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Serum 25-hydroxyvitamin D and risk of breast cancer

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ABSTRACT OF THE DISSERTATION

Serum 25-hydroxyvitamin D and risk of breast cancer

by

Sharif Burgette Mohr

Doctor of Philosophy in Public Health (Epidemiology)

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Globally, a wide range of epidemiological studies have linked low levels of sunlight, ultraviolet B (UVB) irradiance, or serum 25-hydroxyvitamin D [25(OH)], with increased risk of breast cancer. Three studies of different design were employed to assess the relationship between serum 25(OH)D and breast cancer risk. The first study is an ecological analysis of the relationship between UVB irradiance and age-standardized breast cancer mortality rates worldwide. In the regression model, UVB irradiance was inversely associated with mortality rates ($p = 0.04$), after controlling for covariates. The overall model was statistically significant ($R^2 = 0.34$, $p < 0.0001$). The second study was
a case-control analysis of 600 incident cases of female breast cancer and their matched controls that investigated the inverse association between pre-diagnostic serum levels of 25(OH)D and risk of breast cancer in active-duty U.S. military personnel. Conditional logistic regression was used to assess the relationship between serum 25(OH)D concentration and breast cancer risk, while controlling for race and age. In this study, there was an inverse trend between serum 25(OH)D and odds of breast cancer that did not reach statistical significance. However, in 123 pairs for whom serum was collected 90 days or fewer before case diagnosis, women in the highest quintile of serum 25(OH)D had a 70% lower estimated risk of breast cancer (odds ratio 0.30, 95% confidence interval 0.12-0.74, $p \leq 0.01$) compared to those in the lowest quintile. The third study was a pooled analysis of published data from 11 ordinary and nested case-control studies. Data from all 11 studies were combined in order to calculate the pooled odds ratio of the highest vs lowest quintile of 25(OH)D. The pooled odds ratio summarizing the estimated risk in the highest compared to the lowest quintile across all studies was 0.63 (95% confidence interval 0.47, 0.80). These three studies provide compelling evidence that supports a strong inverse relationship between vitamin D status and breast cancer risk. More studies, including randomized controlled trials of higher doses of vitamin D₃ (4,000 IU – 6,000 IU/day), and serum levels of 25(OH)D (60-80 ng/ml), should be performed without delay.
Introduction

The first recognition of the importance of vitamin D in the promotion of human health was made in ancient Greece by Hippocrates. Hippocrates held the belief that living on the south face of a hill, the side that receives the most sunlight, was the healthiest place one could live. Over 2000 years later in Poland, Sniadecki made the observation that children living on farms did not develop rickets. This was in contrast to children living in Warsaw who had a high incidence of the disease. He hypothesized that increased exposure to sunlight in the children living in rural areas prevented them from developing rickets (1).

In 1890, at the height of the rickets epidemic in England, a British medical missionary named Theodore Palm noted that children living in equatorial countries did not develop rickets. This prompted him to write to fellow medical missionaries throughout the world inquiring as to whether children in their areas had rickets. He too attributed the geographical differences in rickets incidence to differences in sunlight exposure (2).

The work of both Sniadecki and Palm remained largely unnoticed until 1918 when Sir Edward Mellanby, searching for a nutritional cure for rickets, performed over 100 experiments on dogs. In these experiments, Mellanby would keep one group of dogs indoors and feed them a diet consisting exclusively of oats, thereby inducing rickets in them (3). He would then feed the rachitic dogs cod liver oil, which would cure them of
rickets within a few months. Through the course of these experiments, cod liver oil became the model for an essential micronutrient.

In the years following, Elmer V. McCollum, a chemist at the University of Wisconsin, discovered the compound that is now known as vitamin D. McCollum decided to take Mellanby’s work a step further by investigating the chemical composition of cod liver oil. At that time, it was known that cod liver oil could prevent night blindness and fractures. McCollum wanted to know if cod liver oil retained its properties with respect to fractures and night blindness after being heated. In a series of experiments, McCollum tested his hypothesis by heating and oxygenating cod liver oil (4). After the heating and oxygenation, McCollum discovered that cod liver oil still prevented fractures but not night blindness (4). This led him to conclude that there were two different active compounds. The compound that was destroyed by the heating process was called vitamin A, and the heat stable component of cod liver oil became known as vitamin D.

Up until 1937, there were two competing theories on the prevention of rickets: use of cod liver oil or the use of UV. Several scientists were able to demonstrate that rickets could be cured by exposure to sunlight or ingestion of UV irradiated food (5-8). The fact that foods containing cholesterol could cure rickets only after being irradiated with UV light led investigators to isolate and identify the precursor of vitamin D. The discovery of 7-dehydrocholesterol (7-DHC), the precursor of vitamin D₃, was made by Windaus in 1937. Windaus isolated 7-DHC from pig skin and was able to induce the formation of
vitamin D₃ by irradiating 7-DHC with ultraviolet radiation (9). Windaus received a Nobel Prize for his work, and more importantly he was able to unify two apparently disparate lines of evidence through the discovery that exposure to ultraviolet light was responsible for vitamin D synthesis in the human body.

The emergence of the epidemiological role of sunlight in cancer prevention began in 1936, when Peller observed that US Navy personnel who experienced skin cancer had a much lower incidence of non-skin cancers (10). This led him to hypothesize that the development of skin cancer conferred protection against other cancers (10). Then, in 1942, Apperly first observed that there were lower mortality rates from internal cancers in sunnier regions of the United States (11). Peller and Apperly’s observations went largely unnoticed until 1970, when the United States government, under the direction of President Richard Nixon, passed the National Cancer Act. The purpose of this act was to appropriate $100 million dollars for the launching of a campaign to develop a cure for cancer. Soon after the passage of this landmark legislation, the National Cancer Institute (NCI) published maps of mortality from several types of cancer in order to better understand the geographical distribution of cancer mortality in the United States.

Through study of the cancer maps published by the NCI, researchers from Johns Hopkins University noticed a strong latitudinal gradient within the United States for colon cancer mortality. They observed that mortality rates were much higher in the Northeastern and Northern parts of the country than in the South and Southwest (Figure 1.1). This observation led to the hypothesis that differences in exposure to UVB and
consequently differences in vitamin D status may be responsible for increased risk of developing colon cancer. The dominant paradigm in cancer research at that time was the search for exposures that were believed to cause the cancers. The idea that cancer might be due to a micronutrient deficiency, namely vitamin D, was a bold departure from the prevailing ideology.

Further exploration of the vitamin D-cancer prevention hypothesis came in 1980 when the seminal paper on the relationship between vitamin D status and colon cancer risk was published in the International Journal of Epidemiology in 1980 (12). In this ecological analysis, vitamin D was proposed as a protective factor against colon cancer (12) and a downward linear correlation between colon cancer mortality and amount of solar radiation in 49 US metropolitan areas was demonstrated.

A similar geographical gradient to colon cancer was also observed for cancer of the breast. This led to the publication of a 1989 study that postulated an association between ultraviolet-B blocking air pollution and increased risk of breast and colon cancer based on inhibition of cutaneous vitamin D photosynthesis, resulting in vitamin D deficiency (13). The investigators examined the association between sulfur dioxide air pollution and other ultraviolet-B-blocking aerosols in 20 Canadian cities, and age-adjusted breast and colon cancer mortality rates. This study demonstrated statistically significant positive associations between measures of air pollution and age-adjusted mortality rates for colon cancer in men and women and breast cancer in women.
Researchers continued their geographic exploration of the inverse association between sunlight and breast cancer in two other studies. In 1990, an inverse association was found between total average annual sunlight energy striking the ground and age-adjusted breast cancer mortality rates in 87 regions of the United States (14). In another study, breast cancer incidence rates in the former soviet republics in relation to sunlight levels were examined. Breast cancer had a threefold range of incidence across the 15 former soviet republics, and total sunlight energy varied from 210 to 400 calories per square centimeter per day. They found a significant negative association between total average annual sunlight energy striking the ground and age-adjusted breast cancer incidence rates in the USSR (15).

Since the publication in 1980 of the seminal paper on vitamin D and cancer prevention, a total of 22 epidemiological studies (13-34) and five systematic reviews or meta-analyses (35-39) of breast cancer risk and markers of vitamin D status or sunlight have been performed. The results of these studies have been largely supportive. In eight of the case-control studies of 25(OH)D status and breast cancer risk, 25(OH)D levels were inversely related to risk of breast cancer (23, 26-33), although four failed to detect a significant association (23-25, 34). In the study performed by Janowsky and colleagues, Caucasian women in the lowest quartile of 1,25(OH)D, the most biologically active vitamin D metabolite, had 5.3 (95% C.I. 2.1, 13.4) times the odds of getting breast cancer compared to women in the highest quartile (17). However, in a different study, there was no statistically significant association between 1,25(OH)2D and breast cancer (16).
Furthermore, of the five meta-analyses performed to date, three confirmed an inverse association between 25(OH)D and risk of breast cancer (35, 37, 39).

The aim of this dissertation is to confirm the findings of previous research on the role of serum 25(OH)D in risk of breast cancer as well as to advance scientific knowledge in this area. This aim will be accomplished by investigating the relationship between breast cancer risk and low vitamin D status through performing three studies utilizing different designs, providing three separate lines of evidence.

Age-standardized annual mortality rates of breast cancer vary considerably in the world. The first study will ecologically assess the association between ultraviolet B irradiance with age-standardized mortality rates of breast cancer. The hypothesis is that mortality rates of breast cancer will be highest at the highest latitudes, intermediate at intermediate latitudes, and lowest at lowest latitudes. It is also hypothesized that a similar association will exist for intensity of UVB irradiance, even after adjustment for covariates.

Among major countries, a low of 2 per 100,000 population occurs in Haiti. This compares to rates of 29.6 per 100,000 in Malta and Cyprus. The mortality rate in the United States is 18.9 per 100,000. The World Health Organization-International Agency for Research on Cancer databases were used to determine mortality rates of breast cancer in 174 countries for the year 2002. These mortality rates were analyzed in terms of key
covariates, including the intensity of ultraviolet B (UVB) irradiance in each country, calculated with methods that account for atmospheric factors, such as cloud cover.

The second study will employ a meta-analysis design. For this investigation, all published case-control or nested case-control studies that examined the association between serum 25-hydroxyvitamin D and risk of breast cancer were identified. The data was extracted from the studies, pooled, and analyzed, in order to assess the effect of high serum 25(OH)D concentration on breast cancer risk across all studies. Moreover, pooling of the data enabled the dose response relationship between risk of breast cancer and serum 25(OH)D level to examined. This was performed as a systematic review and meta-analysis according to the guidelines developed by a consensus committee of meta-analyses of observational studies.

The final study is a nested case-control study of the association between prediagnostic serum 25-hydroxyvitamin D and risk of breast cancer in the Department of Defense. The main objective of this study is to test whether a relationship exists between prediagnostic serum levels of 25-hydroxyvitamin D (25(OH)D) and subsequent risk of breast cancer in an active-duty U.S. military cohort. This study will also quantify the dose-response relationship between serum 25(OH)D and subsequent risk of breast cancer using previously collected sera. This will allow for determination of the optimal circulating 25(OH)D levels needed to reduce breast cancer risk among U.S. military personnel. Although research suggests that maintaining adequate serum vitamin D levels
may reduce the risk of breast cancer, there is a lack of data regarding the dose-responses relationship between serum levels of 25(OH)D and the risk of breast cancer.

Figure 1.1. Breast cancer mortality rates per 100,000, United States, 1990-94
CHAPTER 1

Ultraviolet B irradiance, modeled serum 25-hydroxyvitamin D, and breast cancer mortality: an ecological analysis

by

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ABSTRACT

Background. Breast cancer incidence and mortality rates tend to be higher in areas with low sunlight levels. Exposure of the skin to ultraviolet B radiation through sunlight is the source of 80% to 95% of circulating vitamin D in humans. This study assessed the relationship between ultraviolet B irradiance and age-standardized mortality rates worldwide, while adjusting for other established risk factors for breast cancer.

Methods. Age-standardized mortality rates for 173 countries were obtained from GLOBOCAN and plotted by latitude. Mortality rates were also plotted by modeled serum 25(OH)D levels. Multiple linear regression was employed to investigate the relationship between age-standardized mortality rates of breast cancer and UVB irradiance adjusted for cloud cover, while controlling for covariates in 107 countries.

Results. Age-standardized mortality rates of breast cancer were somewhat higher at latitudes distant from the equator ($R^2 = 0.14, p < 0.001$). There was a modest, inverse dose-response relationship between modeled serum 25(OH)D concentration and mortality rates of breast cancer ($R^2 = 0.16, p < 0.001$). According to the multiple linear regression model, UVB irradiance adjusted for cloud cover was inversely associated with mortality rates ($p = 0.04$), after controlling for covariates. The overall model was statistically significant ($R^2 = 0.34, p < 0.0001$).

Conclusion. There was an association between lower UVB irradiance and higher mortality rates. Further investigation is warranted, particularly studies using prediagnostic serum 25-hydroxyvitamin D levels in individuals.
Introduction

Approximately 450,000 deaths occur globally each year from breast cancer (40), making it the most common cause of death from cancer in women in the world (40). In the US, there were 39,520 deaths from breast cancer in 2011, making it the second most common cause of cancer in women and the third most common cause of death from cancer after lung cancer and colon cancer (41). Breast cancer incidence and mortality rates tend to be higher in areas with low winter sunlight levels, and lower in sunny areas (13, 14).

