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Foundations of Integrative Oncology
COURSE

GRAND ROUNDS CALL
With Dr. Nalini Chilkov
March 13th, 2019
Second Wednesday of Every Month
5:30 PM Pacific / 6:30 PM Mountain / 7:30 PM Central / 8:30 PM Eastern

Clinical Pearl: The VITAL STUDY and Vitamin D Controversies

Take home: Monitoring serum levels of 25 OH Vitamin D and establishing a therapeutic rather than nutritional value is crucial to applications in cancer care.

Questions & Answers

Ana Komazec: **The cytotoxic compound used for 10 days per season, please explain the formulation of it, particularly in addition to the first 3 substances there is Phyto Cyto which I see is Yance's formula, so we would want to include 40 ml of that? Is that correct?**
40 ml *Polygonatum* root
20 ml *Taxus brevifolia*
20 ml *Catharanthus leaf*
40 ml Phyto Cyto???
120 ml total

Dr. Chilkov's Response:
I use PhytoCyto as a base and then increase the amounts of a few cytotoxic herbs as this toxic formula is used under my strict supervision

PhytoCyto
Taxus brevifolia, Catharanthus roseus, Camptotheca acuminata, Viscum album, Asimina triloba, Podophyllum peltatum, Phyolacca americana, Zingiber off, Citrus sinensis

Additions of cytoxic botanicals (do not use unless you are trained and experienced in dosing)

Polygonatum odoratum (Yu Zhu, Solomon's Seal): contains a plant lectin that promotes apoptosis and autophagy, Inhibits Hexokinase 2, a rate limiting enzyme, that inhibits glycolysis and the Warburg effect , suppresses expression of EGF Epidermal Growth Factor, blocks and de-activates EGFR (binding site) blocking EGFR Tyrosine Kinase autophosphorylation and the PI3K pathway, , down regulates bcl2 and upregulates BAX in the mitochondria to initiate apoptosis

Catharanthus (Vinca) rosea, Madagascar Periwinkle leaf
Traditional use diabetes, contains anti tumor vinca alkaloid vinblastine: antimitotic, antiangiogneic, source of the drug Vinorelbine

Taxus brevifolia (Pacific Yew Tree) branch and tips source of Taxanes (a class of diterpenes) from which Paclitaxel/Taxol and Docetaxel/Taxotere chemotherapy drugs are derived, anti-mitotic, cell cycle arrest, widely used in breast, ovarian and lung cancers

Camptotheca (source of camptothecin from which Irinotecan is derived) Topoisomerase inhibitor, inhibits replication and transcription by blocking the topoisomerase 1 enzyme activity and its action upon the DNA (stops the DNA from unwinding and relaxing, linking and unlinking during replication).

Judy Pruzinsky:
You have spoken about not taking some herbs and nutraceuticals during chemotherapy. I believe usually one discontinues only during active treatment time (for a two day dosing of chemo, one would abstain for about five days, depending on days of treatment and half life of substance.)

a. **Is this true for the sulphoraphanes (Broccoprotect) and EGCg?**
b. **Are there any substances you would not suggest a patient taking for the whole duration of chemotherapy?**

Dr. Chilkov Response:
I primarily avoid supplements and botanicals that are strongly active in the Phase 1 and 2 liver detoxification or alter Liver Function, Kidney Function. This assures that we do not interfere with drug metabolism.

General Guidelines
Withhold any agents with potential drug interactions for days 1-6 of chemotherapy cycle

There are some exceptions where we have studies. For example, we know that Milk Thistle (Silybum marianum) is a powerful in liver detoxification. However there are studies showing that it does not interfere with the efficacy of Platinum Chemotherapy drugs and in fact protects the liver, heart and kidneys from platinum drug toxicity.

Where there are no studies, I will do not use Milk Thistle, Sulphoraphanes, N.Acetyl Cysteine, for example so that we err on the side of safety in terms of interfering with drug metabolism

Curcumin interferes with Cyclophosphamide, Ifosfamide
EGCG and bortezomib (Velcade)

Botanicals with known interactions
Milk Thistle/Silybum marianum, Green Tea/EGCG, Echinacea, Curcumin, St. John's wort/Hypericum, Valerian root, and Allium/Garlic concentrates

You must know the duration of action and half life of the chemotherapy agent to determine when you stop and start any nutrient or botanical that would interfere with their oncology treatment.

You must know the oncologist's treatment schedule as well so that you can calculate accurately.

Typically I will wait until day 5 or 6 to reintroduce any supplements or botanicals that potentially interfere with therapeutic actions of IV chemotherapy agents. Remember that the half life of supplements and botanicals is quite short.

There are now **LONG ACTING versions of some chemotherapy drugs.**
There is an emergence of **nanotechnology t**hat has provided new drug delivery systems for docetaxel, which can improve its water solubility, minimize the side effects and increase the tumor-targeting distribution by passive or active targeting (polymer based, lipid based, etc)
For example:
PEGylated (attachment or amalgamation of polyethylene glycol polymer chains to molecules)
Liposomal Doxirubicin (Doxil) commonly used in ovarian cancer

Here you must look up the half life of the drug and be aware of the chemotherapy dosing schedule to avoid interactions

Some chemotherapy drugs are now oral and dosed daily (Xeloda-Capcetabine)
Daily oral supplement recommendations must be adjusted to avoid interactions

Research: Links between diabetes and pancreatic cancer further substantiated

This summary from Clinical Synergy Newsletter 02.20.19

>Recent-onset diabetes is frequently the result of pancreatic cancer

>Long-standing diabetes is a **primary risk factor for this aggressive pancreatic cancer**.

Over 80% of pancreatic cancer cases are diagnosed in late stages, making this one of the **deadliest cancers**—five-year survival rates are just 8%. Any advancements that support an early diagnosis can potentially **improve outcomes** and survival rates for this aggressive disease. New research supports the hypothesis that diabetes, particularly recent-onset diabetes, can be viewed as **both a risk factor as well as a result** of pancreatic cancer. These findings may hopefully lead to more **sophisticated tools for early detection** within this high-risk population.

Diabetes has been associated with pancreatic cancer in a number of studies. This body of data points to a potential twofold increase in the risk of pancreatic cancer among diabetes patients. New research published in the *Journal of the National Cancer Institute* further substantiates this link, with data drawn from a prospective study involving Americans of African and Hispanic descent: two populations with an elevated risk of diabetes.

Risk and Result

Findings demonstrated that recent-onset diabetes was significantly higher in pancreatic cancer cases (16.4%) compared with those with colorectal (6.7%), prostate (5.5 %), and breast (5.3 %) cancer. Analysis demonstrated that patients with **recent-onset diabetes had the highest risk of developing pancreatic cancer**.

Researchers say these findings further support the theory that **recent-onset diabetes is frequently the result of pancreatic cancer,** as well as the idea that **long-standing diabetes** is a **primary risk factor for this aggressive cancer**. Importantly, these results can help researchers identify additional risk predictors and may be used to develop more sophisticated tests for earlier diagnosis of this deadly disease.

