Serum 25-Hydroxyvitamin D and Prevention of Breast Cancer: Pooled Analysis

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Abstract. *Background: Low serum levels of 25-hydroxyvitamin D [25(OH)D] have been associated with a high risk of breast cancer. Since publication of the most current meta-analysis of 25(OH)D and breast cancer risk, two new nested case–control studies have emerged. Materials and Methods: A PubMed search for all case–control studies on risk of breast cancer by 25(OH)D concentration identified 11 eligible studies. Data from all 11 studies were combined in order to calculate the pooled odds ratio of the highest vs. lowest quantile of 25(OH)D across all studies. Results: The overall Peto odds ratio summarizing the estimated risk in the highest compared to the lowest quantile across all 11 studies was 0.61 (95% confidence interval 0.47, 0.80). Conclusion: This study supports the hypothesis that higher serum 25(OH)D levels reduce the risk of breast cancer. According to the review of observational studies, a serum 25(OH)D level of 47 ng/ml was associated with a 50% lower risk of breast cancer.*

Prevention of breast cancer remains one of the greatest public health challenges of our time. In 2010, there were 192,000 cases and 40,000 deaths in the United States from breast cancer, making it the most commonly occurring neoplasm in women, and the second most common cause of death from cancer in women (1). Globally, a wide range of ecological studies have linked low levels of sunlight or ultraviolet B (UVB) irradiance, the main source of circulating vitamin D in humans, with high breast cancer rates (2-7). In another study, women who were regularly exposed to sunlight or consumed above-average amounts of vitamin D were found to have significantly lower incidence rates of breast cancer (8).

Ultraviolet B is needed to make vitamin D, which is synthesized by the skin. Exposure to UVB and supplemental vitamin D intake increase serum 25-hydroxyvitamin D [25(OH)D] levels in a dose-dependent manner (9). Lack of exposure to UVB or everyday use of sunscreens may result in vitamin D deficiency unless there is adequate oral intake (10). A low serum level of 25(OH)D is the main marker of vitamin D deficiency, and has been linked to increased risk of several types of cancer, including cancer of the breast (11).

Normal breast epithelial cells have a vitamin D receptor that is highly sensitive to $1,25(OH)_2D$ the most highly active vitamin D metabolite (12). Numerous laboratory studies have demonstrated the ability of $1,25(OH)_2D$ to promote differentiation and apoptosis in breast cancer cell lines (13, 14).

In 2010, Yin and colleagues extracted data from ten case–control studies on the relationship between serum 25(OH)D levels and risk of breast cancer (15). According to the analysis performed in that study, higher concentrations of serum 25(OH)D were significantly inversely related to breast cancer risk across all studies. The purpose of the present study is to perform an up-to-date pooled analysis of data extracted from all case–control and nested case–control studies of breast cancer and serum 25(OH)D status performed to date.

Materials and Methods

A PUBMED search was conducted by two investigators for observational studies of serum 25(OH)D and risk of breast cancer performed between 1966-2010. The search was performed by using the terms "Vitamin D" or "cholecalciferol" or "calcidiol" or "calcitriol" or "25-hydroxyvitamin D", and "case–control" or

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"epidemiology" and "human" as medical subject heading (MeSH) terms and words in the abstract, combined with the subject term "breast neoplasms". Articles were included if they were published in medical journals, were ordinary case–control, nested case–control, or cohort studies of breast cancer, and included measures of association by quantiles of serum 25(OH)D. Fourteen studies reporting odds ratios for breast cancer by quantiles of serum 25(OH)D in association with breast cancer risk were identified (16-29). One study, a case-control study by Janowsky *et al*., which analyzed the association between 25(OH)D and breast cancer risk, did not report odds ratios but reported no effect (25).

Two studies that investigated the relationship between $1,25(OH)_2D$ and breast cancer risk also were identified, including the above study by Janowsky *et al*., (25) and a case-control study by Hiatt *et al*. (30). Janowsky *et al*. found a strong inverse association with risk of breast cancer (25), while Hiatt *et al*. found no association (30).

The study published by Colston *et al*. (20) did not provide cell frequencies and utilized the same cases and controls that were used by Lowe and colleagues (26). The study by Green *et al*. (24) was left out of the analysis because it was a subset of the larger study by Bertone-Johnson *et al*. (18). Therefore, these studies (20, 24, 26) were excluded, leaving a total of 11 case–control or nested case–control studies on 25(OH)D status and breast cancer risk. Data from these eleven studies were independently extracted by two investigators, pooled, and divided into quintiles according to serum 25(OH)D.

Statistical analysis. A pooled odds ratio for all studies was obtained using Peto's Assumption-Free Method for combining odds ratios (31). This method provides a weighted average of the natural logarithms of the odds ratios from each study. The weights were the inverse of the variances of the logarithms of each odds ratio (32).