Exposure of the skin to ultraviolet B (UVB) rays in sunlight is the source of 80% to 95% of circulating vitamin D and its metabolites in humans, so availability and intensity of sunlight are strong correlates of serum 25-hydroxyvitamin D [25(OH)D], the principal circulating vitamin D metabolite (42). Observational studies have shown that serum 25(OH)D concentrations are lower in populations residing at latitudes more distant from the equator, where less UVB irradiance is present (43, 44). Exposure to UVB and supplemental vitamin D$_3$ intake increase serum 25(OH)D levels in a dose-dependent manner (45). Lack of exposure to sunlight-induced UVB may result in vitamin D deficiency unless there is sufficient oral intake (46). An increased risk of several cancers, including cancer of the breast, has been linked to vitamin D deficiency (47).

Once vitamin D is photosynthesized in the skin by contact with UVB radiation, it is converted in the liver to the main circulating vitamin D metabolite, 25(OH)D (48). Some of the circulating 25(OH)D is further metabolized by the enzyme 1-alpha-
hydroxylase into 1,25 dihydroxyvitamin D \([1,25(\text{OH})_2\text{D}]\), the most biologically active vitamin D metabolite, although present in the circulation in approximately 1/1000\(^{\text{th}}\) the concentration of 25(\text{OH})D (48). The principal site of 1,25(\text{OH})_2\text{D} synthesis is the kidney, but production of the hormone occurs in a wide range of tissue including breast epithelial tissue (49), which has vitamin D receptors that are highly sensitive to 1,25(\text{OH})_2\text{D} (50). Moreover, 1,25(\text{OH})_2\text{D} has been shown to promote differentiation and apoptosis in breast cancer cell lines (51, 52), and inhibit mitosis of breast epithelial cells (53).

In one study of breast cancer mortality rates in the US, there was a distinct latitudinal gradient for mortality, with the highest mortality from breast cancer occurring in the urbanized Northeast and the lowest mortality occurring in the regions with the highest levels of ultraviolet B UVB irradiance: the South and Southwest (14). This geographic gradient in age-standardized mortality rates varied over a 1.8 fold range (14). In another study of 35 countries, ultraviolet B irradiance was independently, inversely associated with breast cancer mortality rates before and after adjustment for other factors thought to be associated with breast cancer risk (18). Furthermore, a statistically significant relationship was found between mortality rates of breast cancer in 20 Canadian cities and levels of sulfate aerosols (13). Sulfate aerosols block UVB radiation and may result in reduced photosynthesis of vitamin D in populations residing in regions with high levels of these pollutants (13).
While some of the geographical gradient for breast cancer may be associated with reproductive factors or lifestyle factors such as alcohol intake, smoking, and physical activity (54), the results from a wide range of studies support the hypothesis that inadequate sunlight exposure and low serum vitamin D metabolite levels may be making a substantial contribution to morbidity and mortality from this disease (13-22, 28, 32, 55-58). Furthermore, in a prospective study of 512 women with breast cancer, women in the highest group of serum 25(OH)D concentration (>30 ng/ml) had a 42% lower risk of death compared to women in lowest group of 25(OH)D concentration (<20 ng/ml) after a mean follow-up time of 11.6 years (59).

The primary aim of this ecological study was to examine the relationship between UVB irradiance adjusted for cloudiness, and breast cancer mortality on global scale, while controlling for several known risk factors for breast cancer. The secondary aim was to assess the relationship between modeled population serum 25(OH)D levels and breast cancer mortality. This is the first study, to the authors’ knowledge, to examine the associations between latitude, UVB irradiance, and modeled serum 25(OH)D with global age-standardized breast cancer mortality rates, while controlling for covariates.

Methods

Data Sources

A data set was created that contained information for each country on age-standardized mortality rates of breast cancer, latitude of the population centroid, UVB irradiance adjusted for cloudiness, per capita health care expenditure in international
dollars, per capita cigarette consumption, per capita alcohol consumption, proportion of female population overweight, and total fertility rate per 1,000 women. Complete data on all variables were available for 107 countries.

Age-standardized mortality rates of breast cancer were obtained for all countries using the International Agency for Research on Cancer (IARC) GLOBOCAN database (40). GLOBOCAN uses national registries and registration of vital events to estimate annual age-standardized mortality rates per 100,000 population in 2002, the latest year for which complete data were available. Mortality rates were age-standardized to the world standard population, using the direct method with 5-year age intervals.

The total solar UVB irradiance at the top of the atmosphere on the winter solstice was calculated using a standard algorithm (60). The total noon solar irradiance at the top of the atmosphere for each country on the date of the winter solstice was calculated using the formula \( A' = A \cdot \cos(x + 23.5 \text{ degrees}) \) in the northern hemisphere; and \( A' = A \cdot \cos(x - 23.5 \text{ degrees}) \) in the southern hemisphere; where \( x \) = latitude of the population centroid of the country in degrees, \( A = \) total solar radiation at the equator in W/m\(^2\) (i.e., the solar constant, 1366 W/m\(^2\) ), and \( A' = \) total solar radiation in W/m\(^2\) for the country on the date of the winter solstice (60). Since UVB irradiance is approximately 0.4% of total solar irradiance, total solar irradiance was multiplied by 0.004 to obtain the estimated UVB irradiance at the top of the atmosphere. Latitude was determined for the population centroid of each country. Population centroids were calculated by the Columbia University Center for International Earth Science Information Network (61).
The cloud cover estimate was based on data from the National Aeronautics and Space Administration (NASA) International Satellite Cloud Climatology Project (ISCCP) satellite (62) that provided cloud cover data for areas corresponding to the size of many countries, rather than population centroids. If there were multiple cloud cover percentages for a particular country, the percentage for the most populous region of the country was used. This differed to a minor degree from the procedure for UVB at the top of the atmosphere, which was estimated for the atmosphere above the population centroids (62). In order to account for the influence of cloud cover on transmission of UVB through the atmosphere, solar UVB at the top of the atmosphere was adjusted for mean cloud cover by multiplying UVB irradiance at the top of the atmosphere by the mean percentage of sky that was not covered by clouds for each country. UVB irradiance was adjusted for cloudiness by the following formula: UVB irradiance * (1 - mean proportion of sky covered by clouds).

Data on per capita alcohol consumption by country were provided in the form of the average number of alcoholic drinks per person per year by the United Nations Food and Agriculture Organization (63). The fertility rate was the mean annual number of babies born per 1,000 women for the time period 2000-2005 (64). The World Health Organization (WHO) provided the proportion of female population overweight in each country for the year 2002 (65). Overweight for females was defined as a BMI > 25 (65). In order to control for disparities in access to and quality of healthcare among the different countries included in the analysis, data on per capita health expenditures in 2001 for each country were used in the regression model. This was expressed as the average
number of international dollars spent by government on healthcare, per citizen (66). Per capita cigarette consumption was the average number cigarettes smoked per person per year in 1980 (67). Smoking data from 1980 were used in this study in order to allow an induction period for breast cancer incidence and mortality to occur (68) between the date of exposure and the year that the mortality rate was estimated (40). Including a time lag was not necessary for solar UVB irradiance at the top of the atmosphere, which does not vary appreciably over time.

Serum 25(OH)D was modeled using measured levels of serum 25(OH)D during winter obtained from 28 regions in 18 countries (Appendix Table 1) as the dependent variable, and UVB irradiance and skin pigmentation levels in the areas where the studies were performed (69). The initial regression model, based on the known values of all variables, provided regression coefficients for use in a separate multiple regression prediction equation that used known UVB and skin pigmentation to estimate mean winter serum 25(OH)D levels in countries where measurements were not available. The prediction equation included a scaling constant that was empirically determined. The measured 25(OH)D levels were used in the final calculations for the 18 countries, and the values modeled from the procedures described above were used for the 155 other countries.

**Statistical analysis**

Age-standardized mortality rates for 173 countries were obtained from GLOBOCAN (40) and plotted by latitude of the population centroid. The best fit to the
data points was obtained using a polynomial trend line. The mortality rates were also plotted by the log of modeled serum 25(OH)D levels using a standard pharmacologic dose-response curve (Prism)(San Diego:GraphPad Software). Multiple linear regression was employed in order to investigate the relationship between age-standardized mortality rates of breast cancer and UVB irradiance adjusted for cloudiness, per capita health care expenditure, per capita alcohol consumption, per capita cigarette consumption, proportion of female population overweight, and total fertility rate per 1,000 women. Complete data for all covariates were available for 107 of the 173 countries in the GLOBOCAN database, therefore the multiple linear regression model consisted of 107 countries. All analyses were performed using SAS Version 9.1 and JMP Version 5.1.2 (Cary NC: SAS Institute).

Results

Age-standardized mortality rates of breast cancer were somewhat higher at latitudes distant from the equator, with a few exceptions ($R^2 = 0.14, p < 0.001$) (Figure 1.2). There was a modest dose-response relationship between modeled serum 25(OH)D and mortality rates of breast cancer ($R^2 = 0.16, p < 0.001$)(Figure 1.3).

In the multiple linear regression model, UVB irradiance adjusted for cloud cover was inversely associated with age-standardized breast cancer mortality rates ($p = 0.04$) (Table 1.1), after controlling for covariates. There was a positive association between total fertility rate and breast cancer mortality ($p = 0.001$) (Table 1.1). There were trends suggestive of positive associations with alcohol consumption, per capita cigarette
consumption, proportion of female population overweight and per capita health expenditures, but the associations were not statistically significant (Table 1.1). The overall model was statistically significant ($R^2 = 0.34$, $p < 0.0001$) (Table 1.1).

**Discussion**

There was a modest association between latitude or modeled serum 25(OH)D with age-standardized breast cancer mortality rates in this analysis. This is not entirely surprising, because under the vitamin D hypothesis, populations at latitudes closer to the equator would be expected to have lower mortality rates from breast cancer due to increased exposure to UVB irradiance, and consequently, higher serum 25(OH)D concentrations. On the other hand, populations who reside at latitudes distant from the equator, where countries tend to have wealthier economies, may benefit from greater access to treatments that might reduce mortality from breast cancer. Furthermore, it may be easier to detect an effect of latitude, with respect to breast cancer mortality, within a country due to global disparities in access and quality of healthcare. For example, a country such as the US spans a wide range of latitudes, and has a high quality of healthcare across most of the country. However, there is a latitudinal gradient in age-standardized mortality rates of breast cancer, with the highest rates in the North and Northeast and lowest rates in the Southwest(14).

Also, as expected, in a multiple linear regression model that included 107 countries, high levels of UVB irradiance were significantly, inversely associated with age-standardized mortality rates of breast cancer. This relationship may be due to reduced
population levels of serum 25(OH)D, the principal circulating vitamin D metabolite, in countries distant from the equator, where UVB irradiance is lower (43). Although it is possible that some of the association could be due to better surveillance in countries distant from the equator, where mortality from breast cancer is highest, the inverse relationship between UVB irradiance and breast cancer mortality rates was independent of per capita health expenditure.

The results of this study were consistent with previous observational studies of breast cancer risk and markers of vitamin D status or sunlight (13-15, 17-22, 26-33) and results of similarly supportive reviews (35-38), including a pooled analysis that found an inverse dose-response relationship between serum 25(OH)D levels and breast cancer risk (70). While the beneficial effect of serum 25(OH)D status on incidence of breast cancer is well documented (70), previous research has also demonstrated a beneficial effect of vitamin D status on breast cancer mortality. In the study performed by Goodwin and colleagues of 512 women with breast cancer, mortality was significantly reduced in women in the highest tertile of 25(OH)D compared to the women in the lowest (59).

The greater explanatory power of UVB irradiance compared to latitude may have occurred because UVB irradiance takes geographic variation in cloud cover into account, while latitude does not. UVB was adjusted for cloud cover because clouds attenuate the UVB irradiance needed to produce vitamin D in the skin (71). Although thin cloud layers transmit almost all UVB, thicker clouds absorb most of the UVB photons that impinge on them (71). As a result, UVB irradiance reaching the ground is, in general, inversely
related to the proportion of the sky covered by overcast or thick clouds. The ISCCP satellite data were weighted toward thick clouds (72), and those data were used to adjust UVB irradiance at the top of the atmosphere for absorption by cloud cover.

UVB irradiance on the winter solstice was included in the multiple linear regression model because vitamin D deficiency is most likely to occur in populations living at latitudes more distant from the equator during winter (45). In addition, individual behaviors that inhibit vitamin D photosynthesis, such as use of heavy clothing that completely covers the skin, as well as spending more time indoors, are more prevalent during the winter months. Therefore, under the vitamin D-cancer prevention hypothesis, inclusion of UVB irradiance at the winter solstice would increase the likelihood of detecting an association between UVB irradiance and breast cancer mortality rates.

A woman’s risk of breast cancer increases the longer the delay between the onset of menarche and the birth of her first child (73). Therefore, the total fertility rate per 1,000 women was included in the model to investigate a possible inverse association. However, this variable had a statistically significant positive association with breast cancer mortality in the regression analysis, contrary to results from previous research (74). One possible explanation is that in the subset of countries included in the regression model, total fertility per 1,000 women was highly correlated with some unknown confounder.
On the other hand, late parity has also been established as a risk factor for breast cancer (75). Since age-standardized total fertility rates were not available for this analysis, it is possible that higher prevalence of late first-births in countries with higher mortality rates of breast cancer may have accounted for the positive association between total fertility rate and breast cancer mortality rates in this study.