Pancreatic Cancer Following Incident Diabetes in African Americans and Latinos: The Multiethnic Cohort.

Setiawan, V. W., Stram, D. O., Porcel, J., Chari, S. T., Maskarinec, G., Marchand, L. L., . . . Monroe, K. R. (2018). Pancreatic Cancer Following Incident Diabetes in African Americans and Latinos: The Multiethnic Cohort**.** *JNCI: Journal of the National Cancer Institute,111*(1), 27-33. doi:10.1093/jnci/djy090

Abstract

BACKGROUND: Diabetes has been proposed to be a risk factor for and a consequence of pancreatic cancer (PC). The relationship between recent-onset diabetes and PC is not well understood, and data in minorities are sparse. We examined the relationships between recent-onset diabetes and PC incidence in

African Americans and Latinos in the Multiethnic Cohort.

METHODS: A total of 48 995 African Americans and Latinos without prior diabetes and cancer at baseline (1993-1996) were included in the study. Questionnaires, Medicare data, and California hospital discharge files were used to identify new diabetes diagnoses. Cox regressions were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for cancer associated with diabetes and with diabetes duration.

RESULTS: A total of 15 833 (32.3%) participants developed diabetes between baseline and 2013. A total of 408 incident PC cases were identified during follow-up. Diabetes was associated with PC (HRage75 = 2.39, 95% CI = 1.91 to 2.98). Individuals with recent-onset diabetes (within three or fewer years of PC diagnosis) had a greater risk compared with those with long-term diabetes across all ages. The HRage75 for recent-onset diabetes was 4.08 (95% CI = 2.76 to 6.03) in Latinos and 3.38 (95% CI = 2.30 to 4.98) in African Americans.

CONCLUSIONS: Diabetes was associated with a more than twofold higher risk of PC in African Americans and Latinos, but recent-onset diabetes was associated with a 2.3-fold greater increase in risk of PC than long-standing diabetes. Our findings support the hypothesis that recent-onset diabetes is a manifestation of PC and that long-standing diabetes is a risk factor for this malignancy.

Research: Another Study Showing that Honey May Minimize Radiation-Induced Oral Mucositis

Charalambous, M., Raftopoulos, V., Paikousis, L., Katodritis, N., Lambrinou, E., Vomvas, D., . . . Charalambous, A. (2018). ***The effect of the use of thyme honey in minimizing radiation - induced oral mucositis in head and neck cancer patients: A randomized controlled trial****. European Journal of Oncology Nursing,34, 89-97. doi:10.1016/j.ejon.2018.04.003*

PURPOSE: Radiation-induced oral mucositis is one of the main side effects during and after the treatment of head and neck cancer patients. The study was designed to provide evidence on the effectiveness of thyme honey on oral mucositis management.

METHODS: This was a randomised controlled trial (RCT) with **72 head and neck cancer patients** who were divided either to the **intervention group (thyme honey rinses) or to the control group (saline rinses).** Oral mucositis was assessed according to the Radiation Therapy Oncology Group (RTOC criteria), and **assessments were performed weekly starting at the 4th week of the radiotherapy for seven weeks and repeated once 6 months later. Additionally, the Oral Mucositis Weekly Questionnaire (OMWQ) was given at 4th week of radiotherapy, 1 month after the completion of radiotherapy and 6 months later.** The ClinicalTrials.gov Identifier for this study is NCT01465308. This paper reports on the findings regarding thyme honey's effectiveness on oral mucositis.

RESULTS: **Generalized estimating equations revealed that patients in the intervention group were graded lower in the objective assessment of oral mucositis (p less than 0,001), maintained their body weight (p less than 0,001) and showed an improvement in their global health (p = 0.001) compared to the control group. Quality of life of the patients in the same group was also statistically significantly higher than that of the patients of the control group (p less than 0,001).**

CONCLUSION: The study provided evidence on the positive effect of thyme honey on the management of radiation-induced oral mucositis and quality of life in head and neck cancer patients.

Research: D-dimer and high-sensitivity C-reactive protein levels to predict venous thromboembolism recurrence after discontinuation of anticoagulation for cancer-associated thrombosis.

Jara-Palomares, L., Solier-Lopez, A., Elias-Hernandez, T., Asensio-Cruz, M. I., Blasco-Esquivias, I., Sanchez-Lopez, V., . . . Otero-Candelera, R. (2018). ***D-dimer and high-sensitivity C-reactive protein levels to predict venous thromboembolism recurrence after discontinuation of anticoagulation for cancer-associated thrombosis.*** *British Journal of Cancer,119(8), 915-921. doi:10.1038/s41416-018-0269-5*

BACKGROUND: Optimal duration of anticoagulation for **cancer-associated thrombosis (CAT**) remains unclear. **This study assessed D-dimer (DD) and high-sensitivity C-reactive protein (hs-CRP) levels after the withdrawal of anticoagulation treatment to predict the risk of venous thromboembolism (VTE) recurrence among patients with CAT.**

METHODS: Prospective, multicentre study to evaluate CAT with ≥3 months of anticoagulation that was subsequently discontinued. Blood samples were taken when patients stopped the anticoagulation and 21 days later to determine the DD and hs-CRP levels. All patients were followed up for 6 months to detect VTE recurrence.

RESULTS: Between 2013 and 2015, 325 patients were evaluated and 114 patients were ultimately enrolled in the study. The mean age was 62 ± 14 years and **nearly 40% had metastasis. Ten patients developed VTE recurrence within 6 months** (8.8%, 95% confidence interval [CI]: 4.3-15.5%). **The DD and hs-CRP levels after 21 days were associated with VTE recurrence.** The subdistribution hazard ratios were 9.82 for hs-CRP (95% CI: 19-52) and 5.81 for DD (95% CI: 1.1-31.7).

CONCLUSIONS: **This study identified that hs-CRP and DD were potential biomarkers of VTE recurrence after discontinuation of anticoagulation in CAT. A risk-adapted strategy could identify low-risk patients who may benefit from discontinuation of anticoagulation.**

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VITAL STUDY

VITAMIN D and Omega 3 Fatty Acid Supplementation for prevention of cancer and cardiovascular disease

DOES VITAMIN D IMPACT CANCER RISK and PROGRESSION??

Dr. Nalini Chilkov, Founder

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Vitamin D Supplements and Prevention of Cancer and Cardiovascular Disease

November 10, 2018, NEJM.org. DOI: 10.1056/NEJMoa1809944

JoAnn E. Manson, M.D., Dr.P.H., Nancy R. Cook, Sc.D., et al

5 Year Randomized, Placebo-Controlled Trial 25,871 participants

Vitamin D3 (cholecalciferol) at a dose of 2000 IU per day
Marine n–3 (omega-3) fatty acids at a dose of 1 g per day

for the prevention of cancer and cardiovascular disease among men 50 years of age or older and women 55 years of age or older in the US.

