)D is related to subsequent breast cancer incidence in 17 prospective cohorts participating in the international Circulating Biomarkers and Breast and Colorectal Cancer Consortium (BBC3). Participant-level data from each cohort were harmonized and pooled centrally. Circulating 25(OH)D was either newly measured using a single, widely accepted assay and laboratory or, if previously measured by the individual cohort, calibrated to this assay. Advantages of our approach include 1) a wide range in vitamin D status due to diversity across cohort populations, 2) calibration of all 25(OH)D data to a single metric, and 3) a sufficiently large sample size (almost 10,500 breast cancer cases and 13,000 controls) to examine risk in population subgroups and by tumor characteristics.

Methods

Study design

Our study population included 17 prospective cohorts in the U.S. and Europe (Table 1). (6–11, 13–16, 27–32) To participate, a cohort needed 100 women who developed breast cancer during follow-up and had either prediagnostic 25(OH)D measurements or stored prediagnostic blood samples available for 25(OH)D assays. Each cohort and the BBC3 coordinating center received approval from its institutional review board.

Cases were defined as women with no history of cancer at study entry who then developed primary invasive breast cancer (International Classification of Diseases [ICD] 9 174.0–174.9; ICD 10 C50.0–C50.9) during follow-up. Cases were identified by the individual cohorts using various combinations of methods depending on the cohort: linkage with cancer and/or mortality registries, review of medical and/or health insurance records, self-report, and follow-up with physicians or next-of-kin. Over 98% of cases were confirmed histologically.

Sixteen cohorts utilized a nested case-control design. One or two controls free of breast cancer at the time of diagnosis of the matched case were individually matched to cases on age at blood collection, usually date of blood collection, and other study-specific factors (Supplementary Table S1). In PLCO, non-cases were frequency matched to cases on age and date at blood collection.(15)

Measurement of circulating 25(OH)D

Our primary exposure was prediagnostic circulating 25(OH)D measured in plasma or serum. For the case and control(s) in each matched set, the blood samples used for vitamin D measurement were collected before the case was diagnosed with breast cancer. For the five breast cancer studies that had not previously quantified circulating 25(OH)D (Table 1), we measured 25(OH)D at Heartland Assays (Ames, IA) using a direct, competitive chemiluminescence immunoassay.(33, 34) For each of the 12 studies that had previously quantified 25(OH)D (Table 1), we calibrated its 25(OH)D measurements to the Heartland Assays chemiluminescence immunoassay to correct for assay and laboratory differences in 25(OH)D measurement. For each calibrated study, blood samples from 29 controls, selected uniformly across deciles of the 25(OH)D distribution in study controls, were re-assayed at Heartland Assays (Supplementary Methods). A study-specific M-estimation robust linear regression model(35) was used to convert its 25(OH)D data to the concentrations expected had the chemiluminescence immunoassay been used.(36, 37) Unless otherwise noted, we present calibrated 25(OH)D values for these 12 studies.

Two types of blinded quality controls were randomly inserted into each assay batch by the individual cohorts: 1) Standard Reference Materials with known 25(OH)D concentrations prepared by the National Institute of Standards and Technology (NIST) and 2) cohort-specific quality controls chosen by the cohort (Supplementary Methods). Coefficients of variation (CVs) for the chemiluminescence immunoassay, including within- and between-batch variability across both newly assayed and calibrated studies, were 13.4%, 7.8%, and 7.8%, respectively, for NIST reference materials at 17.7, 32.3, and 49.8 nmol/L concentrations. CV's, including within- and between-batch variability, ranged from 5.3% to 14.7% for the cohort-specific quality controls inserted into the newly assayed studies.

To adjust for seasonal variation in circulating 25(OH)D, we calculated a season-standardized 25(OH)D value for each participant, which represents her circulating 25(OH)D averaged over the entire year, according to the weekly 25(OH)D variation observed in her study population. For each study, we used sine-cosine functions and data from controls to model 25(OH)D concentrations as a function of week of blood collection.(37, 38) Then for each participant, the residual, i.e., the difference between her observed 25(OH)D concentration and the concentration predicted by the study-specific model for the week in which her blood was collected, was added to the predicted all-year-round mean 25(OH)D for the controls in her study. All 25(OH)D concentrations presented are season-standardized unless otherwise noted.

Harmonization of covariates and breast tumor characteristics

Each cohort provided participant-level data on demographic, lifestyle, menstrual, reproductive, and medical risk factors, based on questionnaires completed by study participants close to the time of blood collection. At the BBC3 coordinating center, covariates were harmonized using uniform definitions across studies.

Each cohort provided information on the tumor characteristics of its individual cases; these data were harmonized at the BBC3 coordinating center (Supplementary Methods).

Statistical analysis

We used a two-stage approach to estimate pooled incidence rate ratios, hereafter called RRs, in most analyses. Study-specific RRs for each nested case-control study were estimated by conditional logistic regression(39) and combined using random-effects models. (40) Heterogeneity in results across studies was evaluated with the Q statistic.(40, 41) For selected analyses, we aggregated the data from all studies into a single dataset (Supplementary Methods).

We modeled circulating 25(OH)D in three ways: 1) consortium-wide deciles based on the 25(OH)D distribution in controls from all studies combined, 2) categories suggested by the IOM recommendations for vitamin D(3), and 3) a continuous variable. All three approaches assume that 25(OH)D concentrations from the individual studies use the same metric, and thus require calibration of previously measured 25(OH)D data to the assay chosen for the newly measured studies. Wald tests for trend across consortium-wide deciles or IOM-based categories assigned the median 25(OH)D in controls to each decile or category. Prior to analyzing 25(OH)D on a continuous scale, we confirmed with restricted cubic spline analyses(42, 43) in the aggregated dataset that the 25(OH)D-breast cancer association was consistent with linearity (P -nonlinearity > 0.05).

For two-stage analyses by consortium-wide deciles, we selected decile 5 as the referent since it permitted us to estimate RRs at low and high concentrations and included cases and controls from each of the participating studies. In order to compare risk between extreme deciles, we combined all studies into a single, aggregated dataset since one study did not contribute cases or controls to decile 1.

Three models were used to control for confounding. Model 1 conditioned on study-specific matching factors, which included age at blood collection and usually date of blood collection (Supplementary Table S1). Alternatively, in unconditional models, age at blood collection, elapsed time from blood collection to assay, and for EPIC and MEC, study center were included as covariates in Model 1. Model 2 added to model 1 body mass index (BMI) and physical activity. For BMI, a variable combining BMI categories with menopausal status at date of blood collection was used because breast cancer risk is inversely associated with premenopausal BMI and positively associated with postmenopausal BMI.(44) Model 3, the model presented unless otherwise noted, was further adjusted for established breast cancer risk factors: race, family history of breast cancer, age at menarche, parity/age at first birth, oral contraceptive use, menopausal hormone therapy, and alcohol intake (Supplementary Methods).

We explored whether the association of circulating 25(OH)D with breast cancer risk varied across population subgroups or by tumor characteristics (Supplementary Methods).

All P values were based on two-sided tests and considered statistically significant if $P < 0.05$. Analyses were conducted using SAS version 9.4 (Cary, NC).

Results

Our study population included 10,484 female breast cancer cases and 12,953 controls from 10 US and 7 European cohorts (Table 1). Median (interdecile range) age was 57 (42–68) years at blood collection, and 63 (49–75) years at breast cancer diagnosis. Median (interdecile range) elapsed time from blood collection to diagnosis was 5.1 (1–13) years. 91% of participants were White.