Previous studies have identified associations between breast cancer risk and obesity (76), smoking (77), and alcohol consumption (78), although these covariates were not significantly associated with breast cancer mortality in this analysis. This may have been due to the lack of resolution at the individual level that is inherent in studies of countries rather than individuals (79).

This study was the first to include age-standardized mortality rates for countries located at widely different latitudes. The analysis allowed assessment of the independent association of UVB irradiance with mortality rates after adjustment for covariates. The regression model accounted 34% of the variation in breast cancer mortality rates among countries. Furthermore, the present study is based on solar irradiance, the main source of vitamin D, rather than oral intake, which is less influential with respect to serum 25(OH)D. In addition, this study included all cases that occurred in each country during a year, regardless of the length of time that had elapsed between the relevant solar exposure and the diagnosis. This allowed the present study, like the case-control study by Knight et al. (80), to assess associations that could have had very long incubation periods.
including associations with vitamin D status that were established in childhood and adolescence.

This was a study of aggregates rather than individual subjects. Findings that apply to aggregates may not apply to individuals (79). For example, all individuals living in areas of high UVB irradiance may not have high exposure to UVB. This could have resulted from urbanization and industrialization. On the other hand, regional solar UVB irradiance is likely to affect a broad range of individuals, and the finding was present despite the possible misclassification of exposure. Systematic assessment errors may produce more pronounced errors in analyses of group-level exposure measures than in analysis of individual-level exposures, increasing the possibility of bias (81-85). It also has been reported that random misclassification in studies of aggregate measures usually, but not always, results in bias toward the null hypothesis (81-85).

A question when trying to generalize from observations of groups to individuals is whether it is appropriate to impute the findings for a group to the individuals in it (79). This was first described for behavioral studies, but may be applicable to measures of precursors of disease. The possibility that characteristics that apply to a group may not apply to the individuals in it should be considered when evaluating this or any analysis based on groups.

There are several other biases that should be evaluated when assessing ecological studies (81-85). These include within-group biases, confounding by group, and effect
modification by group (81). For example, absorption of UVB irradiance by clothing could not be measured in the present study, yet it is possible that the association of UVB with mortality rates of breast cancer could have been influenced by the type of clothing worn. Since there was no systematic source of information available on clothing characteristics according to country, it was not possible to examine this possible interaction.

The possibility of confounding was investigated by performing multiple regression analyses using a model that included the possible confounders for which data were available, i.e., proportion of female population overweight, fertility rates, tobacco and alcohol consumption, and per capita health expenditure. Regression allowed an assessment of the independent contributions of each covariate. Data were not available on prevalence of use of replacement hormones by country, so this could not be used as a covariate. It is possible that the association of UVB with incidence rates could have been confounded by differences in prevalence of use of hormone replacement therapy. Another limitation was the lack of data regarding vitamin D intake from foods, supplements or fortification, which was not available from WHO.

Cigarette smoking and consumption of alcohol have been established as risk factors for breast cancer in previous research (77, 78), necessitating their inclusion in this analysis. However, due to lack of data on these factors for many countries, including these variables in the regression model reduced the sample size from 173 to 107. This may have potentially biased the results of the analysis if countries for which complete
data were not available differed substantially from countries for which complete data were available with respect to prevalence of risk factors for breast cancer incidence and mortality. These exclusions appeared to be somewhat non-differential and were merely due to lack of data on all variables for these countries. The majority of the countries with incomplete data were economically poor countries in sub-Saharan Africa and Central Asia. Most of these countries are located close to the equator, and despite the possibility of having a high prevalence of alcohol and tobacco use, tended to have very low mortality rates of breast cancer (40). Poor countries also tend to have higher fertility rates at younger ages than industrialized countries (64), which could further lower the risk of breast cancer. It is possible that the exclusion of these countries may have weakened an ecological association between UVB irradiance and breast cancer mortality.

In addition, it is possible that geographic variation in per capita cigarette consumption and alcohol intake among women may account for a large part of the association between UVB irradiance and breast cancer mortality rates. However, sex-specific data on alcohol consumption and smoking were not available. Furthermore, the data on per capita cigarette consumption and alcohol intake were for both men and women combined, making it a reasonable approximation of female per capita cigarette consumption and alcohol intake.

Ecological studies should be considered as hypothesis-generating, rather than definitive. They are potentially the source of variables to be investigated with other methods. On the other hand, the diverse geographic distribution of populations in areas
with different levels of UVB irradiance provides a natural experiment on a large scale. Natural experiments are sometimes of value in identifying relevant factors for a disease. For example, ecological comparisons of areas with high fluoride levels in drinking water with areas with low fluoride levels showed that fluoridation could reduce the incidence of dental caries.

In this study, there was an association between low UVB irradiance and breast cancer mortality rates. This could be due to geographic variation in population levels of serum 25(OH)D resulting from differences in UVB irradiance. However, other factors are likely to also contribute to mortality risk. Available evidence from this and other studies suggests that inadequate vitamin D status plays an important role in the etiology of breast cancer. Leadership from the public health community is needed to ensure adequate oral intake of vitamin D3 and to encourage cautious exposure to sunlight in women whose skin type allows them to tan readily and not burn easily. Further investigation is warranted, particularly studies of the association of breast cancer risk and mortality with prediagnostic serum 25-hydroxyvitamin D levels in individuals.
Figure 1.2. Breast cancer mortality rates by latitude, 2002.
Figure 1.3 Dose-response relationship between modeled serum 25(OH)D and breast cancer mortality rates in 173 countries. 2002. Country codes same as in Figure 1.2.
Table 1.1  Solar ultraviolet B irradiance and other covariates in association with age-standardized breast cancer mortality rates, 107 countries, 2002

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Regression coefficient</th>
<th>Standard error</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solar UVB irradiance, Watts/m²&lt;sup&gt;*&lt;/sup&gt;</td>
<td>-0.4631</td>
<td>0.2209</td>
<td>-2.10</td>
<td>0.04</td>
</tr>
<tr>
<td>Per capita alcohol consumption&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.0133</td>
<td>0.0085</td>
<td>1.56</td>
<td>0.12</td>
</tr>
<tr>
<td>Proportion of female population overweight&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>0.0265</td>
<td>0.0308</td>
<td>0.86</td>
<td>0.39</td>
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<tr>
<td>Per capita cigarette consumption&lt;sup&gt;§&lt;/sup&gt;</td>
<td>0.0012</td>
<td>0.0009</td>
<td>1.43</td>
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</tr>
<tr>
<td>Total fertility rate per 1000 women&lt;sup&gt;¶&lt;/sup&gt;</td>
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<td>0.4525</td>
<td>3.37</td>
<td>0.001</td>
</tr>
<tr>
<td>Per capita health expenditure&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.0012</td>
<td>0.0008</td>
<td>1.43</td>
<td>0.16</td>
</tr>
<tr>
<td>Intercept</td>
<td>9.8904</td>
<td>3.1895</td>
<td>3.10</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Table 1.1 continued

\[ R^2 = 0.34, \ p < 0.0001 \]

*Adjusted for cloud cover. Source: Calculated using solar irradiance data from Columbia University (60) and cloud cover data from the NASA International Satellite Cloud Climatology Project (ISCCP) (62).

†Source: United Nations Food and Agriculture Organization (63).

‡Source: World Health Organization (65).

§Source: The Tobacco Atlas (67).

¶Source: World Health Organization (64).

**Source: World Health Organization (66).

Chapter 1 is being prepared for journal submission:

CHAPTER 2

Serum 25-hydroxyvitamin D and breast cancer in the military:

A case-control study utilizing pre-diagnostic serum

by

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Running head: 25-hydroxyvitamin D and breast cancer

Length: Abstract 226 words; Text: 3,497 words; Tables: 2 Figures: 1
ABSTRACT

Background. The objective of this study was to test whether a relationship exists between pre-diagnostic serum levels of 25-hydroxyvitamin D (25(OH)D) and subsequent risk of breast cancer in active-duty U.S. military personnel.

Methods. 600 incident cases of female breast cancer were matched to 600 female controls as part of a case-control study that utilized pre-diagnostic sera. Conditional logistic regression was used to assess the relationship between serum 25(OH)D concentration and breast cancer risk, while controlling for race and age.

Results. The cut points for quintiles of serum 25(OH)D concentration were < 15, 15-21.7, 27.4-35.1, and > 35.1 ng/ml. There was an uneven trend toward an inverse association between serum 25(OH)D and odds of breast cancer that did not reach statistical significance overall. However, in the 123 pairs for whom serum was collected 90 days or fewer before diagnosis of the case, women in the highest quintile of serum 25(OH)D had 70% lower estimated risk of breast cancer (odds ratio 0.30, 95% confidence interval 0.12-0.74, p < 0.01) compared to those in the lowest.

Conclusions. In this study, the favorable (inverse) association of serum 25(OH)D with risk of breast cancer suggests that the influence of serum 25(OH)D on risk of breast cancer in adult women was strongest during the final few doublings of the tumor mass preceding diagnosis. Further epidemiological research, including prospective studies, of the relationship between serum 25(OH)D status and breast cancer risk, and its timing, should be performed to confirm this association.
Introduction

Breast cancer is the most commonly occurring neoplasm among women in the United States with an estimated 192,000 cases and 40,000 deaths occurring in 2010 (86). Previous research has shown a relationship between lower breast cancer incidence or mortality rates and high levels of sunlight or ultraviolet B radiation (UVB) (13, 18, 87-90). Exposure to UVB results in the photosynthesis of pre-vitamin D₃ in the skin (13, 14, 18, 20, 87, 91) and production of 25-hydroxyvitamin D [25(OH)D], the principal circulating vitamin D metabolite, in a dose-dependent manner (45). In addition to the protective effect of increased UVB radiation exposure vis a vis breast cancer, a beneficial effect of increased intake of supplemental vitamin D has also been demonstrated (19, 55, 92-94). Several cancers, including those of the breast (23, 26-33), colon (95), and ovary (96) have been linked to the main marker of vitamin D deficiency: low levels of serum 25(OH)D (97). These findings led to the hypothesis that higher levels of 25(OH)D may reduce a woman’s risk of developing breast cancer.

In an effort to test this hypothesis, several epidemiological studies of the effect of 25(OH)D levels on breast cancer risk have been undertaken. So far, the evidence in favor of vitamin D has been overwhelmingly supportive. Eight epidemiological studies found a significant inverse association between increased levels of serum 25(OH)D and risk of breast cancer (23, 26-33), while four failed to detect a significant association (23-25, 34). Furthermore, a recent meta-analysis of eleven case-control studies found that lower breast
cancer risk was significantly associated with higher serum levels of 25(OH)D across all studies included in the combined analysis (39).

The high prevalence of vitamin D deficiency, combined with the discovery of increased risks of certain types of cancer in those who are deficient, suggests that vitamin D deficiency may account for tens of thousands of cases from breast cancer annually (97). Furthermore, by confirming the association between low serum 25(OH)D levels and increased breast cancer risk, this study will help to raise public awareness about the importance of maintaining adequate serum 25(OH)D levels. As a result, much needless suffering can be prevented, while at the same time achieving substantial economic savings, in both the military and civilian setting (98).

The main objective of this study is to test whether a relationship exists between pre-diagnostic serum levels of 25-hydroxyvitamin D (25(OH)D) and subsequent risk of breast cancer in active-duty U.S. military personnel. This study will also quantify the dose-response relationship between serum 25(OH)D and subsequent risk of breast cancer using previously collected stored sera. This will allow for determination of the optimal circulating 25(OH)D levels needed to reduce breast cancer risk among US military personnel. Although research suggests that maintaining adequate serum vitamin D levels may reduce the risk of breast cancer, there is a lack of data regarding the dose-response relationship between serum levels of 25(OH)D and risk of breast cancer.
Methods

This study employed a case-control design and utilized pre-diagnostic serum from 600 incident cases of female breast cancer and 600 controls. Serum was collected from all active-duty members of the US military who were screened as part of a comprehensive serum screening program conducted during 2002-2008. The samples were collected for military health surveillance and epidemiologic research and were frozen into 0.5 ml aliquots and stored in the Department of Defense Serum Repository (99). The data were stored in the Defense Medical Surveillance System (DMSS), the central repository of medical surveillance data for the US Department of Defense. This secure repository contains current and historical data on hospitalizations and outpatient medical encounters during the military career, and demographic information. It has been used extensively for epidemiological research (99).

Cases were ascertained from a comprehensive database from all Department of Defense (DoD) medical treatment facilities and Tricare civilian hospitals worldwide. A case was identified as an active-duty military member diagnosed with breast cancer (ICD 9-CM 174.0 – 174.9). Both the case and matched control were on active-duty military service during 1994-2009. The case definition required (a) being hospitalized with a discharge diagnosis of breast cancer certified by a physician or (b) 3 or more outpatient medical care visits with a primary diagnosis of breast cancer. Controls were individually matched to cases according to the date the blood sample was drawn (± 2 days); age (± 1 year); length of service (± 30 days); and whether the control was on active duty on the date the case was diagnosed. The most recent serum sample preceding the date of
diagnosis of breast cancer was obtained for each case and its matched control. If more
than one potential control met the criteria, the one whose date of serum collection was
closest to that of the case was selected.