VITAL STUDY

2000 IU VITAMIN D3/day considered “high dose”

Primary end points were
invasive cancer of any type, major cardiovascular events (a composite of myocardial infarction, stroke, or death from cardiovascular causes).

Secondary end points
site-specific cancers, death from cancer additional cardiovascular events.

VITAL STUDY CONCLUSIONS

Supplementation with vitamin D3 at a dose of 2000iu per day for 5 years did not result in a lower incidence of invasive cancer or cardiovascular events than placebo in men over 50 and women over 55 in the US

CANCER
No significant differences between the two groups (Vitamin D group vs Placebo Group) were observed with regard to the incidence of breast, prostate, or colorectal cancer.

VITAL STUDY CONCLUSIONS

However, **there was a suggestive 17% reduction in cancer deaths, which became a 25% reduction in analyses that excluded the first two years of follow-up**

Although vitamin D did not significantly lower the risk of developing cancer in the total study population, **African Americans assigned to vitamin D did experience a suggestive 23% reduction in cancer risk.**

VITAL STUDY | CLINICAL PERSPECTIVE

The findings indicate that **high-dose vitamin D does not lower the risk of developing cancer or cardiovascular disease** in generally healthy men and women, **although it appears to lower the risk of cancer death.**

. "The promising results for cancer mortality need to be confirmed in extended follow-up of the study participants and in future trials,

National guidelines for vitamin D intake from food and/or supplements recommend
600 IU per day for adults up to age 70
800 IU per day for those aged 71 and older.

VITAMIN D

Cancer Related Functions & Mechanisms

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Vitamin D regulates the transcription of more than 60 genes that are responsible for antiproliferative, prodifferentiating, antimetastatic, and proapoptotic effects on cells.

HALLMARKS of CANCER and VITAMIN D

As a hormone, 1,25(OH)2D binds to vitamin D receptor located in nucleus and functions. It is reported to play an important role in

cellular proliferation
differentiation
apoptosis autophagy
angiogenesis
metastasis

All these processes may regulate cancer development and progression

Decreased serum vitamin D levels result in enriched cellular growth, neoangiogenesis, and cancer development. VDR knockout mice show higher rates of preneoplastic mammary lesions

VARIATIONS IN CLINICAL RESPONSE TO VITAMIN D

Single Nucleotide Polymorphisms related to

- Vitamin D Receptor
- Vitamin D binding proteins
- Enzymes that involve activation and degradation of Vitamin D
  CYP2R1, CYP27A1, CYP24A1

- Pre and Post Menopausal Status

- Magnesium Status

- Serum Levels of 25-OH Vitamin D

PLoS One. 2017 May 1;12(5):e0176448. doi: 10.1371/journal.pone.0176448.

Randomized controlled trials of vitamin D and cancer incidence: A modeling study.

The VDR polymorphism case-control studies showed different associations between different VDR polymorphisms and breast cancer risk among different populations

Magnesium status and supplementation influence vitamin D status and metabolism: results from a randomized trial. Qi Dai, Xiangzhu Zhu et al. Am J Clin Nutr 2018;108:1249–1258

Enzymes that synthesize and metabolize vitamin D are magnesium dependent. Recent observational studies found that magnesium intake significantly interacted with vitamin D in relation to vitamin D status and risk of mortality. Accordiing to NHANES, 79% of US adults do not meet their Recommended Dietary Allowance of magnesium.

The mean daily dose of personalized magnesium supplementation was 205.52 mg, with a range from 77.25 to 389.55 mg. 12 week trial

Our findings suggest that optimal magnesium status may be important for optimizing 25(OH)D status

PROSTATE CANCER and VITAMIN D

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REVIEW Circulating vitamin D level and mortality in prostate cancer patients: a dose–response meta-analysis

Zhen-yu Song, Qiuming Yao, et al
Endocrine Connections **(2018)** 7, R294–R303

The summary Hazard Ratio of prostate cancer-specific mortality correlated with an increment of every 20 nmol/L in circulating vitamin D level was 0.91, with 95% CI 0.87–0.97, P = 0.002.

The HR for all-cause mortality with the increase of 20 nmol/L vitamin D was 0.91 (95% CI: 0.84–0.98, P = 0.01).

This meta-analysis suggested that higher 25-hydroxyvitamin D level was associated with a reduction of mortality in prostate cancer patients and vitamin D is an important protective factor in the progression and prognosis of prostate cancer.

J Steroid Biochem Mol Biol. 2013 Jul;136:233-7. doi: 10.1016/j.jsbmb.2012.11.012. Epub 2012 Dec 7.

Vitamin D3 supplementation, low-risk prostate cancer, and health disparities.

Hollis BW, et al

Vitamin D promotes the differentiation of prostate cancer cells, raising the possibility that vitamin D deficiency over time may contribute to the progression from subclinical prostate cancer to clinical disease.

The results of this clinical study suggest that **supplementation with vitamin D3 at 4000IU per day may benefit patients with early stage, low-risk prostate cancer on active surveillance, because of the improved outcome** (**a decreased number of positive cores at repeat biopsy and no increase in Gleason scores**) in more than half of the subjects enrolled in the trial. (1 year study)

BREAST CANCER AND VITAMIN D

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PLoS One. 2017 May 1;12(5):e0176448. doi: 10.1371/journal.pone.0176448.

Randomized controlled trials of vitamin D and cancer incidence: A modeling study.

For breast cancer–controlled studies, case-control studies consistently find an inverse correlation between 25(OH)D and breast cancer risk

This review shows that most of the vitamin D studies support the inverse association between vitamin D level and breast cancer risk, and retrospective and prospective epidemiologic studies revealed that vitamin D deficiency is associated with increased breast cancer risk.

Vitamin D exposure and Risk of Breast Cancer: a meta-analysis

Nuria Estébanez, Inés Gómez-Acebo, Camilo Palazuelos, et al

Sci Rep. 2018; 8: 9039. PMID: 29899554

This systematic review suggests a protective relationship between circulating vitamin D (measured as 25(OH) D)
and breast cancer development in premenopausal women.

Possible mechanism: Vitamin D reduces serum E2

Serum 25-Hydroxyvitamin D Concentrations ≥40 ng/ml Are Associated with >65% Lower Cancer Risk: Pooled Analysis of Randomized Trial and Prospective Cohort Study (women over age 55 x 3.9 years)

PLoS One. 2018; 13(7): e0201078PMID: 30011335 Sharon L. McDonnell,

- Incidence was lower at higher concentrations of 25(OH)D
- **Women with 25(OH)D concentrations ≥40 ng/ml had a 67% lower risk of cancer than women with concentrations <20 ng/ml** (HR = 0.33, 95% CI = 0.12-0.90).25(OH)D
- **Concentrations ≥40 ng/ml were associated with substantial reduction in risk of all invasive cancers combined.**

Breast cancer risk markedly lower with serum 25-hydroxyvitamin D concentrations ≥60 vs <20 ng/ml (150 vs 50 nmol/L): Pooled analysis of two randomized trials and a prospective cohort

PLoS One. 2018; 13(6): e0199265. PMID: 29906273 Sharon L. McDonnell,et al

Studied the Relationship between 25(OH)D concentration and breast cancer risk across a broad range of 25(OH)D concentrations among women aged 55 years and older over 4 years

Higher 25(OH)D concentrations were associated with a dose-response decrease in breast cancer risk with concentrations ≥60 ng/ml being most protective.