For five studies, contributing 14% of participants (1595 cases, 1595 controls), circulating 25(OH)D was newly measured at Heartland Assays (Table 1). For the 12 calibrated studies, which had previously measured 25(OH)D, the change after calibration in median 25(OH)D concentrations among controls ranged from –31 nmol/L to +10 nmol/L (Figure 1). Vitamin D status, based on season-standardized, calibrated data, differed markedly among the participating cohorts, with median 25(OH)D concentrations increasing from 31 to 71 nmol/L across studies (Table 1; Figure 1).

Breast cancer risk by consortium-wide 25(OH)D deciles

Across consortium-wide deciles, 25(OH)D concentrations increased 4-fold, from a median of 22.6 nmol/L in decile 1 to 93.2 nmol/L in decile 10 (Table 2). Breast cancer risk in each decile was not statistically significantly different from that in decile 5 for all three models (RR range = 0.96–1.11; all *P*-trend = 0.20). Comparing women in the highest decile of circulating 25(OH)D to those in the lowest, the RR, fully adjusted for breast cancer risk factors, was 0.98 (95% CI: 0.85–1.12).

Confounding by breast cancer risk factors was minimal; results for the fully adjusted model (model 3) were very similar to those for the two simpler models (Table 2). No statistically significant differences in results were observed across the 17 studies (*P*-heterogeneity = 0.67 and 0.52 for deciles 1 and 10, respectively). Excluding one study at a time revealed that no single study substantially influenced the results (data not shown).

Breast cancer risk by 25(OH)D categories based on IOM clinical guidance

We examined how breast cancer risk was related to the vitamin D guidelines established by IOM.⁽³⁾ Relative to women with sufficient 25(OH)D (50–<75 nmol/L; 36% of consortium controls), RRs were 0.93 (95% CI: 0.83–1.04) for women with deficient levels (<30 nmol/L; 13%) and 1.03 (95% CI: 0.96–1.11) for insufficient levels (30–<50 nmol/L; 34%). For 25(OH)D concentrations beyond sufficient (>75 nmol/L; 16%), RR was 0.95 (95% CI: 0.87–1.03).

We also evaluated finer categories to assess breast cancer risk at very low and very high 25(OH)D concentrations (Figure 2; Supplementary Table S2). Compared with women with 25(OH)D in the lower range of sufficiency (50–<62.5 nmol/L; 21% of consortium controls), statistically non-significant RRs were noted for all 25(OH)D categories, including <20 nmol/L (3%), 100–<125 nmol/L (3%), and ≥125 nmol/L (0.7%). No statistically significant differences in results were observed across the 17 studies (*P*-heterogeneity = 0.57 and 0.25 for <20 and ≥125 nmol/L, respectively).

Breast cancer risk by continuous 25(OH)D

The RR for a 25 nmol/L increase in circulating 25(OH)D was 0.99 (95% CI: 0.95–1.03) (Table 2). Cohort-specific RRs ranged from 0.89 to 1.16, and none reached statistical significance (P -heterogeneity across the 17 studies = 0.63) (Figure 3).

Circulating 25(OH)D-breast cancer associations in population subgroups and by tumor characteristics

The essentially null association between circulating 25(OH)D, modeled on a continuous scale, and breast cancer risk did not vary across subgroups defined by BMI, physical activity, family history of breast cancer, menopausal hormone therapy, latitude, or geographic region; all P -interaction > 0.05 (Figure 4). In addition, results were not statistically significantly different for participants donating blood in the winter vs. summer (P -interaction = 0.83) or for studies in which 25(OH)D was newly measured vs. calibrated (P -interaction = 0.94). Differences by race were not statistically significant (P -heterogeneity = 0.90). For a 25 nmol/L increase in 25(OH)D, RR was 0.98 (95% CI: 0.95–1.02) in Whites (9,579 cases); 1.28 (95% CI: 0.99–1.65) in Blacks (290 cases); and 1.13 (95% CI: 0.76–1.68) in Asians (275 cases).

Substantial numbers of breast cancer subtypes were available for analysis. For example, among the 9,787 breast cancer cases with morphology data (93% of all cases), 7,148 (73%) were ductal only and 1,179 (12%) were lobular only. Of the 8,788 cases with ER data (84% of all cases), 6,949 (79%) were ER-positive (ER+); of the 8,295 cases with PR data (79% of all cases), 5,444 (66%) were PR-positive (PR+). No statistically significant associations with circulating 25(OH)D, modelled on a continuous scale, were observed for any of the tumor subtypes we examined, including those defined by stage, grade, morphology, receptor status, or molecular features(45) (Figure 4; Supplementary Methods). However, a 17% reduction in risk (RR per 25 nmol/L increase = 0.83; 95% CI: 0.68–1.02) was noted for distant disease (stage 4; 319 cases); and an 11% reduction (RR = 0.89; 95% CI: 0.72–1.08), for triple negative disease (ER–/progesterone receptor [PR]–/human epidermal growth factor 2 [HER2]–; 365 cases). Exclusion of cases diagnosed 2 years after blood collection and their matched controls did not attenuate either association (distant disease: RR = 0.77; 95% CI: 0.60–0.98; 269 cases; triple negative disease: RR = 0.87; 95% CI: 0.70–1.08; 310 cases). These two suggestive inverse associations were independent, as only 2% of the women with triple negative disease also presented with distant disease. Of note, risk was not reduced for ER–/PR– or ER–/PR–/HER2+ (HER2-enriched) disease.

We did not observe differences by age at diagnosis (P -heterogeneity = 0.13) (Figure 4). We then evaluated risk of early onset breast cancer (diagnosed <50 y; 1,212 cases; 12%) and late onset breast cancer (diagnosed 75 y; 1,045 cases; 10%) across consortium-wide 25(OH)D deciles. For each subtype, none of the RRs across deciles, relative to decile 5, were statistically significant (data not shown); P -trend was 0.77 for early onset and 0.19 for late onset disease.

Breast cancer risk was not related to elapsed time from blood collection to diagnosis (Figure 4), which suggests that preclinical disease did not influence our findings.

Influence of the season of blood collection

To extend our analysis of the influence of season of blood collection, we explored the 25(OH)D-breast cancer relationship among women who donated blood in winter (defined as weeks 2–14) or summer (defined as weeks 28–40) (Supplementary Methods) using season-specific consortium-wide deciles of circulating 25(OH)D (Figure 5). Median (interdecile range) 25(OH)D was 45 (22–75) and 61 (35–94) nmol/L among controls donating in winter and summer, respectively (Figure 5). There was no apparent change in breast cancer risk over the 25(OH)D ranges observed in each season (P -trend = 0.72 and 0.95 for winter and summer ranges, respectively).

Sensitivity analyses

Results were similar using a two-stage approach with either random-effects or fixed-effects models in the second stage and using an aggregated dataset (Supplementary Table S3). Finer control of BMI for all studies and of physical activity for the 11 studies with sufficiently detailed information did not noticeably change RRs (data not shown). To minimize including women with preclinical disease, we excluded cases diagnosed within the first two or five years after blood collection (2,049 and 5,124 cases, respectively) and their matched controls; our results were essentially unchanged (data not shown). All sensitivity analyses were performed for 25(OH)D modeled as consortium-wide deciles, IOM-based categories, and a continuous measure.

Discussion

Across a wide range in vitamin D status, we saw no evidence that circulating 25(OH)D concentrations were associated with subsequent incidence of invasive breast cancer overall. Breast cancer risk did not differ across consortium-wide deciles of 25(OH)D, even though median 25(OH)D concentrations quadrupled from decile 1 (22.6 nmol/L) to decile 10 (93.2 nmol/L). Relative to women with sufficient vitamin D (50–<75 nmol/L), based on current IOM guidance,(3, 25), breast cancer risk was not different in women considered deficient (<30 nmol/L) or insufficient (30–<50 nmol/L). Even at high circulating 25(OH)D (100–<125 nmol/L and 125 nmol/L), risk was unchanged. Breast cancer risk for a 25 nmol/L increase in 25(OH)D was 0.99 (95% CI: 0.95–1.03).