**Laboratory procedures**

Blood samples were collected in plain tubes, allowed to clot, and serum was
separated from cells using routine centrifugation. Serum was divided into 0.5 mL aliquots
in polypropylene cryogenic vials and frozen at –70°C centigrade. It was stored in Revco
freezers equipped with temperature alarms. Sera were analyzed by a major laboratory
(Ames IA: Heartland Laboratories), using the Diasorin LIAISON radioimmunoassay.
This method is an FDA-approved, direct, competitive chemiluminescence immunoassay
(CLIA) using the DiaSorin LIAISON 25-OH vitamin D total assay (100, 101). This is a
gold standard method for measurement of the serum 25(OH)D concentration (101). It
uses an antibody to 25-hydroxyvitamin D to coat magnetic particles (solid phase), and a
vitamin D analog, 22-carboxy-23,24,25,26,27-pentanorvitamin D₃, linked to an
isoluminol derivative, making it capable of chemiluminescence. 25(OH)D dissociates from
its binding protein in the serum during an incubation period. It then competes with the
isoluminol-labeled analog for binding sites on the antibody. After the incubation, the
unbound material is washed away.

Further reagents are added and a chemiluminescent reaction begins. The intensity
of the light produced is measured by a photomultiplier sensor, and expressed as relative
light units (RLU). Since smaller concentrations of 25(OH)D displace fewer isoluminol-
labeled molecules of the vitamin D analog from the antibody, the intensity of the light is inversely proportional to the concentration of 25-hydroxyvitamin D. The inter-and intra-assay coefficients of variation of this assay are 12.4% and 5.4% respectively, the lower limit of detection is 2.5 ng/ml (101). The laboratory validated its measurements by testing standard aliquots of 25(OH)D provided by the Vitamin D External Quality Assessment Scheme (DQAS), a nonprofit 25(OH)D calibration program (102). The laboratory had no knowledge of case or control status of the samples.

Statistical analysis

Univariate and multivariate analyses were performed using SAS (version 9.2) (Cary NC: SAS Institute). Conditional logistic regression was employed to control for confounding in the multivariate analysis. Quintiles of serum 25(OH)D concentration were defined based on the distribution of 25(OH)D concentrations in the control population. Odds ratios were determined using the lowest quintile of serum 25(OH)D concentration in the control population as the reference category. The criterion for statistical significance was \( p \leq 0.05 \), two-tailed. As a sensitivity analysis, the analyses were repeated after restricting the study population to matched pairs in which the case had their blood drawn no more than 90 days before the diagnosis of breast cancer.

In addition, a dose-response curve was plotted showing the pooled odds ratios for each quintile of the pooled data (103). A least-squares line was drawn to assess the dose-response relationship (104, 105). \( P \)-values for trend were calculated using the Mantel-Haenszel chi-square test (106). 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 0.50. This study was conducted in accordance with the ethical standards of the relevant Department of Defense Institutional Review Board (IRB) and the Helsinki Declaration of 1975, as revised in 1983, and IRB approval was obtained.

Results

There were a total of 600 matched pairs at the beginning and end of this study. In univariate analyses, cases did not significantly differ from controls with respect to mean 25(OH)D levels, distribution of race, rank or age (Table 2.1). When univariate analyses were restricted to pairs where blood draw preceded case diagnosis by up to 90 days, mean 25(OH)D was significantly lower in cases than controls (Table 2.2) and distribution of race was significantly different between cases and controls.

In a multivariate conditional logistic regression model of 600 matched pairs, the relationship between serum 25(OH)D and odds of breast cancer was not statistically significant (Table 2.1) after adjusting for race. In this analysis, race was not significantly associated with odds of breast cancer (Table 2.2). As a sensitivity analysis, the conditional logistic regression model was repeated after restricting the analysis to pairs in which blood draw preceded case diagnosis by up to 90 days. In the sensitivity analysis, a mean serum 25(OH)D level of 35.2 ng/ml was statistically significantly associated with a 70% lower odds of breast cancer compared to the reference category, after controlling
for race (Table 2.3). Results for race were not appreciably different from the main analysis.

The dose-response relationship between serum 25(OH)D and breast cancer risk was estimated after restricting the study population to pairs in which case diagnosis was preceded by blood draw by up to 90 days. Odds ratios for breast cancer according to quintile were calculated using unadjusted data. The mean serum 25(OH)D values in ng/ml for each quintile were as follows: 14.2; 23.2; 28.2; 34.4; and 47.5. The cutpoints for the quintiles in ng/ml were: < 20, 21.1 – 25.5, 25.6 – 30.8, 30.9 – 37.6, and ≥ 37.7. The odds ratios from lowest to highest quintile were: 1.00 (reference), 0.87 (95% confidence interval [CI] 0.3 – 2.5), 0.80 (95% CI 0.3 – 2.4), 0.69 (95% CI 0.2 – 2.0), and 0.47 (95% CI 0.2 – 1.4). Serum 25(OH)D levels accounted for 99% of the variation in breast cancer risk ($R^2 = 0.99$, $p$ for trend = 0.16) (Figure 2.1). According to the dose-response analysis, a mean serum 25(OH)D level of 45 ng/ml (110 nmol/L) was associated with 50% lower risk of breast cancer, compared with a mean level of 14.2 ng/ml (Figure 2.1).

Discussion

In this study, subjects in the highest quintile of 25(OH)D had a 70% lower odds of breast cancer compared to subjects in the lowest quintile when the analysis was restricted to pairs in which blood draw preceded diagnosis of breast cancer in cases by 90 days or less. This finding was statistically significant. When all 600 matched pairs were analyzed, the relationship between high serum levels of 25(OH)D and breast cancer risk was not
statistically significant. There was a perfectly linear, inverse, dose-response relationship between serum 25(OH)D concentration and odds of breast cancer in pairs in which blood draw preceded case diagnosis by up to 90 days (Figure 2.1). This trend was of borderline statistical significance.

The mechanisms by which vitamin D prevents breast cancer are still not completely understood. High serum levels of 25(OH)D may increase serum levels of the most biologically active vitamin D metabolite, 1,25(OH)_2D (49). While the majority of 1,25(OH)_2D synthesis occurs in the kidney and is homeostatically regulated, extrarenal synthesis of 1,25(OH)_2D can occur in a wide range of tissues, including the epithelial tissues of the breast (49). Therefore, a high concentration of serum 25(OH)D may provide a greater amount of substrate for local synthesis of 1,25(OH)_2D in the breast epithelium (49). In addition, vitamin D receptors that are highly sensitive to 1,25(OH)_2D (50) are found in normal breast epithelial cells. In a previous study performed by Janowsky and colleagues, white women in the lowest quartile of 1,25(OH)_2D had an odds of breast cancer that was 5.3 (95% C.I. 2.1, 13.4) times higher than that of women in the highest quartile (17). Moreover, an inverse, albeit non-significant, association was found in other studies (24, 28). However a study by Hiatt and colleagues did not find an association between 1,25(OH)D2 and risk of breast cancer (16).

Evidence from laboratory studies is also supportive of the vitamin D hypothesis. 1,25(OH)_2D has a demonstrated ability to promote differentiation and apoptosis in breast cancer cell lines (51, 52), providing a possible explanation for the apparent protective
effect against breast cancer risk observed in observational studies. Moreover, the ability of 1,25(OH)_{2}D to induce differentiation and apoptosis in existing breast cancer cells may explain the presence of an inverse association in this study and that of Rejnmark and colleagues, (33) as well as numerous, ordinary case-control studies where serum was collected for vitamin D measurement during, or shortly after, diagnosis of breast cancer (23, 26-32).

Among study participants, date of blood draw preceded diagnosis of breast cancer in the case by up to 8 years. Overall, the mean length of time between date of blood draw and diagnosis was 1 year. Therefore, in pairs in which a long period of time elapsed between date of blood draw and diagnosis of the case, the serum 25(OH)D measurement for the case may not have been an accurate representation of 25(OH)D status during the relevant window of time that vitamin D exerts its strongest protective effect against breast cancer. This may also provide a possible explanation of why several nested case-control studies in which serum was collected several years before diagnosis of breast cancer failed to find a statistically significant protective effect of 25(OH)D on breast cancer risk (23-25, 34). Furthermore, in the most recently published nested case-control study on 25(OH)D and breast cancer risk, there was a non-significant, inverse relationship between serum 25(OH)D and breast cancer risk (34). However, the mean time of follow-up between blood collection and diagnosis was 7 years (34).

There was a statistically significant inverse relationship between serum 25(OH)D and odds of breast cancer in pairs in which the case had blood drawn up to 90 days before
a diagnosis of breast cancer. In addition, a strong dose-response relationship in whites, using unadjusted data, was observed. Therefore, it appears that serum 25(OH)D status may be especially influential in the days and weeks preceding the final doubling of the tumor. Another nested-case-control study, in which serum was drawn shortly before diagnosis of breast cancer, had similar findings (33). In the study performed by Rejnmark and colleagues, blood samples were collected shortly before participants underwent mammography; women in the highest quintile of serum 25(OH)D had a 40% lower risk than those in the lowest quantile (33).

One possible explanation for these findings is that 1,25-dihydroxyvitamin D may exert its protective effect at a later stage in the etiology of breast cancer. 1,25(OH)_{2}D has been shown to induce apoptosis and inhibit angiogenesis in breast cancer cells. Therefore, the VDR-mediated anticancer action of 1,25(OH)_{2}D may not begin until after the presence of the tumor mass is well established, which would explain why nested case-control studies with long periods of time between measurement and diagnosis have not found a statistically significant relationship (23-25, 34).

Although the kidneys are the principal site for conversion of 25(OH)D to 1,25(OH)_{2}D via the \(\text{1a} \) hydroxylase, extrarenal synthesis of 1,25(OH)_{2}D also occurs in breast epithelial tissue (108). While renal conversion of 25(OH)D to 1,25(OH)_{2}D is homeostatically regulated, production of 1,25(OH)_{2}D in breast tissue is thought to be largely dependent on circulating 25(OH)D concentration (108). However, in the context of an observational study, the short half-life of 1,25(OH)_{2}D (<24 hours) (109) would
render most informative those measurements of 25(OH)D shortly preceding a diagnosis of breast cancer.

From another perspective, there is a high likelihood that presence of cancerous cells in breast epithelium is ubiquitous in the human population. Therefore, controls with high serum 25(OH)D concentration, and consequently high rates of extrarenal 1,25(OH)_2D synthesis, may have developed tumors that were halted at some critical juncture by the anticarcinogenic action of 1,25(OH)_2D well before such tumors became clinically detectable.

While it is possible that the inverse association in ordinary case-control studies or nested case-control studies where serum was drawn shortly before diagnosis could be due to the disease process itself or a change in lifestyle habits due to the presence of the disease, this is highly unlikely. First, there is no known biological basis for the presence of a tumor in the breast to result in a reduction of circulating serum 25(OH)D. Secondly, most women with undiagnosed breast cancer typically do not exhibit any symptoms that would indicate illness or that would cause a change in lifestyle habits. The overwhelming majority of breast cancer cases are detected through self-exam or mammography, long before the clinical manifestation of any symptoms other than the presence of a lump in the breast or armpit (110).

This study had several advantages. The analytic method used, conditional logistic regression, allows for regression analysis of matched case-control data while controlling
for confounders. This study also had a sample size large enough to be able to detect a statistically significant effect of serum 25(OH)D on breast cancer risk. Moreover, the exposure of interest, serum 25(OH)D concentration, is a biomarker, making its ascertainment more precise than would have been possible through attempting to assess vitamin D status through a questionnaire. In addition, use of pre-diagnostic serum enabled the establishment of a temporal sequence.

The presence of selection bias, a major problem in case-control studies, was minimized in this study by not only matching controls to cases on important factors, but also by the fact that the study cohort was drawn from a military population, which apart from having exposures common to all service members (diet, culture, etc), has physical requirements and standards that apply equally to all members.

This study utilized data from the DMSS, which contains data on hospitalizations and basic demographic information only. Therefore, this study has a number of limitations. The data contained in the DMSS do not include information on subject’s physical activity levels. As a result, we were unable to control for this variable in the regression analysis. Higher levels of physical activity have been associated with lower risk of breast cancer (111). On the other hand, the protective effect of physical activity on breast cancer risk may be a consequence of higher concentrations of serum 25(OH)D due to increased sunlight exposure resulting from exercise performed outdoors. One case-control study on the relationship between ultraviolet sunlight exposure and breast cancer risk, found a significant inverse association between sunlight exposure and breast cancer
risk after adjustment for physical activity and other confounders (112). In another case-control study of 25-hydroxyvitamin D and breast cancer, serum 25(OH)D levels were still significantly, inversely associated with decreased risk of breast cancer after controlling for physical activity (29).

Previous research has suggested that the protective effect of serum 25(OH)D on breast cancer risk is greater in post-menopausal women (26-28, 34). However, because our study population was a relatively young one (mean age 39.6 years), we were unable to assess any interaction between menopausal status and breast cancer risk. Furthermore, due to data limitations, we were unable to include several other variables of interest in studies of breast cancer risk such as parity, menarche, BMI, smoking, breast feeding, and family history of breast cancer. Therefore, we cannot rule out the possibility that the inverse association between 25(OH)D and breast cancer in the present study was not due to a confounder not included in the analysis or due to some form of bias. However, this is unlikely because a significant inverse association was only found in the analysis that was restricted to cases whose blood was collected less than 90 days before diagnosis. Therefore, any confounder or bias would have to also be related to the date of the case’s blood draw relative to diagnosis, which seems unlikely since serum was collected systematically in a military-wide surveillance program, where such measures are usually mandatory.