Sensitivity analysis was performed to assess the effect of inherent differences between case–control and nested case–control studies regarding the point in time of determination of serum 25(OH)D relative to diagnosis of breast cancer in the cases. This analysis was carried out by calculating the pooled odds ratios separately for nested case–control studies of pre-diagnostic serum and ordinary case–control studies. Pooled odds ratios were calculated using random effects models. Since the publication of an earlier meta-analysis by Yin and colleagues (15), two nested case–control studies have been published (22, 29), further necessitating new calculations of pooled odds ratios.

One major study reported finding a statistically significant inverse effect of 25(OH)D in individuals residing at latitudes >37 degrees N (27). Therefore, a further sensitivity analysis was performed in which the pooled odds ratio was calculated for all studies of populations residing at >37 degrees N, regardless of study design.

The *p*-value for the overall summary odds ratio was calculated using a z-score, where the numerator was the natural logarithm of the pooled odds ratio and the denominator was the standard error of the natural logarithm of the pooled odds ratio (31). This is the standard method for calculating the *p*-value using Peto's Assumption-Free Method (31, 33). Odds ratios comparing the highest with the lowest quantiles for each study were displayed in a forest plot (34, 35). Odds ratios (ORs) from the most highly adjusted models were chosen from each study. Confidence intervals (CIs) were computed using the method of Woolf (36, 37). The DerSimonian-Laird statistic was calculated to assess heterogeneity (38). The calculations were performed using Rev Man 5 (Oxford, UK: The Cochrane Collaboration).

Dose–response gradient. A data set was created consisting of one record per participant in each study. The records in this data set identified whether the participant was a case or noncase, the midpoint or median value of the participant's quantile of serum 25(OH)D at baseline, in ng/ml, a study identification number, and a serial number. If the median value for quantiles of 25(OH)D was provided in the study or contributed by the corresponding authors (16, 17, 21-23, 27, 28), it was used. If not (18-20, 24, 26), midpoint values were estimated by computing the arithmetic mean of the upper and lower bounds of the quantiles. If the lower limit of the lowest quantile was not available by inspection or correspondence, the midpoint was calculated using an assumption that the lower bound was zero. If the upper limit of the highest quantile was not available, the lower bound was used as the midpoint value. Data presented in nmol/l were converted to ng/ml using the conversion factor: 1 ng/ml=2.5 nmol/l. The records were put into order by serum 25(OH)D level, then divided into five quintiles using the following cut-off points: 0-10 ng/ml; 11-20 ng/ml; 21-30 ng/ml; 31-40 ng/ml; >40 ng/ml. Odds ratios were then calculated for the association between each quintile of serum 25(OH)D and risk of breast cancer (39). Since raw cell frequencies were used from each study, the odds ratios calculated for the dose–response analysis were unadjusted for potential confounders. The medians of the quintiles were: 10, 15, 25, 35, and 51 ng/ml. The lowest quintile was used as the reference group. Confidence intervals were calculated using the method of Woolf, as for the Forest plots (36, 37).

A dose–response curve was plotted showing the pooled odds ratios for each quintile of the pooled data (36, 37). A least-squares line was drawn to assess the dose–response relationship (40, 41). *P*-values for trend were calculated using the Mantel-Haenszel chi-square test (42). Serum 25(OH)D concentrations associated with a 50% reduction in breast cancer risk, compared to the lowest quintile of 25(OH)D, were obtained by drawing a vertical line from the point on the dose–response curve corresponding to an odds ratio of 0.5. Computations were performed using SAS, Version 9.2 (SAS Institute, Cary, NC, USA).

Results

To date, 12 epidemiological studies have been performed on the relationship between levels of serum 25(OH)D, the main circulating vitamin D metabolite, and risk of breast cancer (16-19, 21-24, 26-29). Of these, eight found a significant association between higher levels of serum 25(OH)D and a reduced risk of breast cancer (16-18, 21, 22, 24, 26, 28), while four failed to detect a significant association (19, 23, 27, 29), although a significant inverse association was present in the unadjusted data reported by one of these studies (19).