Breast cancer risk markedly lower with serum 25-hydroxyvitamin D concentrations ≥60 vs <20 ng/ml (150 vs 50 nmol/L):

The proportion with breast cancer was 78% lower for ≥60 vs <20 ng/ml (P = 0.02).

Women with 25(OH)D concentrations ≥60 ng/ml had an 80% lower risk of breast cancer than women with concentrations <20 ng/ml (HR = 0.20, P = 0.03), adjusting for age, BMI, smoking status, calcium supplement intake, and study of origin.

Breast Cancer Res Treat. 2013 Jan;137(2):599-607.

Association between 25-hydroxyvitamin D concentration and breast cancer risk in an Australian population: an observational case-control study.

Bilinski K[1], Boyages J.

Circulating 25-hydroxyvitamin D (25(OH)D) concentration defined as:
sufficient (≥75 nmol/L),
insufficient (50-74 nmol/L),
deficient (25-49 nmol/L)
severely deficient (<25 nmol/L).

Breast Cancer Res Treat. 2013 Jan;137(2):599-607.

Association between 25-hydroxyvitamin D concentration and breast cancer risk in an Australian population: an observational case-control study.

Bilinski K[1], Boyages J.

Conclusion

25(OH)D concentration below 75 nmol/L at diagnosis was associated with a significantly higher risk of breast cancer.

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ORIGINAL ARTICLE

Vitamin D Supplements and Prevention of Cancer and Cardiovascular Disease

JoAnn E. Manson, M.D., Dr.P.H., Nancy R. Cook, Sc.D., I-Min Lee, M.B., B.S., Sc.D., William Christen, Sc.D., Shari S. Bassuk, Sc.D., Samia Mora, M.D., M.H.S., Heike Gibson, Ph.D., David Gordon, M.A.T., Trisha Copeland, M.S., R.D., Denise D'Agostino, B.S., Georgina Friedenberg, M.P.H., Claire Ridge, M.P.H., Vadim Bubes, Ph.D., Edward L. Giovannucci, M.D., Sc.D., Walter C. Willett, M.D., Dr.P.H., and Julie E. Buring, Sc.D., for the VITAL Research Group*

ABSTRACT

BACKGROUND

It is unclear whether supplementation with vitamin D reduces the risk of cancer or cardiovascular disease, and data from randomized trials are limited.

METHODS

We conducted a nationwide, randomized, placebo-controlled trial, with a two-by-two factorial design, of vitamin D_3 (cholecalciferol) at a dose of 2000 IU per day and marine n–3 (also called omega-3) fatty acids at a dose of 1 g per day for the prevention of cancer and cardiovascular disease among men 50 years of age or older and women 55 years of age or older in the United States. Primary end points were invasive cancer of any type and major cardiovascular events (a composite of myocardial infarction, stroke, or death from cardiovascular causes). Secondary end points included site-specific cancers, death from cancer, and additional cardiovascular events. This article reports the results of the comparison of vitamin D with placebo.

RESULTS

A total of 25,871 participants, including 5106 black participants, underwent randomization. Supplementation with vitamin D was not associated with a lower risk of either of the primary end points. During a median follow-up of 5.3 years, cancer was diagnosed in 1617 participants (793 in the vitamin D group and 824 in the placebo group; hazard ratio, 0.96; 95% confidence interval [CI], 0.88 to 1.06; P=0.47). A major cardiovascular event occurred in 805 participants (396 in the vitamin D group and 409 in the placebo group; hazard ratio, 0.97; 95% CI, 0.85 to 1.12; P=0.69). In the analyses of secondary end points, the hazard ratios were as follows: for death from cancer (341 deaths), 0.83 (95% CI, 0.67 to 1.02); for breast cancer, 1.02 (95% CI, 0.79 to 1.31); for prostate cancer, 0.88 (95% CI, 0.72 to 1.07); for colorectal cancer, 1.09 (95% CI, 0.73 to 1.62); for the expanded composite end point of major cardiovascular events plus coronary revascularization, 0.96 (95% CI, 0.86 to 1.08); for myocardial infarction, 0.96 (95% CI, 0.78 to 1.19); for stroke, 0.95 (95% CI, 0.76 to 1.20); and for death from cardiovascular causes, 1.11 (95% CI, 0.88 to 1.40). In the analysis of death from any cause (978 deaths), the hazard ratio was 0.99 (95% CI, 0.87 to 1.12). No excess risks of hypercalcemia or other adverse events were identified.

CONCLUSIONS

Supplementation with vitamin D did not result in a lower incidence of invasive cancer or cardiovascular events than placebo. (Funded by the National Institutes of Health and others; VITAL ClinicalTrials.gov number, NCT01169259.)

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Long prescribed to prevent and treat bone-related disorders,[1] supplemental vitamin D has been viewed in recent years as a potential strategy for preventing cancer and cardiovascular disease. In the United States, routine assessment of vitamin D status in patients in primary care settings[2] and the use of vitamin D supplements[3] have increased substantially. Ecologic studies have shown lower rates of death from cancer and cardiovascular disease in regions with greater sun exposure than in areas with less sun exposure.[1,4] Such exposure is necessary for cutaneous synthesis of vitamin D. Laboratory studies have shown the presence of vitamin D receptors in many tissues and have suggested plausible vitamin D pathways that may be related to cancer and cardiovascular disease, and observational studies have shown associations between low serum levels of 25-hydroxyvitamin D and increased risks of cancer and cardiovascular disease.[1,4-6] Nevertheless, it is uncertain whether supplementation with vitamin D prevents cancer or cardiovascular disease, because such results cannot establish causality.[1,4,7,8] For example, observational studies are susceptible to confounding by outdoor physical activity (which correlates with sun exposure), adiposity (which may decrease bioavailability of 25-hydroxyvitamin D), general nutritional status, and other factors that may produce spurious protective associations.[1,4]

Data from large-scale randomized trials (involving ≥10,000 participants) of vitamin D in moderate or high doses and designed with cancer or cardiovascular disease as primary outcomes are lacking. Trials examining such outcomes, typically using secondary or post hoc analyses, have usually shown null results, but the use of low doses of vitamin D, insufficient statistical power, short durations, lack of rigorous end-point adjudication, or a combination of these factors limit conclusions.[1,4] However, meta-analyses[9,10] of randomized trial data suggest a stronger benefit of vitamin D with respect to the rate of death from cancer than to the incidence of cancer. The U.S. Preventive Services Task Force concluded that there are insufficient data to evaluate the effectiveness of supplementation with vitamin D for the prevention of cancer or cardiovascular disease.[7] The Institute of Medicine had previously reached this same conclusion and called for new trials of vitamin D (in amounts at least twice the current recommended dietary allowance of 600 to 800 IU per day for bone health) to clarify the benefit–risk balance.[1] The Vitamin D and Omega-3 Trial (VITAL), a large-scale trial that evaluated high-dose vitamin D, was designed to address these knowledge gaps. Included in the trial population were more than 5000 black participants, for whom the question of the effectiveness of vitamin D is particularly relevant because their cutaneous synthesis of vitamin D in response to solar radiation is lower than that in persons in other racial or ethnic groups. VITAL also evaluated n–3 (omega-3) fatty acids; those results are shown in an accompanying article in the *Journal*.[11]