Although the participating cohorts differed by geography, demographics, lifestyle, and diet, results were generally similar across the 17 cohorts. There was no evidence of statistically significant heterogeneity in the results across the studies whether 25(OH)D was modeled as consortium-wide deciles, IOM-based cutpoints, or a continuous variable. In addition, eliminating any single study did not substantially alter results.

Several prospective studies of circulating 25(OH)D and breast cancer did not find statistically significant associations overall but noted relationships limited to subgroups. Statistically significant inverse associations have been reported in younger women,(7, 11) for premenopausal breast cancer,(46) in menopausal hormone therapy users,(14) in leaner women,(47) and at higher latitudes(16) while statistically significant positive associations have been reported in heavier women.(13, 14, 47) By calibrating 25(OH)D data from

multiple studies to a single, widely accepted assay and harmonizing and pooling covariate and outcome data, we could generate more robust RR estimates for population subgroups than individual studies. With almost 10,500 breast cancer cases, more than seven times the number included in the largest study of prediagnostic 25(OH)D and breast cancer published to date,(48) we found null associations across all the subgroups we examined, including those defined by BMI, latitude, and season of blood collection. However, with <300 cases among Blacks or Asians, we had limited power to evaluate risk by race.

Similarly, most studies of circulating 25(OH)D and breast cancer have had too few cases to generate robust RR estimates by tumor characteristics. We found no statistically significant associations by stage, grade, morphology, receptor status, or age at onset. However, we did observe suggestive, independent inverse associations for two breast cancer subtypes with poor prognoses: distant disease (319 cases) and triple negative disease (365 cases). It is unlikely that preclinical disease underlies these findings since each persisted when cases diagnosed 2 years after blood collection and their matched controls were excluded from analysis.

Circulating 25(OH)D fluctuates seasonally since sunlight stimulates its endogenous production. Among consortium controls donating blood in the winter, 24% were deficient (<30 nmol/L) for vitamin D, but only 6% of controls donating in the summer were deficient. It has been hypothesized that women whose circulating 25(OH)D drops to especially low levels in the winter or whose 25(OH)D fails to rise sufficiently in the summer to replenish adipose tissue stores might be at increased breast cancer risk. Two cohorts in our consortium have reported season-specific effects. In NHS, 25(OH)D measured in summer samples (collected May–October) was statistically significantly inversely associated with breast cancer risk, but winter levels (November–April) were not.(48) Similar, but statistically non-significant, findings were reported by NYU WHS.(11) However, in our season-specific analyses, we saw no evidence that 25(OH)D measured during the summer or winter was related to breast cancer risk.

At its initiation, our consortium included nearly all completed prospective studies of circulating 25(OH)D and invasive breast cancer risk (12 cohorts, 8,889 cases), and then increased case numbers by 18% by assaying 25(OH)D in five additional cohorts (1,595 cases). Of the recently published prospective studies of invasive breast cancer not included in our pooling project, six, conducted in Germany (137 cases),(49) Denmark (159 cases), (50) France (233 cases),(47) Japan (239 cases),(46) southeastern Australia (634 cases),(51) and Turkey (57 cases),(52) have reported null associations, consistent with our findings. Three, two in the US (1611(53) and 77(54) cases) and one in western Australia (113 cases),(55) have reported statistically significant inverse associations; and one, in Europe (378 cases),(56) a statistically significant positive association. The largest study to date of prediagnostic circulating 25(OH)D and risk of *in situ* ductal breast cancer (UK; 1340 cases) (57) found no relationship, similar to our study of invasive disease. None of these recent studies have the range in vitamin D status achieved by our international pooling project. In addition, the number of invasive breast cancer cases in all the recent studies combined is less than 35% of the cases included in our pooling project.

Although a number of randomized, placebo-controlled trials of vitamin D supplementation have been published,(58–60) few were sufficiently large to evaluate breast cancer incidence. The Vitamin D and Omega-3 Trial (VITAL) is the largest and longest randomized, placebo-controlled trial of moderate-to-high dose vitamin D supplementation to date.(61) More than 13,000 women, 55 years, were given 2000 IU/day of supplemental vitamin D₃ (cholecalciferol) or placebo and followed for a median of 5.3 years. No statistically significant differences between groups were observed for invasive breast cancer (hazard ratio [HR] = 1.02; 95% CI: 0.79–1.31). Our null results for invasive breast cancer overall concur with and extend these findings. VITAL had limited power to examine breast cancer, with only 246 invasive cases(61) and 10 advanced (metastatic or fatal) cases(62). Our pooling project, with much larger numbers of breast cancers, had more statistical power to explore breast cancer risk overall, by tumor characteristics, and in population subgroups. For example, VITAL noted that vitamin D statistically significantly reduced the incidence of all cancers combined in normal-weight individuals (BMI < 25 kg/m²). (59, 61) However, in our analyses of breast cancer, we found no interaction with BMI.

In our pooling project, we were able to evaluate lower 25(OH)D concentrations than VITAL. Only 13% of VITAL participants had a baseline 25(OH)D <50 nmol/L,(61) the level required for vitamin D sufficiency by IOM guidelines, whereas 48% of our consortium controls were below sufficiency. Conversely, the 25(OH)D concentrations attained through supplementation in VITAL, averaging 102.5 nmol/L,(59) were somewhat higher than in our consortium. Less than 4% of our controls had 25(OH)D concentrations 100 nmol/L.

Recently VITAL reported that advanced cancers (metastatic or fatal) were statistically significantly reduced among those randomized to vitamin D compared to placebo (HR = 0.83; 95% CI: 0.69–0.99),(62) even though all invasive cancers combined were not statistically significantly reduced. Our suggestive protective effects for distant and triple negative breast cancer, subtypes with poor prognoses, concur with these VITAL results.

Our generally null results also agree with those of the WHI vitamin D-calcium trial, the largest trial of low-dose vitamin D supplementation.(22) Over 36,000 postmenopausal women were given 400 IU of vitamin D₃ plus 1000 mg of calcium daily or placebo for a mean of 7.0 years. No statistically significant differences between groups were observed for invasive breast cancer at the end of the trial (HR = 0.96; 95% CI: 0.85–1.09; 1,074 cases)(22) or after five additional years of followup (HR = 1.04; 95% CI: 0.94–1.14; 1,667 cases).(23)

A critical strength of our pooling project is how we addressed the challenges that arise when combining circulating biomarker data from multiple studies. There are often substantial differences in accuracy among assays and laboratories in measuring 25(OH)D(17, 18), as well as other circulating biomarkers. Calibrating previously measured 25(OH)D readings to the widely accepted assay and laboratory chosen for our new 25(OH)D measurements enabled us to use absolute 25(OH)D concentrations, rather than study-specific quantiles, as our metric and confidently estimate RR across the wide range of vitamin D exposure provided by our 17 international cohorts. In addition, to adjust for blood samples being collected throughout the year, we standardized the 25(OH)D value for each woman to

her estimated all-year-round average 25(OH)D, based on models of the weekly 25(OH)D changes in her own cohort.