Eleven studies that analyzed risk of breast cancer by quantiles of serum 25(OH)D were identified through the PubMed search. Six were nested case–control studies and five were regular case–control studies. All of these studies were included in the pooled analysis. There was a downward linear gradient in risk of breast cancer with increasing serum 25(OH)D in the pooled analysis (Figure 1). Serum 25(OH)D levels accounted for 76% of the variation in breast cancer

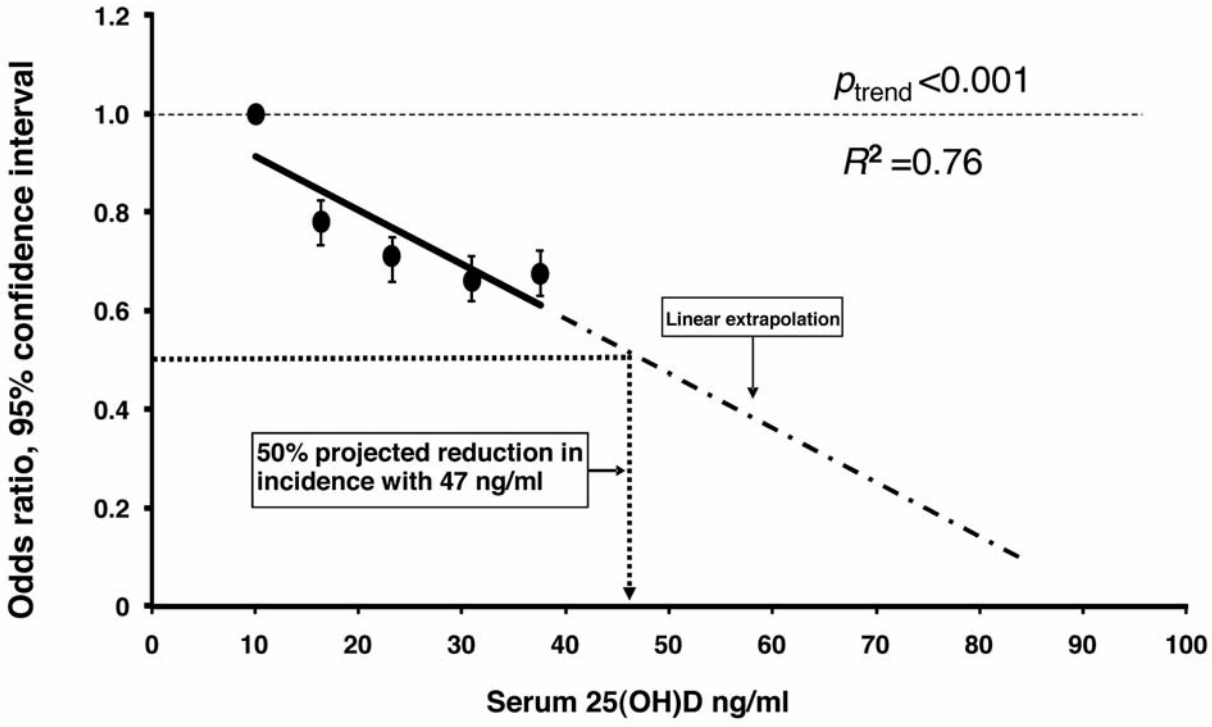

Figure 1. *Pooled analysis of studies of serum 25(OH)D concentration and risk of breast cancer.*

risk (R^2=0.76, *p* for trend <0.001). The ORs for the pooled data from lowest to highest quintile were: 1.00, 0.78, 0.71, 0.66, and 0.67 (*p* for trend <0.001) (Table I). According to the pooled analysis, a serum 25(OH)D concentration of ≥47 ng/ml (110 nmol/l) was associated with a 50% lower risk of breast cancer, compared with <10 ng/ml (Figure 1). This would also correspond to an approximately 10% reduction in risk for every 10 ng/ml increase in 25(OH)D.

The overall Peto OR summarizing the estimated risk in the highest compared to the lowest quantile across all studies was 0.63 (95% CI=0.47-0.80) (Figure 2). In the Forest plot analysis, the 11 studies were heterogeneous (DerSimonian-Laird Chi-square=77.93, df=10, *p*<0.0001). In a sensitivity analysis, 25(OH)D was significantly, inversely related to breast cancer risk in both nested case–control and ordinary case–control studies. In the nested case–control studies, the overall pooled OR was 0.87 (95% CI=0.77–0.99) (Figure 2). The nested case–control studies were homogenous (DerSimonian-Laird chi-square=4.35, df=5, *p*=0.50). In the ordinary case–control studies, the overall pooled OR was 0.41 (95% CI=0.31-0.56) (Figure 2). According to the DerSimonian-Laird test, the case–control studies were heterogeneous (chi-square=14.85, df=4, *p*=0.005). The analyses were repeated using a fixed effects model, and the results were nearly identical.

In a sensitivity analysis of studies performed on individuals residing at a latitude of >37 degrees N, the pooled OR comparing the top with the bottom quintile of 25(OH)D was 0.56 (95% CI=0.50-0.62) (Figure 3). These studies were heterogeneous (Chi-square=58, df=7, *p*=0.001) (Figure 3). According to the results of a funnel plot analysis (not shown), there was no indication of publication bias.