METHODS

TRIAL DESIGN AND OVERSIGHT

We conducted this randomized, double-blind, placebo-controlled trial, with a two-by-two factorial design, to examine the benefits and risks of vitamin D_3 (cholecalciferol) at a dose of 2000 IU per day and marine n–3 fatty acids at a dose of 1 g per day in the primary prevention of cancer and cardiovascular disease among 25,871 men who were 50 years of age or older and women who were 55 years of age or older. The trial protocol has been described elsewhere[4,12] and is available with the full text of this article at NEJM.org.

Participants were recruited throughout the United States, and the groups were balanced according to sex and with a goal to include at least 5000 black participants. Eligible participants had no history of cancer (except nonmelanoma skin cancer) or cardiovascular disease at trial entry, and they were required to agree to limit the use of vitamin D from all supplemental sources, including multivitamins, to 800 IU per day and to complete a 3-month placebo run-in phase. Safety exclusions included renal failure or dialysis, cirrhosis, history of hypercalcemia, or other serious conditions that would preclude participation. Randomization was computer generated within sex, race, and 5-year age groups in blocks of eight.

Baseline questionnaires collected data on risk factors for cancer, cardiovascular disease, and other conditions and included a food frequency questionnaire. Participants received follow-up questionnaires at 6 months and 1 year after randomization and annually thereafter to collect information on adherence to trial regimens, outside use of vitamin D supplements, development of major illnesses, updates on risk factors, and po-

tential side effects of the trial agents. Calendar packs containing the trial capsules of vitamin D or corresponding placebo (and n–3 fatty acids or corresponding placebo) were mailed with questionnaires to the participants.

Blood samples were obtained at baseline during the run-in period from all willing participants — 16,956 of the 25,871 persons who underwent randomization (65.5%). At no cost to the trial, Quest Diagnostics donated and performed serum 25-hydroxyvitamin D assays with the use of liquid chromatography–tandem mass spectrometry on all samples that could be analyzed. Quest had no role in the design of the trial, accrual of the data (other than the assays), analysis of the data (other than assay standards), or preparation of the manuscript. Our trial participated in the vitamin D standardization program of the Centers for Disease Control and Prevention.[13]

The National Institutes of Health, the sponsors of the trial, had a collaborative role in the design and conduct of the trial. Final decisions regarding the data collection, management, and analysis and the review and approval of the manuscript and decision to submit the manuscript for publication resided with trial investigators and the trial research group. The trial was approved by the institutional review board of Partners HealthCare–Brigham and Women's Hospital and was monitored by an external data and safety monitoring board. The trial agents have received Investigational New Drug Approval from the Food and Drug Administration. Pharmavite donated vitamin D and Pronova BioPharma and BASF donated fish oil (Omacor); the companies also donated matching placebos and packaging in the form of calendar packs. None of the donating companies had any role in the design or conduct of the trial, collection or analysis of the data, or preparation or review of the manuscript. The first three authors and the last author had full access to all the trial data and vouch for the completeness and accuracy of the data, for the accuracy of the data analyses, and for the fidelity of the trial to the protocol. All the participants provided written informed consent before enrollment in the trial.

TRIAL END POINTS

The primary end points were invasive cancer of any type and major cardiovascular events (composite of myocardial infarction, stroke, and death from cardiovascular causes). Secondary cancer end points were incident colorectal, breast, and prostate cancers, and death from cancer. Secondary cardiovascular end points were an expanded composite of major cardiovascular events plus coronary revascularization and the individual components of major cardiovascular events. Participants who reported an end-point event were asked to sign a release for medical records, which were reviewed for confirmation by an end-points committee of physicians who were unaware of the trial-group assignments. Cancer was confirmed on the basis of histologic or cytologic data.[14] Myocardial infarction and stroke were confirmed with the use of established criteria,[15,16] coronary revascularization was confirmed by medical record review, and death from cardiovascular causes was confirmed if there was convincing evidence of a cardiovascular event from all available sources. Analyses included only confirmed end points.

For deaths reported by family members, the next of kin was asked for permission to obtain medical records and a copy of the death certificate. Alternatively, the latter was obtained from the state vital records bureau. The end-points committee reviewed the records to assign the cause of death. If records were unavailable (or participants were lost to follow-up), the National Death Index (NDI) Plus was searched for cause of death according to the death-certificate information. Deaths were defined with the use of all these sources; a secondary analysis of cause-specific deaths required medical records or other adjudication of cause of death beyond NDI coding.

STATISTICAL ANALYSIS

Analyses of effect were based on the intention-to-treat principle (all participants who underwent randomization were included). The trial was designed to have a greater than 85% power to detect observed hazard ratios of 0.85 and 0.80 for the primary end points of cancer and cardiovascular disease, respectively.[4] Initial analyses compared baseline characteristics of participants according to trial regimen with the use of t-tests or chi-square tests. Primary analyses compared the main effects of vitamin D on cancer and cardiovascular disease with the use of Cox proportional-hazards models that were controlled for age, sex, and randomization group in the n–3 fatty acid portion of the trial (n–3 fatty acid group or placebo group). Person-time was counted from randomization to