Additional strengths of our study include its size and scope --- almost 10,500 breast cancer cases and 13,000 matched controls from 17 international prospective cohorts. Followup was longer than in most randomized trials. Across our studies, median time from blood collection to diagnosis ranged from 1.7 to 12.4 years, and exceeded 5 years in nine studies. Only studies that collected blood samples before breast cancer diagnosis were included, which reduces the possibility that disease, its diagnosis, or its treatment altered circulating 25(OH)D. We harmonized participant-level data on exposures and outcomes and used a common statistical methodology for analyses. This approach reduces potential heterogeneity across individual studies and facilitates better control for confounding than meta-analyses that combine results, not participant-level data, from published studies. Of note, our consortium's study of circulating 25(OH)D and colorectal cancer, which with 5,706 cases was approximately half the size of our breast cancer study, found a statistically significant inverse association overall, as well as inverse associations in all subgroups.(63) Since the two studies shared the same methodology, same assay, and many of the same cohorts, this comparison suggests our breast cancer study would have seen an association with 25(OH)D if one existed.

We also considered potential limitations of our study. Excess weight and physical inactivity are breast cancer risk factors and inversely correlated with circulating 25(OH)D.(64) Adjustment for both variables did not noticeably change our results, nor did adjustment for other breast cancer risk factors. Although we cannot completely eliminate the possibility of residual confounding, the minimal confounding observed increases our confidence in the validity of our findings. In addition, our results are based on a single blood collection, at a median of 5.1 years before diagnosis, which is an imperfect measure of long-term vitamin D status. However, studies of repeat measures of 25(OH)D have reported relatively stable readings over time, with intraclass correlation coefficients of 0.7 for blood samples collected 3–5 years apart and of 0.5–0.6 for samples collected approximately 10 years apart.(11, 48, 65, 66) Finally, we had limited power to examine associations in non-White populations.

In summary, in our large international cohort consortium, circulating 25(OH)D, measured comparably in 17 cohorts by calibrating to a single assay, was not related to subsequent incidence of invasive breast cancer across a wide range of concentrations (<20 to 100 nmol/L). No statistically significant associations were noted within population subgroups, by tumor characteristics or molecular subtypes, or for winter or summer measurements. Suggestive inverse associations with both triple negative and distant breast cancer, subtypes with poor prognoses, merit further investigation. Our results do not support adoption of routine vitamin D measurement or treatment of low 25(OH)D levels for breast cancer prevention.

Supplementary Material

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Authors

Kala Visvanathan^{1,a}, Alison M. Mondul^{2,a}, Anne Zeleniuch-Jacquotte^{3,a}, Molin Wang^{4,5,6}, Mitchell H. Gail⁷, Shiaw-Shyuan Yaun⁸, Stephanie J. Weinstein⁷, Marjorie L. McCullough⁹, A. Heather Eliassen^{5,6,8}, Nancy R. Cook^{5,10}, Claudia Agnoli¹¹, Martin Almquist¹², Amanda Black⁷, Julie E. Buring^{5,10}, Chu Chen¹³, Yu Chen³, Tess Clendenen³, Laure Dossus¹⁴, Veronika Fedirko¹⁵, Gretchen L. Gierach⁷, Edward L. Giovannucci^{5,6,8}, Gary E. Goodman¹³, Marc T. Goodman¹⁶, Pascal Guénel¹⁷, Göran Hallmans¹⁸, Susan E. Hankinson¹⁹, Ronald L. Horst^{20,b}, Tao Hou⁸, Wen-Yi Huang⁷, Michael E. Jones²¹, Corrine E. Joshu¹, Rudolf Kaaks²², Vittorio Krogh¹¹, Tilman Kühn^{22,23}, Marina Kvaskoff¹⁷, I-Min Lee^{5,10}, Yahya Mahamat-Saleh¹⁷, Johan Malm²⁴, Jonas Manjer²⁵, Gertraud Maskarinec²⁶, Amy E. Millen²⁷, Toqir K. Mukhtar^{28,29}, Marian L. Neuhouser¹³, Trude E. Robsahm³⁰, Minouk J. Schoemaker²¹, Sabina Sieri¹¹, Malin Sund³¹, Anthony J. Swerdlow^{21,32}, Cynthia A. Thomson³³, Giske Ursin^{30,34}, Jean Wactawski-Wende²⁷, Ying Wang⁹, Lynne R. Wilkens³⁵, Yujie Wu⁴, Emilie Zoltick³⁶, Walter C. Willett^{5,8}, Stephanie A. Smith-Warner^{5,8,c}, Regina G. Ziegler^{7,c}

Affiliations

1. Departments of Epidemiology and Oncology, Johns Hopkins Bloomberg School of Public Health and Kimmel Cancer Center, Baltimore, MD, USA.
2. Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, MI, USA.
3. Departments of Population Health and Environmental Medicine, New York University Grossman School of Medicine, New York, NY, USA.
4. Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
5. Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
6. Channing Division of Network Medicine, Department of Medicine, Harvard Medical School, Boston, MA, USA.
7. Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA.
8. Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA, USA.
9. Department of Population Science, American Cancer Society, Atlanta, GA, USA.
10. Division of Preventive Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA.
11. Epidemiology and Prevention Unit, IRCCS National Cancer Institute Foundation, Milan, Italy.
12. Department of Surgery, Skane University Hospital, Lund, Sweden.

- ¹³Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, USA.
- ¹⁴Nutrition and Metabolism Branch, International Agency for Research on Cancer, World Health Organization, Lyon, France.
- ¹⁵Department of Epidemiology, Rollins School of Public Health and Winship Cancer Institute, Emory University, Atlanta, GA, USA.
- ¹⁶Cancer Prevention and Control Research Program, Cedars Sinai Samuel Oschin Comprehensive Cancer Institute, Los Angeles, CA, USA.
- ¹⁷Center for Research in Epidemiology and Population Health (CESP), French National Institute of Health and Medical Research (INSERM), University Paris-Saclay, Villejuif, France.
- ¹⁸Department of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden.
- ¹⁹Department of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts Amherst, Amherst, MA, USA.
- ²⁰Heartland Assays, LLC, Ames, IA, USA.
- ²¹Division of Genetics and Epidemiology, The Institute of Cancer Research, London, UK.
- ²²Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany.
- ²³Institute for Global Food Security, Queen's University, Belfast, Northern Ireland.
- ²⁴Department of Translational Medicine, Lund University, Malmö, Sweden.
- ²⁵Department of Surgery, Skane University Hospital, Lund University, Malmö, Sweden.
- ²⁶Cancer Epidemiology Program, University of Hawai'i Cancer Center, Honolulu, HI, USA.
- ²⁷Department of Epidemiology and Environmental Health, University at Buffalo, Buffalo, NY, USA.
- ²⁸Department of Primary Care and Public Health, Imperial College, London, UK.
- ²⁹Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK.
- ³⁰Cancer Registry of Norway, Institute of Population-Based Cancer Research, Oslo, Norway.
- ³¹Department of Surgical and Perioperative Sciences, Umeå University, Umeå, Sweden.
- ³²Division of Breast Cancer Research, The Institute of Cancer Research, London, UK.

³³.Department of Health Promotion Sciences, Mel and Enid Zuckerman College of Public Health, University of Arizona and University of Arizona Cancer Center, Tucson, AZ, USA.

³⁴.Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway.

³⁵.Population Sciences in the Pacific, University of Hawai'i Cancer Center, Honolulu, HI, USA.

³⁶.Department of Population Medicine, Harvard Medical School, Boston, MA, USA.

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Data availability

The data described in the manuscript will not be made available because we do not have permission to disclose or release the data from the participating cohorts, as specified in their executed data use agreements.