Discussion

Since the publication of the meta-analysis by Yin *et al*. (15), two additional nested case–control studies have been reported that found a beneficial association between 25(OH)D levels and breast cancer risk. In the nested case–control study performed by Almquist *et al*. (29), subjects in the highest category of serum 25(OH)D (37.4 ng/ml) had a 7% lower risk of developing breast cancer (OR 0.93; 95% CI=0.66-1.33) compared to subjects in the lowest category of serum 25(OH)D (18.1 ng/ml) (29), a finding that was not statistically significant. The study performed by Engel and colleagues found that for women <53 years of age, subjects with serum 25(OH)D levels >27 ng/ml had a 40% lower risk of developing breast cancer (OR 0.60; 95% CI=0.37-0.96) than those with a 25(OH)D level <19.8 ng/ml (22). This finding persisted in spite of adjustment for physical activity, a variable

Table I. *Case–control and nested case–control studies of serum 25-hydroxyvitamin D metabolites and risk of cancer of the breast, ICD-CM Code 174, according to PubMed search, 1966-2010.*

Author(s) (ref)	Year	Study design	Country	Matching criteria	Number of cases/controls	Quantile cut-points for 25(OH)D ng/ml	Relative risk	95% Confidence interval	
								Lower	Upper
Engel *et al*. (22)	2010	NCC	France	Age, menopausal status, age at menopause, center and year of blood draw	636/1272	<19.8	1.00	-	-
						19.8-27	0.87	0.68	1.1
						>27	0.80	0.62	1.0
Almquist *et al*. (29)	2010	NCC	Sweden	Age, date of blood collection, menopausal status	764/764	18.1†	1.00	-	-
						24.8	0.84	0.6	1.2
						29.5	0.84	0.6	1.2
						37.4	0.93	0.7	1.3
Abbas *et al*. (16)	2009	CC	Germany	Age, study region	289/595	<12	1.00	-	-
						12-18	0.68	0.4	1.1
						18-24	0.59	0.4	0.9
						>24	0.45	0.3	0.7
Crew *et al*. (21)	2009	CC	USA	Age	1026/1075	<20	1.00	-	-
						20-29	0.80	0.6	1.0
						30-39	0.83	0.6	1.1
						>40	0.56	0.4	0.8
Rejnmark *et al*. (28)	2009	NCC	Denmark	Age, menopausal status, season of blood draw	142/420	<24	1.00	-	-
						24-33.6	0.94	0.6	1.5
						>33.7	0.52	0.3	0.9
McCullough *et al*. (27)	2009	NCC	USA	Age, race, date of blood draw	516/516	<14.7	1.00	-	-
						14.7-19.1	1.29	0.9	1.9
						19.9-24.3	1.14	0.8	1.7
						24.3-29.2	1.44	1.0	2.2
						>29.2	1.09	0.7	1.7
Abbas *et al*. (17)	2008	CC	Germany	Age, study region	1394/1365	<12	1.00	-	-
						12-18	0.57	0.5	0.7
						18-24	0.49	0.4	0.6
						24-30	0.43	0.3	0.6
						>30	0.31	0.2	0.4
Chlebowski *et al*. (19)	2008	NCC	USA	Age, latitude of clinic, race, date of blood draw	895/898	9.44†	1.00	-	-
						15.4	0.96	0.7	1.3
						19.7	1.08	0.8	1.4
						24.4	0.93	0.7	1.4
						32.8	1	0.6	1.3
Freedman *et al*. (23)	2008	NCC	USA	Age, year of entry	1005/1005	<18.3	1.00	-	-
						18.3-23.4	1.02	0.75	1.4
						23.5-28.2	1.36	0.99	1.9
						28.3-33.6	1.13	0.82	1.6
						>33.7	1.04	0.75	1.5
Bertone-Johnson *et al*. (18)	2005	NCC	USA	Age, date of blood draw, time of blood draw, PMH use, menopausal status, fasting status	701/701	<22†	1.00	-	-
						25.8	0.95	0.7	1.4
						31.7	0.74	0.5	1.1
						37.6	0.77	0.5	1.1
						41.7	0.73	0.5	1.1
Lowe *et al*. (26)	2005	CC	UK	Age, date of blood draw, menopausal status	179/179	<20	1.00	-	-
						20-40	0.34	0.2	0.6
						40-60	0.31	0.2	0.6
						>60	0.20	0.1	0.5

CC, Case–control; NCC, nested case–control; †median values, cut-off points were not provided.

that has been shown to have considerable influence on 25(OH)D status (43).