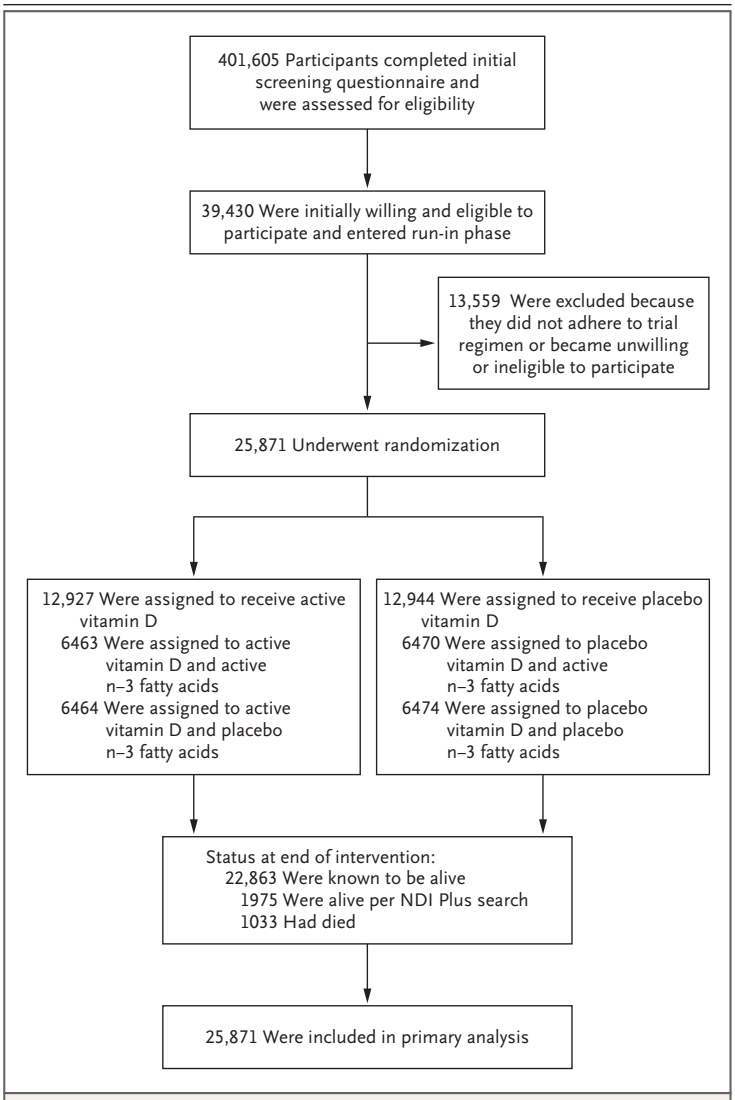

Figure 1. Screening, Randomization, and Follow-up of the Participants.
NDI denotes National Death Index.

the end point, to death, or to the end of the trial on December 31, 2017. Cumulative-incidence plots and interactions with time were used to examine whether effects varied over time. Prespecified analyses of the primary outcomes excluding events that occurred during the first year and the first 2 years of follow-up assessed latent effects. Adherence effects were estimated by censoring follow-up data when the participant discontinued trial capsules or began taking more than 800 IU per day of outside vitamin D.

Possible variations in the effect according to race or ethnic group, age, sex, body-mass index (BMI, the weight in kilograms divided by the square of the height in meters), baseline 25-hydroxyvitamin D level, concurrent randomization to the n–3 group, outside use of vitamin D supplements, and baseline risk factors for cancer and cardiovascular disease were specified a priori. However, there was no control for multiple hypothesis testing, and no formal adjustment was made to the P values or confidence intervals. Thus, results regarding secondary and exploratory end points, as well as those regarding subgroups, should be interpreted with caution. The incidence of potential side effects according to randomly assigned group was also compared.

RESULTS

TRIAL PARTICIPANTS

Randomization to receive vitamin D, n–3 fatty acids, both active agents, or both placebos took place from November 2011 through March 2014. The trial intervention ended as planned on December 31, 2017, which yielded a median follow-up of 5.3 years (range, 3.8 to 6.1). A total of 401,605 persons were screened for eligibility to participate, and 25,871 persons ultimately underwent randomization (Fig. 1).

Table 1 shows baseline characteristics of the trial participants (further details are provided in Table S1 in the Supplementary Appendix, available at NEJM.org). Of the 25,871 participants, 51% were women. The mean age of the participants was 67.1 years. The cohort was racially diverse and included 71% self-declared non-Hispanic white participants and 20% black participants; the rest were members of other racial or ethnic groups. Characteristics of the participants were balanced between the two groups.

Among the 15,787 participants who had blood samples that could be analyzed, the mean (±SD) serum total 25-hydroxyvitamin D level at baseline was 30.8±10.0 ng per milliliter (77 nmol per liter); 12.7% had levels below 20 ng per milliliter (50 nmol per liter), and 32.2% had levels from 20 to less than 30 ng per milliliter (50 to <75 nmol per liter). In a subgroup of 1644 participants with repeat measurements after 1 year, mean 25-hydroxyvitamin D levels increased from 29.8 ng per milliliter (74 nmol per liter) at baseline to 41.8 ng per milliliter (104 nmol per liter) at 1 year (a 40% increase) in the vitamin D group and changed minimally (mean, −0.7 ng per milliliter [−2 nmol per liter]) in the placebo group. Base-

Table 1. Characteristics of the Participants at Baseline, According to Randomized Assignment to Vitamin D or Placebo.*

Characteristic	Total (N=25,871)	Vitamin D Group (N=12,927)	Placebo Group (N=12,944)
Female sex — no. (%)	13,085 (50.6)	6547 (50.6)	6538 (50.5)
Age — yr	67.1±7.1	67.1±7.0	67.1±7.1
Race or ethnic group — no./total no. (%)†			
Non-Hispanic white	18,046/25,304 (71.3)	9013/12,647 (71.3)	9033/12,657 (71.4)
Black	5106/25,304 (20.2)	2553/12,647 (20.2)	2553/12,657 (20.2)
Nonblack Hispanic	1013/25,304 (4.0)	516/12,647 (4.1)	497/12,657 (3.9)
Asian or Pacific Islander	388/25,304 (1.5)	188/12,647 (1.5)	200/12,657 (1.6)
Native American or Alaskan native	228/25,304 (0.9)	118/12,647 (0.9)	110/12,657 (0.9)
Other or unknown	523/25,304 (2.1)	259/12,647 (2.0)	264/12,657 (2.1)
Body-mass index‡	28.1±5.7	28.1±5.7	28.1±5.8
Current smoking — no./total no. (%)	1836/25,485 (7.2)	921/12,729 (7.2)	915/12,756 (7.2)
Hypertension treated with medication — no./total no. (%)	12,791/25,698 (49.8)	6352/12,834 (49.5)	6439/12,864 (50.1)
Current use of cholesterol-lowering medication — no./total no. (%)	9524/25,428 (37.5)	4822/12,700 (38.0)	4702/12,728 (36.9)
Diabetes — no./total no. (%)	3549/25,828 (13.7)	1812/12,903 (14.0)	1737/12,925 (13.4)

* Plus–minus values are means ±SD. Percentages may not sum to 100 because of rounding. There were no significant differences between the groups with regard to the baseline characteristics.
† Race and ethnic group were reported by the participants.
‡ The body-mass index is the weight in kilograms divided by the square of the height in meters. Data were missing for 2.4% of the participants.

line 25-hydroxyvitamin D levels varied according to age, sex, race or ethnic group, and BMI (Fig. S1A in the Supplementary Appendix), but most groups had 25-hydroxyvitamin D levels close to, or above, 40 ng per milliliter (100 nmol per liter) after 1 year of supplementation with vitamin D (Fig. S1B in the Supplementary Appendix).

The mean rate of response to questionnaires was 93.1%, and follow-up regarding mortality was greater than 98% over the 5.3-year follow-up period. The mean rate of adherence to the trial regimen (the percentage of participants who reported taking at least two thirds of the trial capsules) was 82.0% in the vitamin D group and 80.3% in the placebo group during this time (Table S2 in the Supplementary Appendix). At 2 years, the prevalence of outside use of vitamin D (>800 IU per day) was 3.8% in the vitamin D group and 5.6% in the placebo group; at 5 years, the rates were 6.4% and 10.8%, respectively. These results probably reflect outside screening during the trial for 25-hydroxyvitamin D levels and the initiation of supplementation in some participants who had low levels.