Consistent with previous research, in pairs in which serum was collected less than 90 days before case diagnosis, women whose serum 25(OH)D concentration was high
(42 ng/ml) had approximately half the odds of breast cancer as those whose serum 25(OH)D was low (11 ng/ml) (Table 2.3). The trend was similar after adjustment for race. The favorable association of serum 25(OH)D with risk of breast cancer was apparently strongest during the final few doublings of the tumor mass preceding diagnosis in this population. Further epidemiological research, including prospective studies, on the relationship between serum 25(OH)D status and breast cancer risk should be undertaken to confirm this association.

A serum 25(OH)D concentration of 42 ng/ml can be achieved with vitamin D3 intake of 4000 IU/day. This is the safe daily upper level intake of the National Academy of Sciences-Institute of Medicine (NAS-IOM December 2010 monograph). Such intake should be recommended as a useful tool for prevention of breast cancer. Serum 25(OH)D should be monitored on a routine basis, when feasible, to ensure that at least 40 ng/ml is maintained throughout the year, and 150 ng/ml is not exceeded. This hygienic measure could prevent many cases of breast cancer in the US.
Figure 2.1. Serum 25(OH)D level and risk of breast cancer, whites, case serum drawn ≤ 90 days before diagnosis
Table 2.1 Characteristics of cases and controls, all subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=600)</th>
<th>Controls (n=600)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>24.8 (12.2)</td>
<td>25.9 (12.3)</td>
<td>0.12</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>314 (26.2)</td>
<td>350 (29.2)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>216 (18)</td>
<td>186 (15.5)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>70 (5.9)</td>
<td>64 (5.3)</td>
<td>0.11</td>
</tr>
<tr>
<td>Rank (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enlisted</td>
<td>370 (30.9)</td>
<td>368 (30.7)</td>
<td></td>
</tr>
<tr>
<td>Officer</td>
<td>230 (19.2)</td>
<td>232 (19.3)</td>
<td>0.91</td>
</tr>
<tr>
<td>Age in years (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-35</td>
<td>156 (13)</td>
<td>152 (12.7)</td>
<td></td>
</tr>
<tr>
<td>36-40</td>
<td>160 (13.3)</td>
<td>164 (13.7)</td>
<td></td>
</tr>
<tr>
<td>41-45</td>
<td>159 (13.3)</td>
<td>156 (13)</td>
<td></td>
</tr>
<tr>
<td>&gt; 45</td>
<td>125 (10.4)</td>
<td>128 (10.7)</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Table 2.2. Characteristics of cases and controls, restricted to pairs where blood draw preceded case diagnosis by up to 90 days

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cases (n=123)</th>
<th>Controls (n=123)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>23.2 (12)</td>
<td>26.7 (12.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>Race (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>59 (23.6)</td>
<td>79 (32.5)</td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>51 (20.8)</td>
<td>30 (12.2)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>13 (5.3)</td>
<td>14 (5.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>Rank (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enlisted</td>
<td>78 (31.3)</td>
<td>73 (30.8)</td>
<td></td>
</tr>
<tr>
<td>Officer</td>
<td>45 (18.3)</td>
<td>50 (20.3)</td>
<td>0.58</td>
</tr>
<tr>
<td>Age in years (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-35</td>
<td>24 (9.8)</td>
<td>23 (9.4)</td>
<td></td>
</tr>
<tr>
<td>36-40</td>
<td>28 (11.5)</td>
<td>29 (11.9)</td>
<td></td>
</tr>
<tr>
<td>41-45</td>
<td>39 (16)</td>
<td>39 (16)</td>
<td></td>
</tr>
<tr>
<td>&gt; 45</td>
<td>31 (12.7)</td>
<td>31 (12.7)</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Table 2.3. Conditional logistic regression analysis of serum 25(OH)D and risk of breast cancer, analysis of all 600 pairs

<table>
<thead>
<tr>
<th>Regression Variable</th>
<th>Regression Coefficient</th>
<th>Standard Error</th>
<th>OR</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean serum 25(OH)D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 14.9 ng/ml</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15 - 21.7 ng/ml</td>
<td>-0.1944</td>
<td>0.1902</td>
<td>0.82</td>
<td>0.57, 1.2</td>
<td>0.31</td>
</tr>
<tr>
<td>21.8 - 27.3 ng/ml</td>
<td>-0.2305</td>
<td>0.2042</td>
<td>0.79</td>
<td>0.53, 1.19</td>
<td>0.26</td>
</tr>
<tr>
<td>27.4 - 35.1 ng/ml</td>
<td>-0.0539</td>
<td>0.2023</td>
<td>0.94</td>
<td>0.34, 1.41</td>
<td>0.79</td>
</tr>
<tr>
<td>&gt;35.2 ng/ml</td>
<td>-0.1737</td>
<td>0.2159</td>
<td>0.84</td>
<td>0.55, 1.3</td>
<td>0.42</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Black</td>
<td>0.1968</td>
<td>0.1517</td>
<td>1.22</td>
<td>0.90, 1.63</td>
<td>0.19</td>
</tr>
<tr>
<td>Other</td>
<td>0.2201</td>
<td>0.2057</td>
<td>1.25</td>
<td>0.83, 1.86</td>
<td>0.28</td>
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</tbody>
</table>
Table 2.3 continued, analysis of 123 matched pairs where blood draw preceded case diagnosis by up to 90 days

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Coefficient</th>
<th>Standard Error</th>
<th>OR</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean serum 25(OH)D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 14.9 \text{ ng/ml} )</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15 - 21.7 ng/ml</td>
<td>-0.5685</td>
<td>0.4195</td>
<td>0.56</td>
<td>0.25, 1.29</td>
<td>0.18</td>
</tr>
<tr>
<td>21.8 - 27.3 ng/ml</td>
<td>-0.6807</td>
<td>0.4245</td>
<td>0.51</td>
<td>0.22, 1.16</td>
<td>0.11</td>
</tr>
<tr>
<td>27.4 - 35.1 ng/ml</td>
<td>-0.2653</td>
<td>0.4091</td>
<td>0.77</td>
<td>0.34, 1.71</td>
<td>0.51</td>
</tr>
<tr>
<td>( \geq 35.2 \text{ ng/ml} )</td>
<td>-1.2038</td>
<td>0.4575</td>
<td>0.30</td>
<td>0.12, 0.74</td>
<td>0.01</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>-</td>
<td>-</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Black</td>
<td>0.2226</td>
<td>0.2146</td>
<td>1.25</td>
<td>0.82, 1.9</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Chapter 2 is being prepared for journal submission:

CHAPTER 3

Serum 25-hydroxyvitamin D and prevention of breast cancer: Pooled analysis

by

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Running head: 25-hydroxyvitamin D and breast cancer

Length: Abstract 150 words; Text: 4,250 words; Tables: 1 Figures: 3

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ABSTRACT

Background/Aim: Low serum levels of 25-hydroxyvitamin D [25(OH)D] have been associated with 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 review of observational studies, a serum 25(OH)D level of 47 ng/ml was associated with a 50% lower risk of breast cancer.
Introduction

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 (86). 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 (13, 14, 18, 20, 87, 91). 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 (55).

Ultraviolet B is needed to make vitamin D, which is photosynthesized by the skin. Exposure to UVB and supplemental vitamin D intake increase serum 25(OH)D levels in a dose-dependent manner (45). Lack of exposure to UVB through sunlight or everyday use of sunscreens may result in vitamin D deficiency unless there is adequate oral intake (46). 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 cancers including cancer of the breast (47).

Normal breast epithelial cells have a vitamin D receptor that is highly sensitive to 1,25(OH)₂D (50). Numerous laboratory studies have demonstrated the ability of 1,25(OH)₂D to promote differentiation and apoptosis in breast cancer cell lines (51, 52).
In 2010, Yin and colleagues extracted data from nine case control studies on the relationship between serum 25(OH)D levels and risk of breast cancer (36). 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 or nested case-control studies on 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 “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 (17, 23-34, 56). One study that examined the association between 25(OH)D and breast cancer risk did not report odds ratios but reported no effect (17). Two studies that investigated the relationship between 1,25(OH)_{2}D and breast cancer risk were also identified. Of these, one found a strong
inverse association with risk of breast cancer (17) and the other found no association (16).

The study published by Colston and colleagues did not provide cell frequencies and utilized the same cases and controls that were used by Lowe and colleagues. The study by Green and colleagues (31) was left out of the analysis because it was a subset of the larger study by Bertone-Johnson and colleagues (28). Therefore, these studies were excluded (31, 32, 56), leaving a total of eleven case-control or nested case-control studies on 25(OH)D status and breast cancer risk. Data from these studies were independently extracted by two investigators, pooled, and divided into quintiles of 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 (113). 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 (114).

Sensitivity analysis was performed to assess an association 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 done 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, two nested case-control studies have been published (30, 31). This further necessitated new calculations of pooled odds ratios according to study design.

One major study reported finding a statistically significant inverse effect of 25(OH)D in individuals residing at latitudes > 37 degrees N (25). 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. This is the standard method for calculating the $p$-value using Peto’s Assumption-Free Method (113). Odds ratios comparing the highest with the lowest quantiles for each study were displayed in a forest plot (115, 116). Odds ratios from the most highly adjusted models were chosen from each study. Confidence intervals were computed using the method of Woolf (103). The DerSimonian-Laird statistic was calculated to assess heterogeneity (117). The calculations were performed using Rev Man 5 (Oxford, England: 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 non-case, the
midpoint or median value of the participant’s quantile of serum 25(OH)D 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 (24-27, 29, 30, 33), it was used. If not (23, 28, 31, 32, 56), 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 cutpoints: 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 quintile of serum 25(OH)D and risk of breast cancer (118). 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 computed using the method of Woolf (103).

A dose-response curve was plotted showing the pooled odds ratios for each quintile of the pooled data (103). A least-squares line was drawn to assess the dose-response relationship (104, 105). $P$-values for trend were calculated using the Mantel-Haenszel chi-square test (106). 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 0.50. Computations were performed using SAS, Version 9.2 (Cary NC: SAS Institute).

Results

To date, twelve 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 (23-34). Of these, eight found a significant association between higher levels of serum 25(OH)D and a reduced risk of breast cancer (26-33), while four failed to detect a significant association (23-25, 34).

Eleven studies that analyzed risk of breast cancer by quartiles 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 3.1). Serum 25(OH)D levels accounted for 76% of the variation in breast cancer risk ($R^2 = 0.76$, $p$ for trend < 0.001). The odds ratios for the pooled data from lowest to highest quintile were: 1.00, 0.78, 0.71, 0.66, and 0.67 ($p$ trend < 0.001)(Table 3.1). According to the pooled analysis, a serum 25(OH)D $\geq 47$ ng/ml (110 nmol/L) was associated with 50% lower risk of breast cancer risk, compared with < 10 ng/ml (Figure 3.1). This would also correspond to an approximately 10% reduction in risk for every 10 ng/ml increase in 25(OH)D.
The overall Peto odds ratio summarizing the estimated risk in the highest compared to the lowest quantile across all studies was 0.61 (95% C.I. 0.47, 0.80) (Figure 3.2). In the Forest plot analysis, the eleven 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 odds ratio was 0.87 (95% C.I. 0.77, 0.99) (Figure 3.2). 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 odds ratio was 0.41 (95% C.I. 0.31, 0.56) (Figure 3.2). 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 \( \geq 37 \) degrees North, the pooled odds ratio comparing the top with the bottom quintile of 25(OH)D was 0.56 (95% C.I. 0.50, 0.62)(Figure 3.3). These studies were heterogeneous (Chi-square = 58, df = 7, \( p = 0.001 \))(Figure 3.3). 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 and colleagues, 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 and colleagues, subjects in the highest category of serum 25(OH)D (37.4ng/ml) had a 7% lower risk of developing breast cancer (OR 0.93; 95% C.I. 0.66, 1.33) compared to subjects in the lowest category of serum 25(OH)D (18.1 ng/ml) (34), 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% C.I. 0.37, 0.96) than subjects with a 25(OH)D level < 19.8 ng/ml (30). This finding persisted in spite of adjustment for physical activity, a variable that has been shown to have considerable influence on 25(OH)D status (119).

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, subjects in the highest category of 25(OH)D had an overall reduction in risk of breast cancer of approximately 39% compared to subjects in the lowest category of serum 25(OH)D (Figure 3.2). 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 3.1).

When pooled odds ratios 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 case-control and nested case control studies (Figure 3.1). This finding differed in magnitude, but not direction, from those of Yin and
colleagues. 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 performed their meta-analysis.

Pooled odds ratios were calculated using random effects models, which provide a more conservative estimate of the pooled odds ratio. The random effects model incorporates inter-study variance into the estimate and is the most appropriate method when dealing with heterogeneous studies (113). 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 (36). 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 changes in dietary and other lifestyle habits as a result of the disease, possibly creating a spurious association (36).

However, nearly 80% of incident cases of breast cancer are discovered as a result of self-examination when a lump is found in the breast or armpit (110). 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 and colleagues, 25(OH)D was measured shortly before diagnosis, when changes in lifestyle habits that could influence 25(OH)D were unlikely to have occurred. In this study, women in the highest quintile of serum 25(OH)D had a 40% lower risk than those in the lowest quantile (33).