In the present study higher levels of 25(OH)D were significantly inversely associated with breast cancer risk. When comparing the highest *vs*. lowest quintile of serum 25(OH)D across all studies, individuals in the highest category of 25(OH)D had an overall reduction in risk of breast cancer of approximately 39% compared to those in the

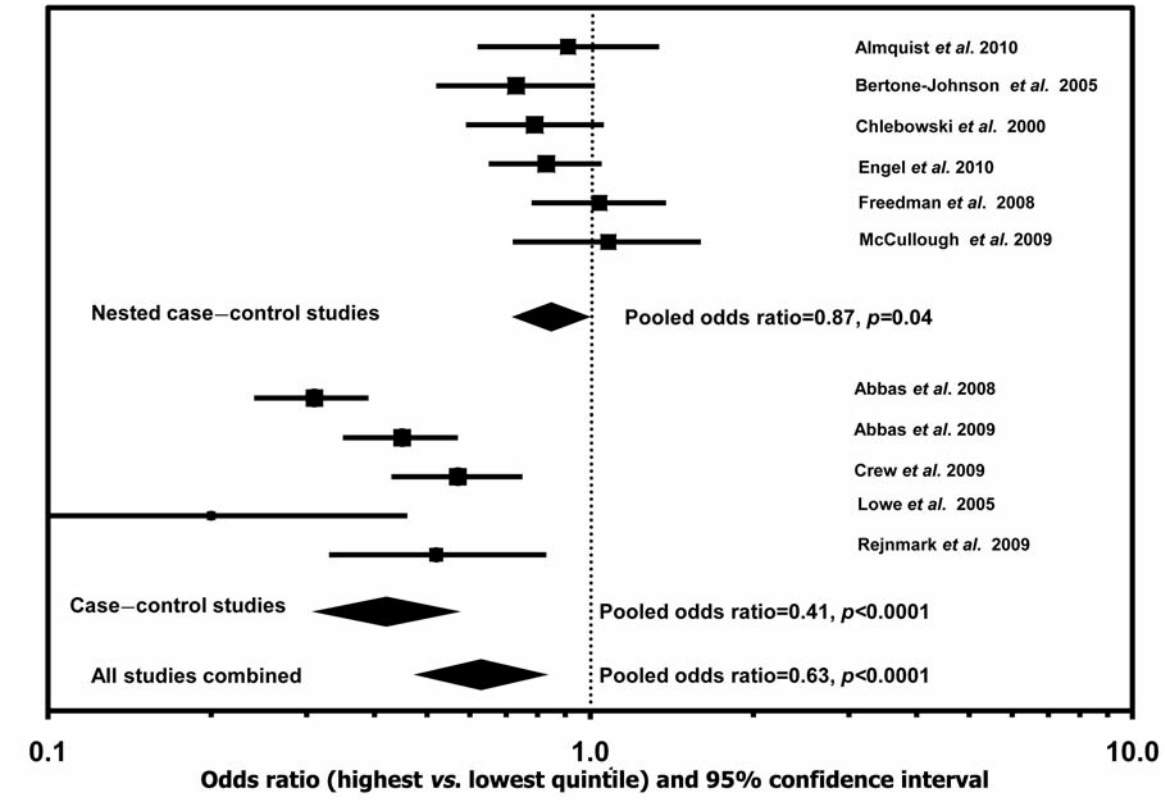

Figure 2. *Pooled odds ratios of breast cancer risk according to serum 25-hydroxyvitamin D, 1966-2010, comparing highest to lowest quintile.*

lowest category of serum 25(OH)D (Figure 2). Data from all 11 studies provided an estimate of the dose–response relationship between serum 25(OH)D concentration and breast cancer risk. This revealed that a serum level of 44 ng/ml would cut risk of breast cancer by 50%, compared to a median level of 9.7 ng/ml (Figure 1).

When pooled ORs were calculated separately according to study design (case–control *vs*. nested case–control) higher serum 25(OH)D levels were associated with a lower risk of breast cancer in both ordinary case–control and nested case–control studies (Figure 1). These findings differed in magnitude, but not direction, from those of Yin *et al*. (15). However, the current pooled analysis benefited from the pooling of two additional nested case–control studies that had not been published at the time Yin and colleagues (15) performed their meta-analysis.

Pooled ORs were calculated using random effects models, which provide a more conservative estimate of the pooled OR. The random effects model incorporates inter-study variance into the estimate and is the most appropriate method when dealing with heterogeneous studies (31). The nested case–control design is superior to the standard case–control design, since it establishes a temporal sequence.

In a previous meta-analysis performed by Yin and colleagues, serum 25(OH)D was not significantly associated with breast cancer risk in nested case–control studies utilizing pre-diagnostic serum (15). However, 25(OH)D was significantly inversely associated with breast cancer risk in regular case–control studies in which serum 25(OH)D levels were measured shortly after diagnosis. The authors attributed the association in the regular case–control studies to 25(OH)D concentrations being diminished as a result of the disease process itself, or of changes in dietary and other lifestyle habits as a result of the disease, possibly creating a spurious association (15).