CANCER

The primary end point of invasive cancer of any type developed in 1617 participants, with similar event rates in the vitamin D group and the placebo group (793 and 824 participants with cancer, respectively; hazard ratio, 0.96; 95% confidence interval [CI], 0.88 to 1.06; P=0.47) (Table 2). No significant differences between the two groups were observed with regard to the incidence of breast, prostate, or colorectal cancer. During follow-up, 341 participants died from cancer, with 154 such deaths in the vitamin D group and 187 in the placebo group (hazard ratio, 0.83; 95% CI, 0.67 to 1.02).

The cumulative incidence of invasive cancer of any type (Fig. 2A and Table 2) and death from cancer (Table 2, and Fig. S2D in the Supplementary Appendix) did not differ significantly between the two groups. No significant differences between the two groups were observed with regard to preplanned analyses of the primary end point of cancer, excluding the first 1 and 2 years of follow-up. However, the test for proportionality over time was significant for the rate of death

Table 2. Hazard Ratios and 95% Confidence Intervals for the Primary, Secondary, and Other End Points, According to Randomized Assignment to Vitamin D or Placebo, in Intention-To-Treat Analyses.*

End Point	Vitamin D Group (N = 12,927)	Placebo Group (N = 12,944)	Hazard Ratio (95% CI)
	no. of participants with event		
Cancer			
Primary end point: invasive cancer of any type	793	824	0.96 (0.88–1.06)
Breast cancer	124	122	1.02 (0.79–1.31)
Prostate cancer	192	219	0.88 (0.72–1.07)
Colorectal cancer	51	47	1.09 (0.73–1.62)
Death from cancer	154	187	0.83 (0.67–1.02)
Cardiovascular disease			
Primary end point: major cardiovascular event†	396	409	0.97 (0.85–1.12)
Cardiovascular event in expanded composite end point‡	536	558	0.96 (0.86–1.08)
Myocardial infarction	169	176	0.96 (0.78–1.19)
Stroke	141	149	0.95 (0.76–1.20)
Death from cardiovascular causes	152	138	1.11 (0.88–1.40)
Other cardiovascular end point§			
PCI	182	188	0.97 (0.79–1.19)
CABG	73	98	0.75 (0.55–1.01)
Death from myocardial infarction	24	15	1.60 (0.84–3.06)
Death from stroke	19	23	0.84 (0.46–1.54)
Death from any cause	485	493	0.99 (0.87–1.12)
Analyses excluding the first 2 yr of follow-up			
Invasive cancer of any type	490	522	0.94 (0.83–1.06)
Death from cancer	112	149	0.75 (0.59–0.96)
Major cardiovascular event	274	296	0.93 (0.79–1.09)
Death from any cause	368	384	0.96 (0.84–1.11)

* Analyses were from Cox regression models that were controlled for age, sex, and n–3 fatty acid randomization group. Analyses were not adjusted for multiple comparisons.
† This end point was a composite of myocardial infarction, stroke, or death from cardiovascular causes.
‡ This end point was a composite of major cardiovascular events and coronary revascularization (percutaneous coronary intervention [PCI] or coronary-artery bypass grafting [CABG]).
§ These events were not prespecified as primary or secondary outcomes.

from cancer. In both an analysis that excluded 1 year of follow-up and an analysis that excluded 2 years of follow-up, neither of which was specified in the protocol, the rate of death from cancer was significantly lower with vitamin D than with placebo (hazard ratio, 0.79 [95% CI, 0.63 to 0.99], and hazard ratio, 0.75 [95% CI, 0.59 to 0.96], respectively). In analyses restricted to 153 deaths from cancer in patients with medical records or other adjudication of the cause of death beyond the NDI coding, the hazard ratios were 0.72 (95% CI, 0.52 to 1.00) over the total follow-up period and 0.63 (95% CI, 0.43 to 0.92) after the first 2 years were excluded. Preliminary analyses of cancer stage at diagnosis showed slightly fewer advanced cancers, metastatic cancers, or both among patients assigned to vitamin D than among those assigned to placebo, but differences were not significant (data not shown). The cumulative incidence rates of site-specific cancers and of death from cancer (prespecified secondary end points) are shown in Figure S2 in the Supplementary Appendix.

Results of prespecified subgroup analyses are

presented in Table 3. The findings suggest that BMI may have modified the effect of vitamin D on cancer.

CARDIOVASCULAR DISEASE AND ALL-CAUSE MORTALITY

During follow-up, there were 805 major cardiovascular events (myocardial infarction, stroke, or cardiovascular death), with events in 396 participants in the vitamin D group and 409 participants in the placebo group (hazard ratio, 0.97; 95% CI, 0.85 to 1.12; P=0.69) (Table 2). Supplementation with vitamin D also did not affect the risk of secondary cardiovascular end points (Table 2). There were no significant differences between the two groups with respect to the cumulative incidence of major cardiovascular events (Fig. 2B) and no significant effect modification according to baseline characteristics or randomization to the n–3 fatty acid intervention (Table 3) or according to traditional cardiovascular risk factors (Table S3 in the Supplementary Appendix). There were 978 deaths from any cause; the numbers of these deaths were similar in the vitamin D group and the placebo group (485 and 493 deaths, respectively; hazard ratio, 0.99; 95% CI, 0.87 to 1.12). Analyses that censored data for nonadherence did not materially alter the results. No meaningful change in the rates of major cardiovascular events or death from any cause occurred after data from the first 2 years of follow-up were excluded (Table 2).

ADVERSE EVENTS

There were no significant differences between the two groups with respect to incident diagnoses of hypercalcemia, kidney stones, or gastrointestinal symptoms (Table S4 in the Supplementary Appendix).