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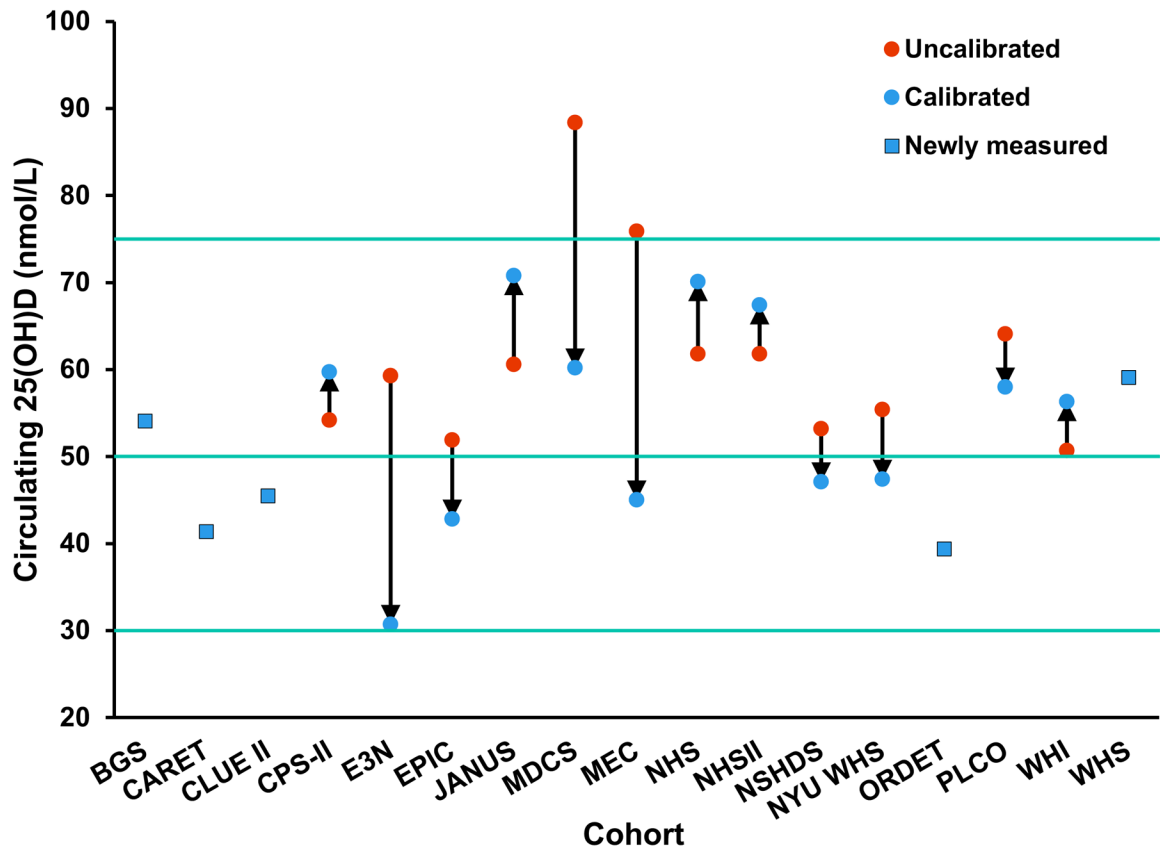


Fig. 1. Median circulating 25(OH)D concentrations among controls for the 12 calibrated studies, before and after calibration, and for the five newly measured studies. For the calibrated studies, median 25(OH)D before calibration is indicated by red circles and after calibration by blue circles. For the newly measured studies, median 25(OH)D is indicated by blue squares. All 25(OH)D concentrations are season-standardized. The relative change after calibration is +10% for CPS-II, -47% for E3N, -18% for EPIC, +16% for JANUS, -31% for MDCS, -41% for MEC, +14% for NHS, +9% for NHSII, -11% for NSHDS, -14% for NYU WHS, -9% for PLCO, and +11% for WHI. Horizontal green lines indicate IOM-based clinical guidance for circulating 25(OH)D: deficiency at <math>< 30</math> nmol/L, insufficiency at 30-<math>< 50</math> nmol/L, sufficiency at 50-<math>< 75</math> nmol/L, beyond sufficiency at 75 nmol/L. The full names of the 17 cohorts are given in Table 1. 25(OH)D: 25-hydroxyvitamin D; IOM: Institute of Medicine.

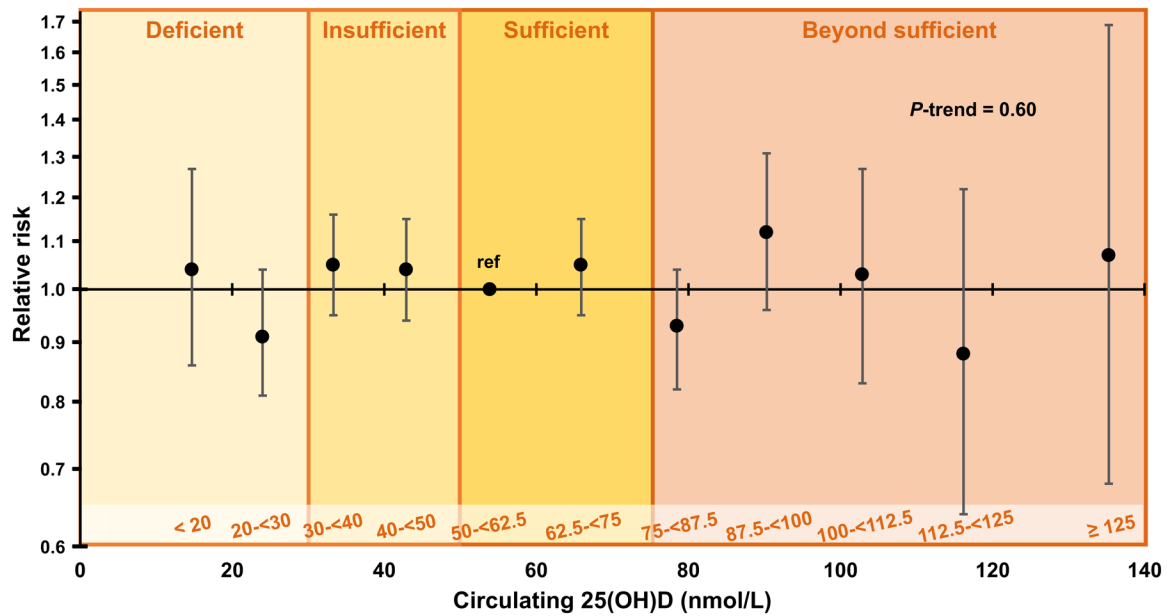


Fig 2.

RRs of breast cancer by 25(OH)D categories based on IOM clinical guidance for vitamin D. RRs (solid circles) of breast cancer and 95% CIs (vertical lines) are plotted on a log scale for 11 categories of circulating 25(OH)D. These 25(OH)D categories expand those established by the IOM. The categories $<$ 20 (3.1% of controls) and 20- $<$ 30 (10.4%) nmol/L are considered deficient; 30- $<$ 40 (15.7%) and 40- $<$ 50 (18.5%) nmol/L are considered insufficient; 50- $<$ 62.5 (21.2%) (the referent) and 62.5- $<$ 75 (14.9%) nmol/L are considered sufficient; and 75- $<$ 87.5 (9.0%), 87.5- $<$ 100 (3.9%), 100- $<$ 112.5 (1.8%), 112.5- $<$ 125 (0.9%), and \geq 125 nmol/L (0.7%) are considered beyond sufficient. The RR and 95% CI for each category are plotted at the median concentration of 25(OH)D among controls in that category. The model used, model 3, conditioned on study-specific matching factors, which included age at blood collection and usually date of blood collection, and adjusted for BMI (using a variable combining BMI and menopausal status at blood collection), physical activity, race, family history of breast cancer, age at menarche, parity and age at first birth, oral contraceptive use, menopausal hormone therapy, and alcohol intake. The *P*-trend across categories was calculated with a Wald test that assigned the median 25(OH)D in controls to each category. 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CI: confidence interval; IOM: Institute of Medicine; ref: referent; RR: relative risk.