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 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 (59). The analyses of serum 25(OH)D concentrations by Goodwin and colleagues 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, or behavioral changes resulting from the diagnosis, influenced 25(OH)D concentration, it is highly unlikely that Goodwin and colleagues 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 (120, 121). Furthermore, Abbas and colleagues 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 (25, 27).
All of the ordinary case-control studies on 25(OH)D and breast cancer risk were performed in populations residing in a single, well-defined geographic area above 37 degrees North latitude. All of these studies found a beneficial association between serum 25(OH)D and breast cancer risk (Figure 3.3). One possible explanation is that measurement of serum 25(OH)D at a single point in time, 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 (25). The case-control study performed by McCullough and colleagues, was nested in the Cancer Prevention Study (CPS) II cohort, with volunteers from throughout the United States participating. In that study, subjects 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 North, a finding that was highly statistically significant (25).

In order to explore the effect of latitude, we performed a sub-analysis in which the pooled odds ratio was calculated for studies performed at latitudes >37 degrees North, including the data from the study done by McCullough and colleagues (Figure 3.3). Seven studies were included in this analysis. The data from McCullough and colleagues’ sub-analysis of latitude was included as a separate study. All seven studies found a significant reduction in breast cancer risk in women in the highest quintile of 25(OH)D compared to women in the lowest quintile. The overall pooled odds ratio was 0.50 (95% C.I. 0.38, 0.65). Interestingly, the nested case-control studies performed in fixed
populations residing at a latitude >37 degrees North, found a statistically significant inverse association between 25(OH)D levels and breast cancer risk (25, 30, 33).

The nested case-control studies performed by Bertone-Johnson and colleagues and Green and colleagues found an inverse association between 25(OH)D and breast cancer risk, despite the studies being done in a cohort of individuals spread throughout the country. One possible explanation for this is that in both studies, the participants were nurses. Vitamin D insufficiency is common among healthcare professionals, even those living at very sunny latitudes (122, 123).

In the McCullough study, an association of 25(OH)D with breast cancer risk was not present in women residing at latitude < 37 degrees North. The investigators acknowledged that while 95% of the subjects residing in the north were born in the north, 36% of the women living in the south were born in the north (25). One possible reason that the other nested case-control study, performed by Freedman and colleagues (24) did not find an association between 25(OH)D and breast cancer risk is that it was performed in a cohort 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 (124).
This 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 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 (23, 26-30), 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 in 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 in 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 (124). Furthermore, although the authors obtained the most highly adjusted odds ratios (adjusted for BMI, use of HRT, age at menarche etc), 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. First, 25(OH)D plays an important role in the up-regulation of e-cadherin (125), a glue-like substance that keeps cells bound tightly together, and in a well-differentiated state. Second, high serum levels of 25(OH)D may provide a greater amount of substrate for synthesis of 1,25(OH)₂D, the most biologically active vitamin D metabolite (126). 1,25(OH)₂D is synthesized in a wide range of tissues, including the epithelial tissues of the breast (49).

In the estimation of the dose-response gradient, a serum 25(OH)D level of > 44 ng/ml would be associated with 50% lower risk of breast cancer, compared to serum 25(OH)D < 9.7 ng/ml (Figure 3.1). Classical dose-response curves for micronutrients are either linear (127) or have a predominantly linear middle segment (104, 105). This
appears to be true for most functions of vitamin D (128, 129). 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 (130). The median minimum threshold for toxicity in all studies was nearly twice this value, 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 Adverse Health Effect Level of 10,000 IU/day of vitamin D have been established by the Institute of Medicine (131). Also, the National Academy of Sciences reported that no hypercalcemia from vitamin D intoxication has been described for vitamin D intakes < 10,000 IU/day (132). Another review reported that no cases of toxicity were documented at doses < 40,000 IU per day (128).

A vitamin D$_3$ 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 (132) would increase median serum 25(OH)D by only 4-6 ng/ml (42), 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 (133). Therefore, an increase of vitamin D intake to 2,000 – 4,000 IU/day of vitamin D₃ 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 (42). 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 (42). 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 (128-130, 132, 134, 135). Increasing serum 25(OH)D to 50 – 60 ng/ml would be most efficiently achieved by intake of vitamin D₃ (cholecalciferol) rather than vitamin D₂ (ergocalciferol) (136).

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 (13, 14, 18, 20, 87, 91), observational studies linking deficient serum 25(OH)D levels with increased risk (19, 80), studies linking low oral intake of vitamin D with increased risk, and laboratory studies illuminating the mechanisms in vivo and vitro (51, 52). Also, vitamin D receptor polymorphisms that interfere with vitamin D are associated with increased risk of breast cancer, particularly in combination with low levels of serum
25(OH)D (32). 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 both 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 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 metabolites 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 D₃ (cholecalciferol) as a breast cancer primary prevention strategy would be highly effective and safe (132).
Figure 3.1. Pooled analysis of studies of serum 25(OH)D concentration and risk of breast cancer

- $P_{\text{trend}} < 0.001$
- $R^2 = 0.76$
- 50% projected reduction in incidence with 47 ng/ml
Figure 3.2. Pooled odds ratios of breast cancer risk according to serum 25(OH)D, 1966-2010, comparing highest to lowest quintile.
Figure 3.3. Pooled odds ratios of breast cancer risk according to serum 25-hydroxyvitamin D, 1966-2010, comparing highest vs lowest quintile, studies based on populations residing at >37 degrees N latitude
Table 3.1 All known studies of serum 25-hydroxyvitamin D metabolites and risk of cancer of the breast, ICD-CM Code 174, according to PubMed search, 1966 - 2010

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<th>Country</th>
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<td>Age</td>
</tr>
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<td>USA</td>
<td>Age, race, date of blood draw</td>
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*Abbreviations: CC, case-control; NCC, nested case-control

†Median values; cutpoints were not provided
Chapter 3 has been published:

CHAPTER 4

Does the evidence for an inverse relationship between serum vitamin D status and breast cancer risk satisfy the Hill criteria?

by

Sharif B. Mohr, M.P.H. 1,2

Edward D. Gorham, M.P.H., Ph.D., John E. Alcaraz, Ph.D., Christopher I. Kane, M.D., Caroline A. Macera, Ph.D., J. Kelley Parsons, M.D., Deborah L. Wingard, Ph.D., Cedric F. Garland, Dr.P.H., F.A.C.E.

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Running head: 25-hydroxyvitamin D and breast cancer

Length: Abstract 150 words; Text: 4,250 words; Tables: 1 Figures: 3

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ABSTRACT

A wide range of epidemiologic and laboratory studies combined provide compelling evidence of a protective role of vitamin D on risk of breast cancer. This review evaluates the scientific evidence for such a role in the context of the A.B. Hill criteria for causality, in order to assess the presence of a causal, inverse relationship, between vitamin D status and breast cancer risk. After evaluation of this evidence in the context of Hill’s criteria, it was found that the criteria for a causal relationship were largely satisfied. Studies in human populations and the laboratory have consistently demonstrated that vitamin D plays an important role in the prevention of breast cancer. Vitamin D supplementation is an urgently needed, low cost, effective, and safe intervention strategy for breast cancer prevention that should be implemented without delay. In the meantime, randomized controlled trials of high doses of vitamin D₃ for prevention of breast cancer should be undertaken to provide the necessary evidence to guide national health policy.
In agreement with previous epidemiological research on the relationship between serum 25(OH)D concentration and breast cancer risk including ecological studies, case-control studies, and several pooled analyses, the results of these three studies indicate the presence of a strong protective effect of high serum 25(OH)D levels on breast cancer risk.

**Ecological analysis (Chapter 1)**

In the ecological analysis, intensity of UVB irradiance, the principal source of circulating vitamin D in humans, was independently, inversely associated with mortality rates of breast cancer in the 107 countries for which complete data were available. This association persisted after adjustment for several confounders known to be important in the etiology of breast cancer, such as per capita cigarette consumption, prevalence of obesity, and per capita alcohol consumption. Furthermore, the findings in this analysis were consistent with those of previous ecological studies that examined the association between latitude or UVB irradiance and incidence or mortality rates of breast cancer (13, 14, 18, 87).

**Case-control study (Chapter 2)**

In the case-control study that assessed the relationship between serum 25(OH)D concentration and breast cancer risk using pre-diagnostic sera in an active duty military
population, there was an inverse association between serum 25(OH)D and estimated risk of breast cancer, but it did not reach statistical significance ($p = 0.42$). However, in a subgroup analysis restricted to pairs in which serum was collected 90 days or less before diagnosis of the case, there was a 70% reduction in estimated risk of breast cancer in women in the highest quintile of 25(OH)D concentration compared to women in the lowest quintile ($p = 0.01$). Furthermore, there was a strong linear, inverse gradient of odds of breast cancer with increasing serum 25(OH)D concentration in this group (Manuscript 3, figure 1).

The finding in the subgroup analysis suggests that the influence of serum 25(OH)D on risk of breast cancer in adult women may be strongest during the final few doublings of the tumor mass preceding diagnosis. A forest plot from the pooled analysis also demonstrated that the inverse association was much stronger in ordinary case-control studies than in nested case-control studies (Manuscript 2, figure 2).

One possible explanation is that the inverse association between serum 25(OH)D and risk of breast cancer observed in the present nested case-control study could have been due to an effect of vitamin D that occurs mainly when the tumor is actively recruiting blood vessels and, possibly, lymphatics. Vitamin D has been shown to prevent this recruitment of vasculature in endothelial cells (138). Addition of 1,25(OH)$_2$D to tissue culture prevented neo-vascularization of endothelial cells in a model where angiogenesis was stimulated by Vascular Endothelial Growth Factor (VEGF) (138).
1,25(OH)₂D also caused regression of blood vessels that had begun to spread from the tumor (138). This inhibition of neo-vascularization has not, to the author’s knowledge, been examined in breast epithelial cells, but the effect in endothelium provides an intriguing analogy for a similar effect on breast epithelium. In the absence of an adequate concentration of anti-carcinogenic vitamin D metabolites, recruitment of a rich blood supply could produce a growth spurt in the tumor, allowing it to grow rapidly to the threshold diameter for reliable mammographic detection, which is 1-2 cm (139). It might also provide the growing tumor with access to the circulation, and possibly the lymphatic circulation, creating a pathway for remote metastasis.

If there is no inhibition of neo-angiogenesis, breast cancers grow at a logarithmic rate (140). Doubling time is the product of the inverse of the growth rate multiplied by the natural logarithm of 2. The most commonly observed doubling time is 3 months, although the mean is slightly longer (5 months). Therefore, the median time interval observed in the subgroup analysis in the case-control study between blood draw and diagnosis, roughly equals the most common time interval required for the tumor mass to double. For tumors of 0.5 cm diameter, the lesion usually requires 3 months to become detectable (140). It is possible that the anti-carcinogenic action of vitamin D metabolites is greatest in preventing the last doubling before clinical detection of the tumor. Such a phenomenon would produce the type of association that has been previously reported: namely, a strong, dose-dependent association for serum 25(OH)D during the period, measured in months rather than years, preceding clinical detection.
An alternative explanation for the strong inverse association observed in ordinary case-control studies is that of reverse causation. In other words, that the tumor itself is somehow responsible for lower serum 25(OH)D levels through a biological and/or behavioral pathway. For example, the neoplasm could result in lower 25(OH)D concentration by consuming an excess of circulating 25(OH)D. However, there is no evidence that such a phenomenon exists. Furthermore, the vast majority of incident breast cancers do not initially produce clinical symptoms other than the presence of a lump. Therefore, it seems unlikely that reverse causation would be due to behavioral changes, such as people with an occult tumor avoiding the sun (spending time indoors convalescing, for example) as a result of symptoms associated with having breast cancer.

**Meta-analysis (Chapter 3)**

In the meta-analysis of ordinary case-control and nested case-control studies on the association between serum 25(OH)D concentration and breast cancer risk, data from 11 epidemiological studies were abstracted, pooled, and re-analyzed. In addition, the pooled dose-response relationship between 25(OH)D and breast cancer risk was determined. In this analysis, there was a 39% lower estimated risk of breast cancer comparing the highest versus lowest category of 25(OH)D concentration across all studies ($p<0.0001$). When the analysis was stratified according to latitude and study design, results were even more remarkable. Across ordinary case-control studies, there was a 59% lower estimated risk for subjects in the highest versus lowest quintile ($p<0.0001$). When the analysis was restricted to studies that were performed in populations residing at latitude $>37$ degrees N, which included both ordinary and nested case-control
designs, there was a 44% lower risk ($p < 0.0001$). There was a 13% lower estimated risk across nested-case control studies ($p < 0.04$).

After using pooled data to plot odds ratios by median serum 25(OH)D level, clear evidence of a dose-response relationship emerged after fitting a trend line to the data points (Figure 3.1). According to this dose-response analysis, 76% of the variation in breast cancer risk could be explained by serum 25(OH)D concentration. According to this analysis, there would be a projected 50% reduction in breast cancer incidence if population serum 25(OH)D levels were raised to 47 ng/ml.

Since publication of the meta-analysis in 2011 by Mohr and colleagues (39), another nested case-control study was published that did not show a statistically significant effect of serum 25(OH)D on breast cancer risk. However, similar to the nested case-control studies included in the pooled analysis, the mean duration of the interval between blood draw and diagnosis was about 5 years (137). This may have resulted in misclassification of exposure due to the vitamin D measurement not being an accurate indication of 25(OH)D levels during the relevant time period that vitamin D status is most influential in preventing cancer.