However, the majority of incident cases of breast cancer are discovered as a result of self-examination when a lump is found in the breast or armpit, or by mammography (44). Therefore, most incident cases lack the severe symptoms characteristic of a more advanced stage of the disease that might be likely to cause a drastic change in lifestyle habits. For example, in the study performed by Rejnmark *et al*., 25(OH)D was measured shortly before diagnosis, when changes in lifestyle habits that could influence 25(OH)D were unlikely to have occurred. Women in the Rejnmark *et al*. study in the highest tertile of serum 25(OH)D had half the risk as those in the lowest (28).

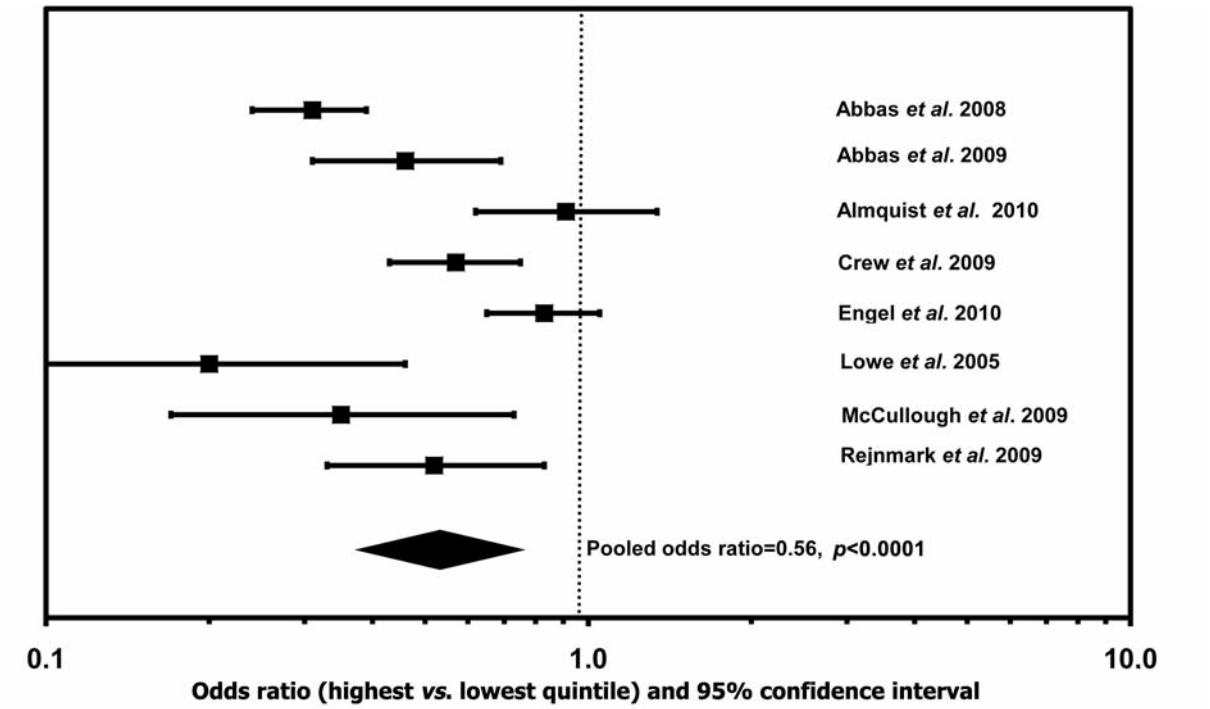

Figure 3. *Pooled odds ratios of breast cancer risk according to serum 25-hydroxyvitamin D, 1966-2010, comparing highest to lowest quintile, studies based on populations residing at latitude >37 degrees N.*

Serum 25(OH)D levels may not be greatly affected by a diagnosis of breast cancer unless the disease itself was somehow responsible for reducing serum 25(OH)D levels, a concept for which there is no known biological basis. One study performed by Goodwin and colleagues found that in women with breast cancer, mortality was significantly reduced in women in the highest tertile of 25(OH)D compared to the lowest (45). The Goodwin *et al*. analyses of serum 25(OH)D concentrations indicate that, from a biological standpoint, having breast cancer does not necessarily influence the serum 25(OH)D concentration. If the presence of breast cancer itself, rather than behavioral changes as a result of the diagnosis, influenced 25(OH)D concentration, it is highly unlikely that Goodwin *et al*. would have found an significant inverse association between 25(OH)D status and breast cancer mortality. Two additional studies found that serum 25(OH)D concentration did not markedly change in breast cancer patients after receiving chemotherapy (46, 47). Furthermore, Abbas *et al*. performed sensitivity analyses excluding cases who underwent chemotherapy before measurement of 25(OH)D or had their 25(OH)D measured greater than 6 months after the diagnosis, with no appreciable effect on the results (17, 27).