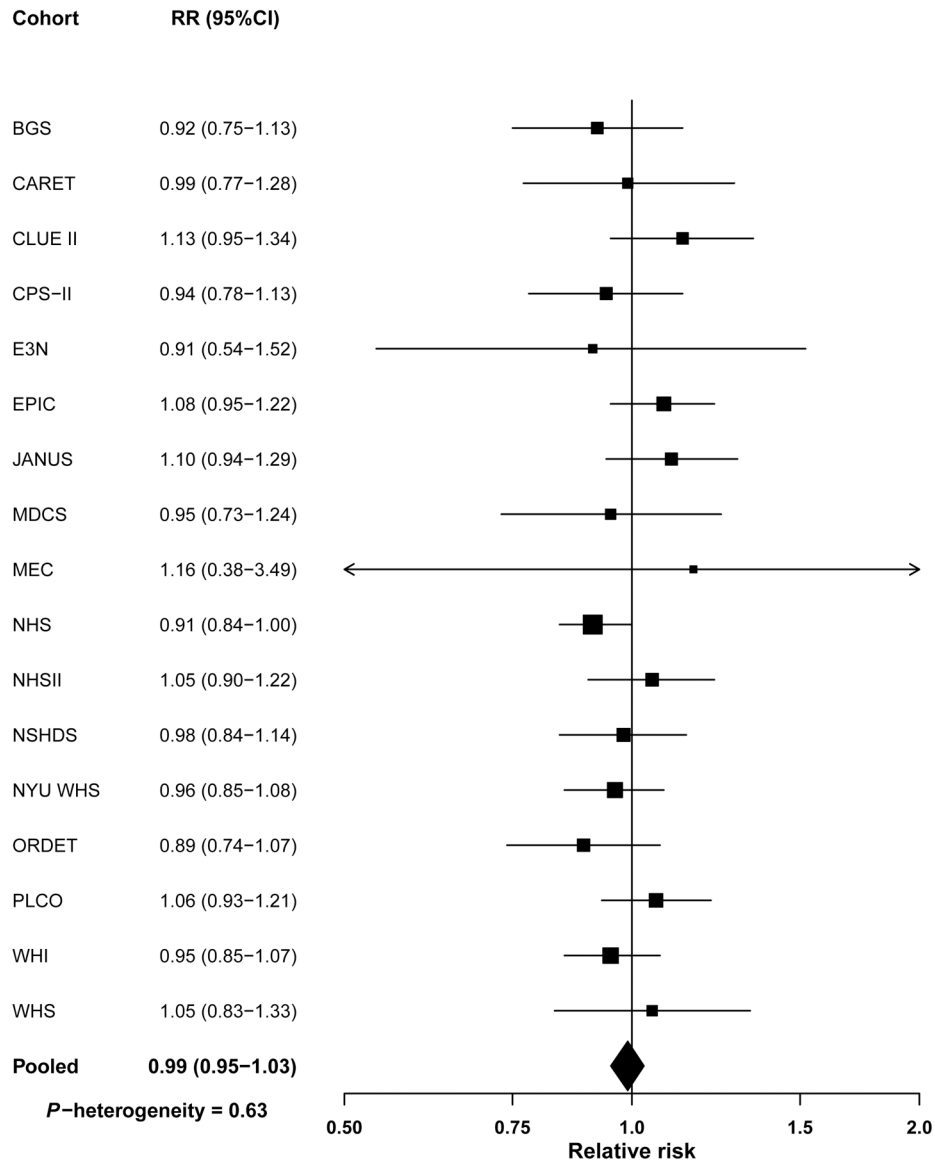


Fig 3. Cohort-specific and pooled RRs of breast cancer per 25 nmol/L increase in circulating 25(OH)D. Cohort-specific RRs of breast cancer per 25 nmol/L increase in 25(OH)D are plotted on a log scale and indicated by solid squares with the size proportional to the cohort’s contribution to the pooled RR. Horizontal lines indicate 95% CIs. The pooled RR is indicated by a solid diamond. A RR of 1.0, marked by the vertical line, indicates no association. The model used, model 3, conditioned on study-specific matching factors, which included age at blood collection and usually date of blood collection, and adjusted for BMI (using a variable combining BMI and menopausal status at blood collection), physical activity, race, family history of breast cancer, age at menarche, parity and age at first birth, oral contraceptive use, menopausal hormone therapy, and alcohol intake. The P heterogeneity *in* the results across cohorts was calculated using the Q statistic. The full

names of the 17 cohorts are given in Table 1. 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CI: confidence interval; ref: referent; RR: relative risk.

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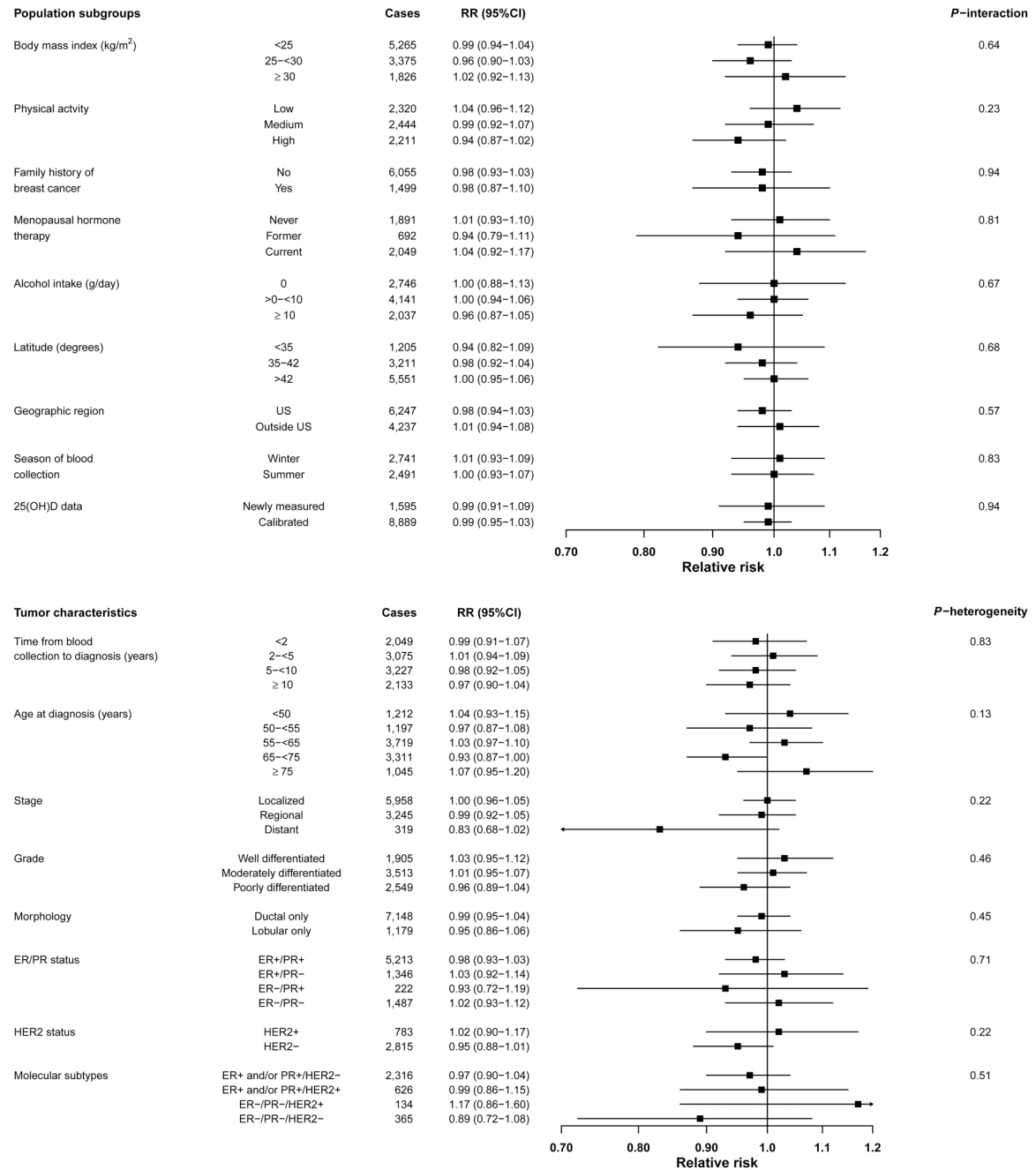