The role of vitamin D in prevention of breast cancer is strongly supported by six important lines of evidence. These lines of evidence all intersect at the conclusion that vitamin D and its metabolites play a paramount role in the prevention of breast cancer:
1. Four studies that found a positive association between latitude or UVB irradiance and breast cancer (13-15, 18).

2. Seven ordinary case-control studies of serum 25(OH)D and breast cancer risk (26, 27, 29, 32, 33).

3. Two nested case-control studies of serum 25(OH)D and breast cancer risk (28, 30).

4. Substantial evidence from laboratory studies (141).

5. Five studies of oral intake of vitamin D and risk of breast cancer in humans (19, 55, 93, 142, 143).

6. A randomized controlled trial that identified a 77% reduction in overall incidence of all invasive cancers in postmenopausal women, including breast cancer (144).

Furthermore, when the scientific evidence regarding the role of serum 25-hydroxyvitamin D concentration in prevention of breast cancer is taken as a whole and framed in the context of Hill’s criteria, there is a compelling case for a causal, inverse relationship. In epidemiology, the nine criteria postulated by Hill are used to determine whether or not a causal relationship exists between a given exposure and disease. These criteria are:

1. Presence of a temporal relationship. The exposure must precede the disease
2. **Strength of the association.** This is the magnitude of the relationship between the exposure and disease, usually expressed by the relative risk or odds ratio in epidemiological studies.

3. **Presence of a dose-response relationship.** Increasing or decreasing exposure to a given factor results in a corresponding increase or decrease in risk of the disease.

4. **Consistency.** The results of studies investigating the relationship between a given exposure and disease are consistent across most or all studies.

5. **Biological plausibility.** The relationship between a given exposure and disease fits with current scientific knowledge of the biological mechanisms of that disease.

6. **Consideration of alternative hypotheses.** Alternative hypotheses regarding the cause of a given disease must be considered and ruled out before inferring a causal relationship between the disease and the exposure of interest.

7. **Experiment.** The disease can be prevented or treated by administration of the appropriate agent or lack thereof.

8. **Specificity.** The disease of interest is the result of one exposure or agent.

9. **Coherence.** The association between the exposure and disease of interest does not conflict with current knowledge of the etiology of the disease.
Temporal relationship

The first criterion in establishing causality is the presence of a temporal relationship. In other words, if a given exposure is thought to cause a disease, then the exposure must precede the onset of disease. In studies of serum 25(OH)D and breast cancer, this criterion is satisfied. In the randomized controlled trial performed by Lappe and colleagues (144), 1,179 cancer-free women receiving 1,100 IU/day of vitamin D₃ experienced a 77% lower risk from all cancers (including breast cancer) over a four year period. Overall, there were 9 cases of cancer in the vitamin D group compared to 15 cases in the placebo group.

In the meta-analysis of 11 case-control and nested case control studies by Mohr and colleagues (39), there was a 13% reduction in risk of breast cancer in women in the highest quantile vs. the lowest quantile of serum 25(OH)D across all studies that used pre-diagnostic sera to measure vitamin D status ($p = 0.04$). In all of the nested case-control studies, serum 25(OH)D was measured before case diagnosis, with mean time between serum draw and case diagnosis ranging from 3-7 years. Therefore, serum 25(OH)D measurements may not have been representative of 25(OH)D levels during the relevant window of time in which vitamin D is most active against the development of a tumor. Although the Rejnmark study was not considered a nested case-control study in the meta-analysis performed by Mohr and colleagues, blood samples for vitamin D measurement were obtained before diagnosis of breast cancer via mammography and biopsy (33). In the Rejnmark study, women with serum 25(OH)D concentrations greater
than 34 ng/ml had a 48% lower estimated risk compared to women with less than 24
ng/ml.

In contrast, the effect of serum 25(OH)D concentration on risk was much stronger
in ordinary case-control studies where serum 25(OH)D levels were measured during or
shortly after diagnosis. While an alternative explanation for the strong inverse association
observed in these studies is that the breast neoplasm may be responsible for lower serum
25(OH)D levels, this is highly unlikely and there is no biological basis or scientific
evidence to support it. Furthermore, in the study performed by Abbas and colleagues,
there was a 50% lower risk of breast cancer in women in the highest quartile of serum
25(OH)D compared to the lowest ($p = 0.001$). This result did not change when cases
whose 25(OH)D concentration was measured greater than six months after diagnosis
were excluded (26).

**Strength of Association**

A strong relationship between exposure and disease is necessary to satisfy this
criterion. The inverse association between serum 25(OH)D and risk of breast cancer
ranged from an odds ratio of 0.20 (95% C.I. 0.1 – 0.5) (32), corresponding to an 80%
lower risk for the highest vs lowest quantile of 25(OH)D concentration, to a non-
statistically significant odds ratio of 1.20 (95% C.I. 0.9 to 1.6) (137). However, in the
meta-analysis performed by Mohr and colleagues, there was an overall 46% lower risk
(pooled odds ratio 0.63, $p < 0.0001$) across all studies, including ordinary and nested
case-control designs (39). When the analysis was restricted to ordinary case-control
studies, there was an even lower risk of breast cancer when comparing subjects in the highest vs lowest quantile of 25(OH)D concentration (pooled odds ratio 0.41, \( p < 0.0001 \)). In addition, there was a 13% reduction in risk (pooled odds ratio 0.87, \( p < 0.04 \)) when only nested case-control studies were included in the pooled analysis. This is important because the effect was still present in these studies despite that serum 25(OH)D levels were often measured years before case diagnosis and may not have been an accurate representation of 25(OH)D levels during the relevant period of time for cancer prevention, resulting in non-differential misclassification of exposure, which would increase the tendency toward a null finding.

The inverse relationship between serum 25(OH)D concentration and breast cancer risk is of a sufficiently high magnitude to satisfy this criterion. Moreover, by linear extrapolation, the estimated reduction in risk for breast could possibly be as high as 90% at a serum 25(OH)D concentration of 85 ng/ml, a level that is still well below the threshold at which adverse effects are observed (145).

**Presence of a dose-response relationship**

Seven of the eleven published case-control studies of 25(OH)D levels and breast cancer risk demonstrate a biological gradient with increased levels of serum 25(OH)D resulting in a linear decrease in risk (26-30, 32, 33). This is similar to the inverse, dose-response relationship observed between risk of colorectal cancer and serum 25(OH)D levels (146). In the meta-analysis by Mohr and colleagues (39), the dose-response relationship was estimated using data from eleven case-control or nested case-control
studies. In that study, a downward linear trend was observed with increasing concentration of 25(OH)D ($p < 0.001$).

**Consistency**

An inverse association between serum 25(OH)D levels and breast cancer risk has been observed across a wide range of published studies including 4 ecological (13, 14, 18, 87), 7 case-control (26-33), and one randomized controlled trial (144). To date, no cohort studies of serum 25(OH)D concentration and incidence of breast cancer have been published.

**Biological plausibility**

In addition to the abundant evidence from observational studies, the powerful anti-carcinogenic properties of vitamin D metabolites, especially 1,25(OH)$_2$D, have been demonstrated in numerous laboratory studies. Studies have shown that 1,25(OH)$_2$D helps to maintain breast epithelial cells in a well differentiated state and down-regulates expression of aromatase through several mechanisms such as inhibiting production of the COX-2 enzyme (147). Expression of aromatase is also required for synthesis of estrogen and may therefore play a significant role in the prevention of estrogen receptor (ER) positive breast cancers (147).

In human breast cancer cells, 1,25(OH)$_2$D has been shown to induce apoptosis and also to inhibit factors that aid in cell proliferation (141). It has also been shown that 1,25(OH)$_2$D can prevent angiogenesis by inhibiting synthesis of pro-angiogenic factors
such as the signal protein Vascular Endothelial Growth Factor (VEGF), effectively blocking tumor cells from recruiting blood vessels from local tissue. Furthermore, COX-2 has also been shown to increase angiogenesis. By down-regulating expression of COX-2, $1,25(OH)_2D$ further blocks angiogenesis (141, 147).

Several mechanisms have been proposed for the prevention of human breast cancer through vitamin D sufficiency. One of the main attributes of malignancy in breast cancer is the loss of adhesion between cells in the terminal ductal epithelium of the mammary gland (148). This loss of adhesion can be partly attributed to the down-regulation of e-cadherin that occurs in vitamin D deficiency. E-cadherin is a glycoprotein that serves as a sort of glue that helps to keep cells in a well-differentiated state. Research has shown that breast cancer prognosis is significantly worse in the total absence of e-cadherin expression due to loss of differentiation and an increase in metastatic behavior (149).

**Consideration of alternative hypotheses**

This should probably be considered one of the weakest of Hill’s criteria because in the face of strong epidemiologic evidence supporting a causal relationship between a disease and exposure of interest, the presence or lack of alternative hypotheses, resting upon disparate mechanisms, or consideration of such hypotheses, may be largely irrelevant. There are several well established risk factors for development of breast cancer. These include alcohol consumption (150), exogenous estrogen (151), ionizing radiation (152), and in postmenopausal women, obesity (152). Obesity is associated with
lower risk of premenopausal breast cancer, but higher risk of postmenopausal breast cancer (153).

Studies have also demonstrated a protective effect of physical activity on risk of breast cancer (154). However, it is difficult to separate the effect of these risk factors from that of serum 25(OH)D concentration. Much of the physical activity measured in those studies may have actually been performed outdoors, and epidemiological investigations of the effect of physical activity on cancer risk rarely differentiate between indoor physical activity and outdoor physical activity. Furthermore, obesity is independently associated with low serum 25(OH)D. A reduced capacity to produce 25(OH)D in obese persons has been found in previous studies (155). Interestingly, in the studies performed by Bertone-Johnson and colleagues, Crew and colleagues, and Engel and colleagues, serum 25(OH)D concentration was significantly, inversely associated with breast cancer risk after controlling for physical activity (28-30).

According to a recent meta-analysis of studies on the relationship between alcohol consumption and breast cancer risk, excess risk associated with alcohol consumption was estimated to be approximately 22% (156). This leaves a large amount of excess risk unexplained. Although a possible association between red meat consumption and breast cancer incidence has been investigated in epidemiological studies, the evidence from these investigations is inconclusive (157). Yet another risk factor that was thought to modify breast cancer risk is intake of dietary fat, possibly by modifying levels of endogenous estrogen. However, in the Women’s Health Initiative study population, there
was no effect of a low fat diet on risk of breast cancer (158). While exogenous estrogen in the form of hormone replacement therapy (HRT) increases risk of breast cancer in postmenopausal women (159), use of HRT at the population level is still not enough to account for the majority of breast cancer cases that occur every year.

None of the aforementioned risk factors can unilaterally account for all the variation in breast cancer risk. Although these risk factors, when taken together, make a substantial contribution to breast cancer risk at the population level, they still do not account for all of the excess risk. Exposure to the main determinant of circulating 25(OH)D concentration, ultraviolet B irradiance, tends to be ubiquitous at the population level and depends chiefly on latitude, culture, and health behaviors that are widespread across large groups of people. Therefore, vitamin D status may be able to account for a greater proportion of excess risk for breast cancer.

In previous case-control studies on serum 25(OH)D concentration and breast cancer risk, up to an 80% reduction in risk of breast cancer has been observed in subjects with the highest levels of serum 25(OH)D (32). Using data on US population serum 25(OH)D levels from the NHANES III study and risk estimates from case-control studies (45), the estimated population attributable risk of vitamin D insufficiency could be as high as 70% for breast cancer. Results from studies on serum 25(OH)D and breast cancer risk have also demonstrated a clear dose-response relationship. In the meta-analysis by Mohr and colleagues, data from 11 studies were used to estimate the dose-response curve, with a 50% projected reduction in breast cancer risk occurring at 47 ng/ml (39).
There is no evidence to suggest a threshold effect at which higher concentrations of serum 25(OH)D become less effective at preventing breast cancer. By making the logical assumption that the dose-response curve continues on a linear, downward trend, up to 80% of breast cancers could be prevented by raising population serum 25(OH)D levels to 80 ng/ml, a level that is still well below the National Academy of Sciences No Adverse Effect Level of over 100 ng/ml (131).

Experiment

This criterion is satisfied by the only published experimental study of serum 25(OH)D concentration and risk of breast cancer in humans: a randomized controlled trial performed by Lappe and colleagues (144). In this study, women in the treatment group received 1,100 IU of vitamin D_3_ and 1,450 mg of calcium per day over 4 years. By the end of the 4 year follow-up period, subjects in the treatment group experienced a 77% reduction in risk from all cancers (mainly lung and breast) compared to women in the placebo group ($p < 0.05$).

Specificity

Under the vitamin D-cancer prevention hypothesis, breast cancer occurs in several distinct phases that can be explained by a theoretical model termed the Disjunction-Initiation-Natural selection-Overgrowth-Metastasis-Involution-Transition (DINOMIT) model (Figure 4.1). In the first phase of the DINOMIT model, vitamin D deficiency causes the expression of e-cadherin to be down regulated, resulting in loss of adhesion
and a poorly differentiated state (160). Expression of e-cadherin may be highly regulated by 25(OH)D concentration (160). High levels of circulating 25(OH)D could provide substrate for conversion to 1,25(OH)_{2}D. 1,25(OH)_{2}D is synthesized via hydroxylation of 25(OH)D by the 1α hydroxylase (108). Although the principal site of this synthesis is the kidney, 1α hydroxylase is produced in a wide range of tissue, including breast epithelial tissue (108). 1,25(OH)_{2}D locally synthesized in breast epithelium is free to bind with the nuclear vitamin D receptor (VDR), unmasking the portion of the cell’s DNA that contains the instructions for assembly of e-cadherin (160, 161).