All of the ordinary case–control studies on 25(OH)D and breast cancer risk were performed in populations residing in a well-defined geographic area above a latitude of 37 degrees N. All of these studies found a beneficial association between serum 25(OH)D and breast cancer risk (Figure 3). One possible explanation is that measurement of serum 25(OH)D in populations residing at higher latitudes, may be a better indication of lifetime 25(OH)D status since migration from north to south is far more common in the US than migration from south to north (27). The case–control study performed by McCullough *et al*. was nested in the Cancer Prevention Study (CPS) II cohort, with volunteers from throughout the United States participating. In that study, individuals in the highest category of 25(OH)D had a 65% lower risk than those in the lowest category when the analysis was restricted to women residing at a latitude of >37 degrees N, a finding that was highly statistically significant (27).

In order to explore the effect of latitude, we performed a sub-analysis in which the pooled OR was calculated for studies performed at latitudes >37 degrees North, including the data from the McCullough *et al*. study (Figure 3). Eight studies were included in this analysis. The data from McCullough and colleagues' sub-analysis of latitude was included as a separate study. All eight studies found significantly lower breast cancer risk in women in the highest quantile of 25(OH)D compared to women in the lowest quantile. The overall pooled OR was 0.56 (95% CI=0.38, 0.65). Interestingly, the nested case–control studies performed in fixed populations residing at a latitude >37

degrees N, found a statistically significant inverse association between 25(OH)D levels and breast cancer risk (22, 27, 28).

The nested case–control study performed by Bertone-Johnson *et al*. found an inverse association between 25(OH)D and breast cancer risk, despite the study being carried out in a cohort of individuals who were spread throughout the country. One possible explanation for this is that the participants were nurses. Vitamin D insufficiency is common among healthcare professionals, even those living at very sunny latitudes (48, 49).

In the McCullough *et al*. study, an association of 25(OH)D with breast cancer risk was not present in women residing at latitude <37 degrees N. The investigators acknowledged that while 95% of those residing in the north were born in the north, 36% of the women living in the south were also born in the north (27). One possible reason that the other nested case–control study performed by Freedman *et al*. (23) did not find an association between 25(OH)D and breast cancer risk is that it was performed in cohorts where many people may have migrated from north to south. Therefore, the measurement of vitamin D status in these studies may not have been a good indicator of lifetime vitamin D status, or of vitamin D status during the relevant window of time during which vitamin D would exert its protective effect. This non-differential misclassification would most likely result in underestimation of the true relative risk (50).

The present study had several advantages. All known published case–control or nested case–control studies of serum 25(OH)D and risk of breast cancer were included, to the Authors' knowledge. An advantage of serum studies is that they are free from most of the uncertainties of collecting questionnaire data. An advantage of pooled analysis is that by combining the results of many studies, statistical power is increased, making it easier to detect an association between exposure and disease. In addition, the pooling of data from many studies in this analysis allowed for estimation and display of the dose–response gradient.

There were also some important limitations to the present study. One limitation was the inability to study menopausal status as a modifier of the relationship between serum vitamin D levels and risk of breast cancer. Previous studies have found that the relationship between serum 25(OH)D levels and breast cancer risk may be modified by menopausal status (16-19, 21, 22), however, this study was unable to investigate the effect of menopausal status on risk because not all studies provided cell frequencies according to 25(OH)D quantile and menopausal status.

It was also not possible to obtain data on serum 25(OH)D concentrations for each individual for the pooled analysis. Therefore, the median value for quantiles of 25(OH)D, where possible, or the mean of the upper and lower bounds of the quantiles, were used as the serum 25(OH)D value for each of the individual records in the pooled dataset. This may have resulted in a loss of precision for this variable, and possibly, the measures of association based on it.

In addition, the individual studies used different methods of measuring serum 25(OH)D, which may have introduced non-differential misclassification of exposure in both cases and controls. However, non-differential misclassification tends to obscure associations rather than strengthen them (50). Furthermore, although the Authors obtained the most highly adjusted ORs (such as adjusted for BMI, use of HRT, and age at menarche), confounders may have been measured and controlled for differently across studies. Finally, despite conducting a thorough literature search of the PubMed database, we cannot rule out the possibility that this analysis may have excluded a pertinent study.

High concentrations of serum 25(OH)D most likely prevent breast cancer through two key mechanisms. Firstly, 25(OH)D plays an important role in the up-regulation of E-cadherin (51), a glue-like substance that keeps cells bound tightly together, and in a well-differentiated state. Secondly, high serum levels of 25(OH)D provide a greater concentration to serve as substrate for synthesis of $1,25(OH)_2D$, the most biologically active vitamin D metabolite (52). $1,25(OH)_2D$ is synthesized in a wide range of tissues, including the epithelial tissues of the breast (53).