Fig. 4. RRs of breast cancer per 25 nmol/L increase in circulating 25(OH)D in population subgroups and by tumor characteristics. RRs (solid squares) of breast cancer and 95% CIs (horizontal lines) per 25 nmol/L increase in 25(OH)D are plotted on a log scale. A RR of 1.0, marked by the vertical line, indicates no association. Differences in RR by participant characteristics, such as breast cancer risk factors, latitude, and season of blood collection, were examined using a two-stage approach and unconditional logistic regression. Differences in RR by study characteristics, such as geographic region and calibration (newly measured vs. calibrated 25(OH)D data), were examined using a two-stage

approach and conditional logistic regression. Differences in associations by participant and study characteristics were tested for statistical significance using meta-regression. Heterogeneity by time elapsed from blood collection to diagnosis, age at diagnosis, and tumor characteristics were assessed in an aggregated dataset with conditional analyses, and tested for statistical significance with a contrast test. Conditional models were conditioned on study-specific matching factors, which included age at blood collection and usually date of blood collection; while unconditional models included as covariates age at blood collection, elapsed time from blood collection to assay, and study center (EPIC and MEC only). In addition, all models were further adjusted for BMI (using a variable combining BMI and menopausal status at blood collection), physical activity, race, family history of breast cancer, age at menarche, parity and age at first birth, oral contraceptive use, menopausal hormone therapy, and alcohol intake. Analyses by use of menopausal hormone therapy were restricted to postmenopausal and perimenopausal women. For analyses stratified by season of blood collection, winter was defined as weeks 2–14; summer as weeks 28–40; and circulating 25(OH)D was not season-standardized. In the analyses by molecular subtype, ER+ and/or PR+/HER2- tumors are considered luminal A-like, ER+ and/or PR+/HER2+ tumors are considered luminal B-like, ER-/PR-/HER2+ tumors are considered HER2-enriched, and ER-/PR-/HER2- tumors are considered triple negative. Additional information on how we defined and harmonized breast tumor characteristics is presented in Supplementary Methods. 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CI: confidence interval; ER: estrogen receptor; HER2: human epidermal growth factor receptor 2; PR: progesterone receptor; ref: referent; RR: relative risk; +; positive; -: negative.

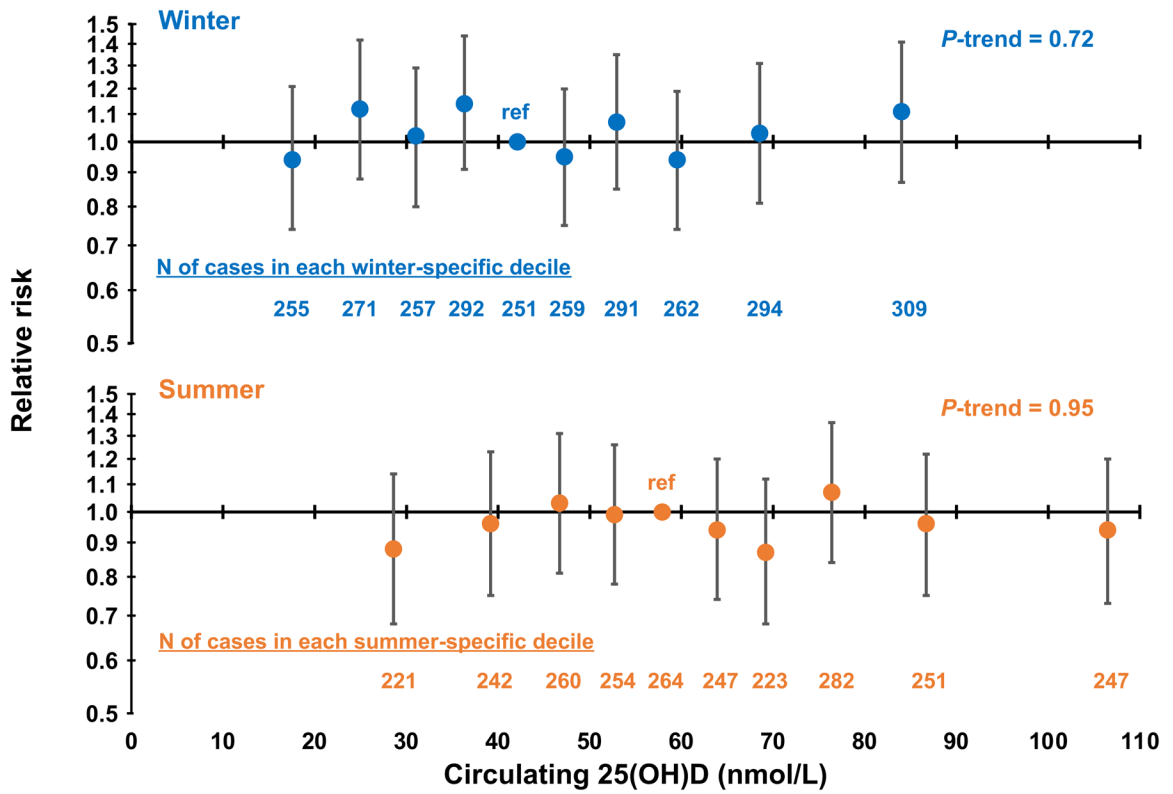


Fig 5. RRs of breast cancer by season-specific deciles of circulating 25(OH)D. RRs (solid circles) of breast cancer and 95% CIs (vertical lines) are plotted on a log scale by winter-specific and summer-specific, consortium-wide deciles of circulating 25(OH)D. Results for the participants who donated blood in the winter (defined as weeks 2–14; 2,741 cases) are shown in blue; results for those who donated in the summer (defined as weeks 28–40; 2,491 cases) are shown in orange. Participants donating blood in the spring and fall were excluded from these analyses. 25(OH)D concentrations were not season-standardized. Decile cutpoints, based on the 25(OH)D distributions in controls, were for winter <22, 22-<28, 28-<34, 34-<39, 39-<45, 45-<50, 50-<56, 56-<63, 63-<75, and 75 nmol/L and for summer <35, 35-<43, 43-<50, 50-<55, 55-<61, 61-<67, 67-<72, 72-<81, 81-<94, and 94 nmol/L. The RR and 95% CI for each decile was plotted at the median 25(OH)D concentration among the controls in that decile. Unconditional logistic regression models in an aggregated dataset were adjusted for study, age at blood collection, elapsed time from blood collection to assay, study center (EPIC and MEC only), BMI (using a variable combining BMI and menopausal status at blood collection), physical activity, race, family history of breast cancer, age at menarche, parity and age at first birth, oral contraceptive use, menopausal hormone therapy, and alcohol intake. The *P*-trend across categories was based on a Wald test that assigned the median 25(OH)D in controls to each category. 25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CI: confidence interval; N: number; ref: referent.