In the second phase of the model, Initiation, DNA is modified either through uncorrected errors that occur during replication or through exposure to mutagens such as ionizing radiation or free radicals. These changes in the DNA, especially ones that occur in an environment where cells are in a poorly differentiated state, set the stage for malignancy and unchecked cell division.

The next phase is Natural Selection. In this phase, due to the operation of evolutionary forces, malignant cells with a just a 1% competitive advantage will eventually overtake a tissue compartment. In the overgrowth phase, the tumor cells grow outside the basement membrane of the tissue compartment in which they originated, due to increasing scarcity of essential resources necessary to fuel further growth and cell division.
As the tumor continues to grow, a few malignant cells will break off from the tumor mass and be transported by the lymphatic system or bloodstream where they will colonize remote tissue sites. This is known as the metastasis phase. During the next phase, Involution, the growth of the tumor mass is temporarily halted by a seasonal rise in serum 25(OH)D concentration. This is supported by research that has demonstrated that diagnosis for breast cancer is highest in winter when population serum 25(OH)D levels are lowest (162).

Under the vitamin D-cancer prevention hypothesis, this process can be stopped at almost any point in the DINOMIT model by restoring vitamin D sufficiency in the organism. Beyond the DINOMIT model, evidence from laboratory studies have demonstrated a powerful anti-cancer effect of vitamin D metabolites on three critical phases in the development of a breast tumor: differentiation, apoptosis, and angiogenesis (141). Therefore, because vitamin D exerts such a powerful effect over a broad spectrum of processes essential for the development of a breast neoplasm, the specificity criterion is well satisfied.

Coherence

This criterion is broadly satisfied. To the author’s knowledge, there is no conflict between what is known about the natural history and biology of breast cancer and the existence of an inverse relationship between serum levels of vitamin D metabolites and risk of breast cancer. Laboratory evidence is overwhelmingly supportive and fits well with the vitamin D-cancer prevention hypothesis and the proposed DINOMIT model.
Conclusion

Study after study, in both human populations and the laboratory, has demonstrated that vitamin D plays an important role in the prevention of breast cancer. There have been over 30 studies performed on toxicity of vitamin D. These studies have shown that at oral intakes of up to 10,000 IU per day of vitamin D$_3$ or serum 25(OH)D concentrations below 100 ng/ml, no adverse health effects have been observed (130, 163, 164). Furthermore, the Institute of Medicine recently established 4,000 IU per day as the tolerable upper limit of safe intake (131).

Further epidemiological studies of the effect of vitamin D status on breast cancer risk should be performed. At minimum, randomized controlled trials of oral intake of 4,000 IU/day of vitamin D$_3$, should be undertaken. There should be separate trials for premenopausal and post menopausal women residing at latitudes > 37 degrees north. This would enable researchers and policymakers to directly observe the effect of a moderate dose of oral vitamin D$_3$ on cancer incidence. Vitamin D supplementation is an urgently needed, low cost, effective, and safe intervention strategy for breast cancer prevention that should be implemented without delay.
<table>
<thead>
<tr>
<th>Phase</th>
<th>Diagram</th>
<th>Process</th>
<th>Preventive or therapeutic Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D Replete (Normal)</td>
<td></td>
<td>Tight junctions intact</td>
<td>Maintain 25(OH) D level of 40-60 ng/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intercellular communication, growth inhibition and cell cycle normal non-mitotic</td>
<td></td>
</tr>
<tr>
<td>1. Vitamin D Insufficiency</td>
<td></td>
<td>Tight junctions weak or absent. Cells separate from each other very slightly. Cadherins lost or weak. Contact inhibition lost. Beta-catenins relocate. Natural selection begins.</td>
<td>Upregulation of tight junctions and cadherins by vitamin D metabolites Vitamin D maintains tight junctions, contact inhibition, and normal growth and cell cycle</td>
</tr>
<tr>
<td>Disjunction</td>
<td></td>
<td>Natural selection favors reproduction of rapidly mitotic, aggressive cells. These appear as new stem cells</td>
<td></td>
</tr>
<tr>
<td>2. Natural Selection</td>
<td></td>
<td>Rapidly mitotic, aggressive progeny predominate, a 1% advantage will fill compartment in 9000 generations</td>
<td>Vitamin D favors apoptosis and normal cell cycle Vitamin D inhibits lysis of basement membrane, Promotes sharing of micronutrients; Maintains intercellular junctions and desmosomes</td>
</tr>
<tr>
<td>3. Clonal Expansion</td>
<td></td>
<td>Most aggressive cells compete for nutrients and oxygen, and penetrate basement membrane</td>
<td></td>
</tr>
<tr>
<td>4. Lysis and Penetration of Basement Membrane</td>
<td></td>
<td>Invasion of Stroma</td>
<td>Re-establish tight junctions between cancer cells</td>
</tr>
<tr>
<td>5. Stromal Phase</td>
<td></td>
<td>Lymph vessel invasion</td>
<td>Re-establish tight junctions Prevent lymphatic entry</td>
</tr>
<tr>
<td>6. Lymphatic Entry Phase</td>
<td></td>
<td>Lymph node colonization</td>
<td>Re-establish tight junctions Confine malignancy to lymph nodes</td>
</tr>
<tr>
<td>7. Lymphatic Growth Phase</td>
<td></td>
<td>Lymphatic transport to brain, lung, liver, bone</td>
<td>None</td>
</tr>
<tr>
<td>8. Lymphatic Transport Phase</td>
<td></td>
<td>Malignant cells colonize remote host site</td>
<td>If VDR still present, re-establish tight junctions, downregulate VEGF, reduce growth rate, restore contact inhibition</td>
</tr>
<tr>
<td>9. Metastasis (colonization) Phase</td>
<td></td>
<td></td>
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**Figure 4.1** DINOMIT model of cancer prevention
Chapter 4 has been published:

APPENDIX

Appendix Table 1. Mean 25(OH)D levels from 28 published studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Location</th>
<th>25(OH)D ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mazess et al.</td>
<td>1985</td>
<td>Alaska, USA</td>
<td>16.6</td>
</tr>
<tr>
<td>Oliveri et al.</td>
<td>1990</td>
<td>Argentina</td>
<td>19.0</td>
</tr>
<tr>
<td>Xue et al.</td>
<td>1991</td>
<td>Beijing, China</td>
<td>17.7</td>
</tr>
<tr>
<td>Chailuikit et al.</td>
<td>1996</td>
<td>Thailand</td>
<td>67.4</td>
</tr>
<tr>
<td>Chapuy et al.</td>
<td>1997</td>
<td>France</td>
<td>17.9</td>
</tr>
<tr>
<td>Aloia al.</td>
<td>1998</td>
<td>New York, USA</td>
<td>27.5</td>
</tr>
<tr>
<td>Harris et al.</td>
<td>1998</td>
<td>Boston, USA</td>
<td>25.0</td>
</tr>
<tr>
<td>Bettica et al.</td>
<td>1999</td>
<td>Italy</td>
<td>18.7</td>
</tr>
<tr>
<td>Guillemant et al.</td>
<td>1999</td>
<td>Paris, France</td>
<td>8.6</td>
</tr>
<tr>
<td>Kristal-Boneh et al.</td>
<td>1999</td>
<td>Israel</td>
<td>25.4</td>
</tr>
<tr>
<td>Goswami et al.</td>
<td>2000</td>
<td>New Dehli, India</td>
<td>19.6</td>
</tr>
<tr>
<td>Brot et al.</td>
<td>2001</td>
<td>Denmark</td>
<td>26.2</td>
</tr>
<tr>
<td>Mishal et al.</td>
<td>2001</td>
<td>Amman, Jordan</td>
<td>14.4</td>
</tr>
<tr>
<td>Nakamura et al.</td>
<td>2001</td>
<td>Japan</td>
<td>14.1</td>
</tr>
<tr>
<td>Vieth et al.</td>
<td>2001</td>
<td>Toronto, Canada</td>
<td>24.1</td>
</tr>
<tr>
<td>Looker et al.</td>
<td>2002</td>
<td>USA</td>
<td>26.2</td>
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Appendix Table 1 continued

<table>
<thead>
<tr>
<th>Study</th>
<th>Year</th>
<th>Location</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nesby-O'Dell et al.</td>
<td>2002</td>
<td>USA</td>
<td>32.9</td>
</tr>
<tr>
<td>Rucker et al.</td>
<td>2002</td>
<td>Calgary, Canada</td>
<td>23.8</td>
</tr>
<tr>
<td>Tangpricha et al.</td>
<td>2002</td>
<td>USA</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Buenos Aires,</td>
<td></td>
</tr>
<tr>
<td>Fassi et al.</td>
<td>2003</td>
<td>Argentina</td>
<td>22.0</td>
</tr>
<tr>
<td>Arya et al.</td>
<td>2004</td>
<td>Lucknow, India</td>
<td>12.3</td>
</tr>
<tr>
<td>Hashemipour et al.</td>
<td>2004</td>
<td>Tehran, Iran</td>
<td>8.6</td>
</tr>
<tr>
<td>MacFarlane et al.</td>
<td>2004</td>
<td>Brussels, Belgium</td>
<td>13.8</td>
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<td>Premaor et al.</td>
<td>2004</td>
<td>Porto Algere, Brazil</td>
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<td>2005</td>
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<td>Rockell et al.</td>
<td>2005</td>
<td>Dunedin, New Zealand</td>
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Appendix Table 2. Country names for two letter codes used in ecological analysis:

AF, Afghanistan; AL, Albania; DZ, Algeria; AO, Angola; AR, Argentina; AM, Armenia; AU, Australia; AT, Austria; AZ, Azerbaijan; BS, Bahamas; BH, Bahrain; BD, Bangladesh; BB, Barbados; BY, Belarus; BE, Belgium; BZ, Belize; BJ, Benin; BT, Bhutan; BO, Bolivia; BA, Bosnia Herzegovena; BW, Botswana; BR, Brazil; BN, Brunei; BG, Bulgaria; BF, Burkina Faso; BI, Burundi; KH, Cambodia; CM, Cameroon; CA, Canada; CV, Cape Verde; CF, Central African Republic; TD, Chad; CL, Chile; CN, China; CO, Colombia; KM, Comoros; CD, Congo; CG, Congo Brazzaville; CR, Costa Rica; CI, Cote d'Ivoire; HR, Croatia; CU, Cuba; CY, Cyprus; CZ, Czech Republic; DK, Denmark; DJ, Djibouti; DO, Dominican Republic; EC, Ecuador; EG, Egypt; SV, El Salvador; GQ, Equatorial Guinea; ER, Eritrea; EE, Estonia; ET, Ethiopia; FJ, Fiji; FI, Finland; FR, France; GA, Gabon; GM, Gambia; GE, Georgia; DE, Germany; GH, Ghana; GR, Greece; GU, Guam; GT, Guatemala; GN, Guinea; GW, Guinea-Bissau; GY, Guyana; HT, Haiti; HN, Honduras; HU, Hungary; IS, Iceland; IN, India; ID, Indonesia; IR, Iran, Islamic Republic of; IQ, Iraq; IE, Ireland; IL, Israel; IT, Italy; JM, Jamaica; JP, Japan; JO, Jordan; KZ, Kazakhstan; KE, Kenya; KP, Korea, Democratic Republic of; KR, Korea, Republic of; KW, Kuwait; KG, Kyrgyzstan; LA, Lao People Democratic Republic; LV, Latvia; LB, Lebanon; LS, Lesotho; LR, Liberia; LY, Libya; LT, Lithuania; LU, Luxembourg; MK, Macedonia; MG, Madagascar; MW, Malawi; MY, Malaysia; ML, Mali; MT, Malta; MR, Mauritania; MU, Mauritius; MX, Mexico; FM, Micronesia; MD, Moldova; MN, Mongolia; MA, Morocco; MZ, Mozambique; MM, Myanmar; NA, Namibia; NP, Nepal; NL, Netherlands; NZ, New Zealand; NI, Nicaragua; NE, Niger; NG, Nigeria; NO, Norway; OM, Oman; PK, Pakistan; PA, Panama; PG, Papua New
Guinea; PY, Paraguay; PE, Peru; PH, Philippines; PL, Poland; PT, Portugal; PR, Puerto Rico; QA, Qatar; RO, Romania; RU, Russian Federation; RW, Rwanda; WS, Samoa; SA, Saudi Arabia; SN, Senegal; YU, Serbia and Montenegro; SL, Sierra Leone; SG, Singapore; SK, Slovakia; SI, Slovenia; SB, Solomon Islands; SO, Somalia; ZA, South African Republic; ES, Spain; LK, Sri Lanka; SD, Sudan; SR, Suriname; SZ, Swaziland; SE, Sweden; CH, Switzerland; SY, Syrian Arab Republic; TJ, Tajikistan; TZ, Tanzania; TH, Thailand; TG, Togo; TT, Trinidad and Tobago; TN, Tunisia; TR, Turkey; TM, Turkmenistan; UG, Uganda; UA, Ukraine; AE, United Arab Emirates; GB, United Kingdom; US, United States of America; UY, Uruguay; UZ, Uzbekistan; VU, Vanuatu; VE, Venezuela; VN, Viet Nam; YE, Yemen; ZM, Zambia; ZW, Zimbabwe
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