In the estimation of the dose–response gradient, a serum 25(OH)D level of >47 ng/ml would be associated with 50% lower risk of breast cancer, compared to serum 25(OH)D <9.7 ng/ml (Figure 1). Classical dose–response curves for micronutrients are either linear (54) or have a predominantly linear middle segment (40, 41). This appears to be true for most functions of vitamin D (55, 56). More studies of effects at higher vitamin D intakes are needed.

According to an analysis of 30 studies reporting any adverse effect of high serum 25(OH)D in adults, no reproducible toxicity was reported below 100 ng/ml (57). The median minimum threshold for toxicity in all studies was nearly twice this value, at 197 ng/ml. Therefore, the projected serum 25(OH)D level of approximately 44 ng/ml that would be associated with 50% lower breast cancer risk would be below the threshold for minimal toxicity by a safety factor of 4-5. An upper level of 4,000 IU/day and a no observed adverse health effect level of 10,000 IU/day of vitamin D have been established by the Institute of Medicine (IOM) (58). The IOM also reported that no hypercalcemia from vitamin D intoxication has been described for vitamin D intakes <10,000 IU/day (59). Another review reported that no cases of toxicity were documented at doses <40,000 IU per day (55).

A vitamin D3 intake of 2,000 – 4,000 IU/day, and a target of 45 ng/ml of serum 25(OH)D, are the most practical estimates now available for decision-makers who must weigh the potential benefits of actions that could reduce incidence of breast cancer. The current recommended daily intake of

the Institute of Medicine of 600-800 IU/day for mature adults (59) would increase median serum 25(OH)D by only 4-6 ng/ml (60), which would be insufficient to raise the median population serum 25(OH)D levels into the range for cancer prevention.

The results of the NHANES 2001-2004 survey revealed that the mean serum 25(OH)D value for the US population was 24 ng/ml (61). Therefore, an increase of vitamin D intake to 2,000-4,000 IU/day of vitamin D_3 would boost serum 25(OH)D by approximately 14-28 ng/ml, raising the estimated median level in the population to 38-52 ng/ml (60). According to the findings of this pooled analysis, the optimal oral vitamin D intake would also be 4,000 IU/day since this would be the dose required to raise median serum 25(OH)D levels from 24 ng/ml to 52 ng/ml (60). A serum 25(OH)D concentration of 52 ng/ml is far below the concentration that would be associated with hypercalcemia or adverse health effects, which ranges in different sources from 195-300 ng/ml (55-57, 59, 62, 63). Increasing serum 25(OH)D to these levels would be most efficiently achieved by intake of vitamin D3 (cholecalciferol) rather than vitamin D2 (ergocalciferol) (64).

This pooled analysis strongly supports the theory that there is an inverse association between serum 25(OH)D and risk of breast cancer. Although confounding is possible, there are four lines of epidemiological evidence that would support the association being causal: the geographic gradient with latitude and solar UVB irradiance (2-7), observational studies linking deficient serum 25(OH)D levels with increased risk (65, 66), studies linking low oral intake of vitamin D with increased risk, and laboratory studies illuminating the mechanisms *in vivo* and *in vitro* (13, 14). Vitamin D receptor polymorphisms that interfere with vitamin D are also associated with increased risk of breast cancer, particularly in combination with low levels of serum 25(OH)D (26). It seems unlikely that a single confounder could account for all these lines of evidence.

This pooled analysis provides the most current epidemiological evidence to investigate the relationship between serum vitamin D levels and breast cancer risk. The results support the hypothesis that higher serum 25(OH)D concentrations reduce the risk of breast cancer. The relationship was present in ordinary case–control studies and to a statistically significant, but lesser degree in nested case–control studies. Researchers may wish to consider the effect of geography on serum 25(OH)D concentrations in future nested case–control studies of serum 25-hydroxy vitamin D levels and breast cancer risk.

Numerous laboratory studies and observational studies have demonstrated the presence of a protective effect of serum 25(OH)D against breast cancer risk. The findings overall are consistent and vary only in degree, not direction. Now is the time to translate the accumulated knowledge from decades of research into public health policy. Vitamin D supplementation with 4000 IU/day of vitamin D_3 (cholecalciferol) as a breast cancer primary prevention strategy would be highly effective and safe (59).

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Note added in proof: While this study was in proof, another nested case–control study was released that studied the association between serum 25(OH)D and risk of breast cancer. It found no association. This finding would not substantially affect the results that were based on the 11 studies that were included here, except possibly for a slight flattening of the dose–response curve. The reference to this study is: Eliassen AH, Spiegelman D, Hollis BW, Horst RL, Willett WC, Hankinson SE: Plasma 25-hydroxyvitamin D and risk of breast cancer in the Nurses' Health Study II. Breast Cancer Res *13*: R50, 2011.

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