Table 1
Descriptive characteristics of studies participating in pooled analyses of circulating 25(OH)D and breast cancer

Cohort	Country or continent	Breast cancer cases / controls	25(OH)D data ^a	Median (10%–90%) season-standardized 25(OH)D in controls (nmol/L) ^b	Median (10%–90%) age at blood collection (y) ^c	Years of blood collection ^c	Median (10%–90%) time from blood collection to diagnosis (y)
<i>U.S.</i>							
Beta-Carotene and Retinol Efficacy Trial (CARET)	U.S.	195 / 195	New	41 (19–73)	60 (54–68)	1987–1996	5.5 (1.2–10.3)
CLUE II: Campaign Against Cancer and Heart Disease (CLUE II)	U.S.	447 / 447	New	46 (23–74)	55 (37–71)	1989	9.6 (2.1–17.0)
Cancer Prevention Study-II (CPS-II)	U.S.	413 / 413	Calibrated	60 (33–88)	70 (37–71)	1998–2001	2.3 (0.6–4.9)
Multithemic Cohort Study (MEC)	U.S.	556 / 556	Calibrated	45 (34–58)	67 (58–79)	1994–2006	3.2 (0.6–6.4)
Nurses' Health Study (NHS)	U.S.	1,368 / 1,368	Calibrated	70 (43–101)	57 (47–66)	1989–1991	7.4 (1.8–15.8)
Nurses' Health Study II (NHSII)	U.S.	415 / 824	Calibrated	67 (42–96)	45 (38–50)	1996–1999	4.6 (0.9–8.4)
New York University Women's Health Study (NYU WHS)	U.S.	893 / 1,642	Calibrated	47 (25–73)	52 (39–63)	1985–1991	11.5 (3.7–17.9)
Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial (PLCO)	U.S.	761 / 975	Calibrated	58 (34–82)	63 (57–70)	1993–2001	3.6 (0.9–7.3)
Women's Health Initiative (WHI) ^d	U.S.	1,003 / 1,003	Calibrated	56 (31–86)	63 (55–73)	1994–2009	3.6 (0.8–6.8)
Women's Health Study (WHS)	U.S.	196 / 196	New	59 (34–92)	49 (46–53)	1992–1995	6.0 (1.4–9.2)
<i>Outside U.S.</i>							
Breakthrough Generations Study (BGS) ^e	U.K.	311 / 311	New	54 (30–82)	58 (48–68)	2004–2009	1.7 (0.4–3.6)
Etude Epidémiologique auprès de femmes de l'Education Nationale (E3N)	France	582 / 1,074	Calibrated	31 (20–47)	56 (49–66)	1994–1999	3.8 (0.8–7.1)
European Investigation into Cancer and Nutrition (EPIC)	Europe	1,229 / 1,229	Calibrated	43 (23–68)	50 (39–61)	1992–2002	4.5 (1.1–8.6)
JANUS Serum Bank (JANUS)	Norway	388 / 388	Calibrated	71 (42–103)	41 (39–52)	1973–2002	12.4 (3.6–20.3)
Malmö Diet and Cancer Study (MDCS)	Sweden	590 / 590	Calibrated	60 (47–77)	56 (48–66)	1991–1996	7.2 (1.8–12.1)
Northern Sweden Health and Disease Study (NSHDS)	Sweden	691 / 1,296	Calibrated	47 (29–70)	56 (50–67)	1995–2006	6.1 (0.8–11.9)
Hormones and Diet in the Etiology of Breast Cancer (ORDET)	Italy	446 / 446	New	39 (24–66)	49 (39–62)	1987–1992	9.1 (2.0–15.3)
<i>Total</i>		10,484 / 12,953		51 (27–83) ^f	57 (42–68)	1973–2009	5.1 (1.1–13.4)

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25(OH)D: 25-hydroxyvitamin D.

^dNew studies: Circulating 25(OH)D concentrations were measured in cases and controls using a direct, competitive chemiluminescence immunoassay at Heartland Assays (Ames, IA) between November 2011 and April 2013. Calibrated studies: In these studies, circulating 25(OH)D concentrations had been previously measured and needed to be calibrated. For each study, blood samples from 29 controls selected uniformly across deciles of the 25(OH)D distribution in the study were re-assayed during 2011–2013 with the same assay and in the same laboratory used for the new studies.

^eIncludes newly measured and calibrated circulating 25(OH)D data. To convert circulating 25(OH)D from nmol/L to ng/mL, divide values by 2.496.

^cFor cases and controls combined.

^dWHI randomized trial of calcium and vitamin D.

^eNow called Generations Study.

^fFor all cases combined, median (interdecile range) circulating 25(OH)D was 52 (26–84) nmol/L.

Table 2
RRs of breast cancer by consortium-wide 25(OH)D deciles and continuous 25(OH)D

<i>Deciles of 25(OH)D (nmol/L)</i> ^a	Cases / controls	Median 25(OH)D in controls (nmol/L)	RR (95% CI)	Model 1 ^b	Model 2 ^c	Model 3 ^d
<27	976 / 1,295	22.6		1.04 (0.91–1.20)	1.02 (0.88–1.17)	1.03 (0.89–1.19)
27 - <34	978 / 1,296	31.0		1.02 (0.89–1.16)	1.01 (0.88–1.15)	1.01 (0.89–1.16)
34 - <40	1,065 / 1,295	37.5		1.10 (0.97–1.24)	1.09 (0.96–1.23)	1.11 (0.98–1.26)
40 - <46	1,027 / 1,295	43.0		1.02 (0.90–1.14)	1.01 (0.89–1.14)	1.01 (0.89–1.14)
46 - <51	1,036 / 1,295	48.5		1.0 (ref) ^e	1.0 (ref) ^e	1.0 (ref) ^e
51 - <57	1,078 / 1,296	54.0		1.04 (0.92–1.17)	1.04 (0.93–1.18)	1.04 (0.91–1.18)
57 - <63	1,105 / 1,295	60.1		1.05 (0.93–1.18)	1.08 (0.96–1.22)	1.11 (0.98–1.26)
63 - <71	1,096 / 1,295	66.9		1.00 (0.88–1.13)	1.02 (0.90–1.16)	1.04 (0.92–1.19)
71 - <83	1,044 / 1,295	76.5		0.96 (0.85–1.10)	1.00 (0.88–1.15)	1.00 (0.87–1.15)
83	1,079 / 1,296	93.2		1.01 (0.88–1.16)	1.05 (0.92–1.21)	1.05 (0.91–1.21)
<i>P</i> -trend ^f				0.20	0.83	0.64
<i>P</i> -heterogeneity for decile 1 ^g				0.90	0.89	0.67
<i>P</i> -heterogeneity for decile 10 ^g				0.55	0.63	0.52
<i>Continuous 25(OH)D</i>						
per 25 nmol/L				0.98 (0.94–1.02)	1.00 (0.96–1.03)	0.99 (0.95–1.03)
<i>P</i> -heterogeneity ^g				0.35	0.68	0.63

25(OH)D: 25-hydroxyvitamin D; BMI: body mass index; CI: confidence interval; ref: referent; RR: relative risk.

^aConsortium-wide decile cut-points were based on the 25(OH)D distribution in controls from all studies combined.

^bModel 1 conditioned on study-specific matching factors, which included age at blood collection and usually date of blood collection. The matching factors for each study are provided in Supplementary Table S1.

^cModel 2 added to model 1 BMI (using a variable combining BMI and menopausal status at blood collection) and physical activity.

^dModel 3 further adjusted for established breast cancer risk factors: race, family history of breast cancer, age at menarche, parity/age at first birth, oral contraceptive use, menopausal hormone therapy, and alcohol intake.

^eDecile 5 was chosen as the referent category since it permitted us to estimate RR at low and high concentrations and included cases and controls from each of the participating studies.

f P -trend was calculated with a Wald test that assigned the median 25(OH)D in controls to each decile.
 g P -heterogeneity in the results across studies was evaluated using the Q statistic.

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