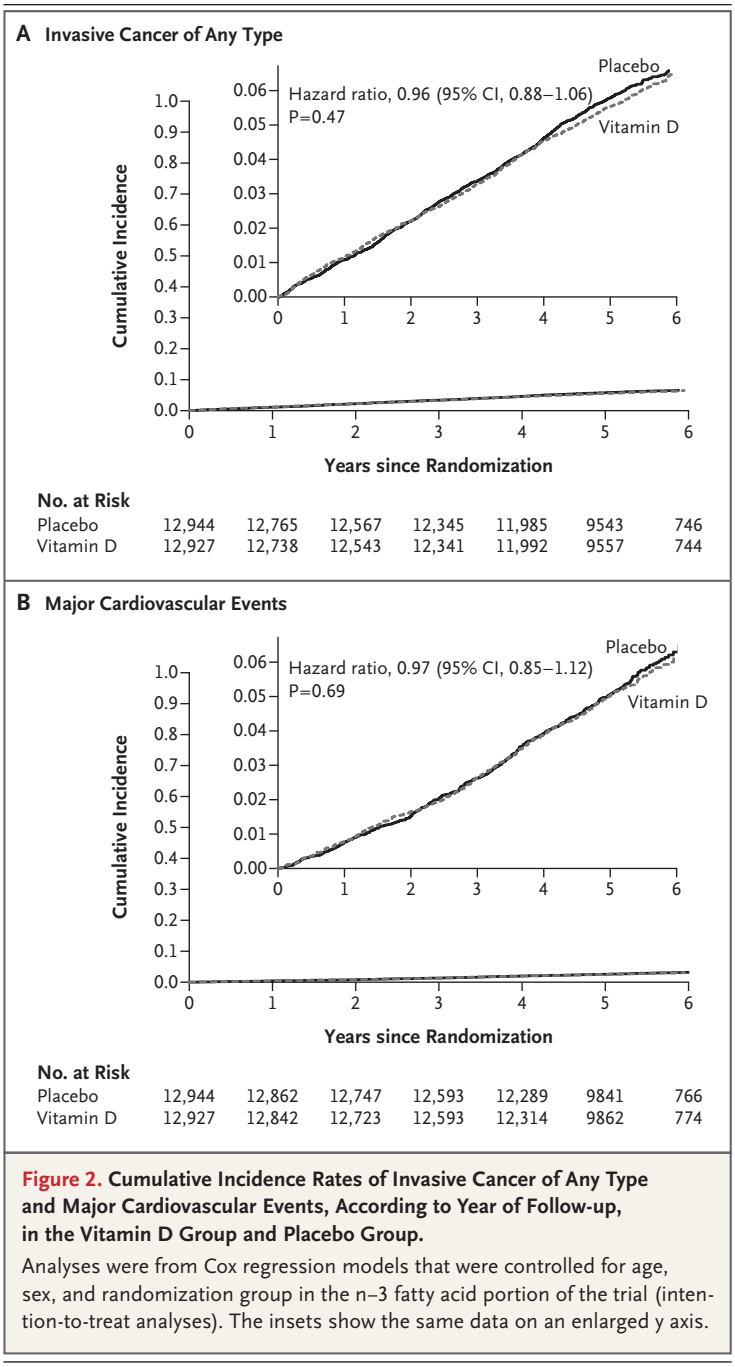

Figure 2. Cumulative Incidence Rates of Invasive Cancer of Any Type and Major Cardiovascular Events, According to Year of Follow-up, in the Vitamin D Group and Placebo Group.

Analyses were from Cox regression models that were controlled for age, sex, and randomization group in the n–3 fatty acid portion of the trial (intention-to-treat analyses). The insets show the same data on an enlarged y axis.

DISCUSSION

In this large primary-prevention trial, supplementation with vitamin D_3 (at a dose of 2000 IU per day) did not lead to a significantly lower incidence of invasive cancer of any type or a composite of major cardiovascular events (myocardial infarction, stroke, and death from cardiovascular causes) than placebo. The intervention also did not lead to a lower incidence of total deaths from cancer or a lower incidence of breast, prostate, or colorectal cancer than placebo.

Effects did not vary according to baseline serum 25-hydroxyvitamin D levels. The use of vitamin D did not lead to a significant difference in any of the secondary cardiovascular end points or in the rate of death from any cause in the overall cohort or in subgroups.

In analyses excluding early follow-up data, there was also no significant between-group difference in the incidence of invasive cancer of any type or

Table 3. Hazard Ratios of the Primary Outcomes According to Subgroup, Comparing the Vitamin D Group with the Placebo Group.*

Subgroup	No. of Participants	Invasive Cancer of Any Type				Major Cardiovascular Events			
		Vitamin D	Placebo	Hazard Ratio (95% CI)	P Value for Interaction	Vitamin D	Placebo	Hazard Ratio (95% CI)	P Value for Interaction
		no. of participants with event				*no. of participants with event*			
Age	25,871				0.73				0.31
<Median of 66.7 yr	12,859	302	322	0.95 (0.81–1.11)		140	131	1.07 (0.85–1.36)	
≥Median of 66.7 yr	13,012	491	502	0.98 (0.86–1.11)		256	278	0.93 (0.78–1.10)	
Sex	25,871				0.38				0.57
Male	12,786	452	488	0.93 (0.82–1.06)		223	223	1.01 (0.84–1.21)	
Female	13,085	341	336	1.02 (0.87–1.18)		173	186	0.93 (0.76–1.14)	
Race†	25,304				0.21				0.37
Non-Hispanic white	18,046	626	632	0.99 (0.89–1.11)		280	301	0.93 (0.79–1.10)	
Black	5,106	98	126	0.77 (0.59–1.01)		69	76	0.91 (0.65–1.26)	
Other	2,152	53	52	1.03 (0.70–1.51)		32	24	1.36 (0.80–2.31)	
Body-mass index	25,254				0.002				0.66
<25	7,843	206	278	0.76 (0.63–0.90)		117	115	1.07 (0.83–1.38)	
25 to <30	10,122	338	323	1.04 (0.90–1.21)		152	162	0.93 (0.74–1.16)	
≥30	7,289	228	199	1.13 (0.94–1.37)		120	120	0.98 (0.76–1.26)	
Body-mass index category	25,254				0.026				0.89
<Median of 27.1	12,582	361	421	0.86 (0.75–0.99)		189	193	0.99 (0.81–1.21)	
≥Median of 27.1	12,672	411	379	1.08 (0.94–1.24)		200	204	0.97 (0.80–1.18)	
Baseline serum 25-hydroxyvitamin D	15,787				0.99				0.75
<20 ng/ml	2,001	58	63	0.97 (0.68–1.39)		34	34	1.09 (0.68–1.76)	
≥20 ng/ml	13,786	459	464	0.98 (0.86–1.12)		218	216	1.00 (0.83–1.21)	
Baseline serum 25-hydroxyvitamin D category	15,787				0.57				0.42
<Median of 31 ng/ml	7,812	251	252	1.02 (0.86–1.21)		128	139	0.94 (0.74–1.20)	
≥Median of 31 ng/ml	7,975	266	275	0.95 (0.80–1.12)		124	111	1.09 (0.84–1.41)	

Baseline vitamin D use‡	25,871			0.64				0.71
Yes	11,030	370	376	0.99 (0.86–1.14)	165	164	1.00 (0.81–1.25)	
No	14,841	423	448	0.94 (0.83–1.08)	231	245	0.95 (0.79–1.14)	
Randomization in the n–3 fatty acids portion of trial	25,871			0.56				0.56
Placebo group	12,938	385	412	0.94 (0.82–1.08)	210	209	1.01 (0.83–1.22)	
Active-agent group	12,933	408	412	0.99 (0.87–1.14)	186	200	0.93 (0.76–1.14)	

* Analyses were from Cox regression models that controlled for age, sex, and n–3 fatty acid randomization group (intention-to-treat analyses). Analyses were not adjusted for multiple comparisons. To convert the values for 25-hydroxyvitamin D to nanomoles per liter, multiply by 2.5.
† Race was reported by the participants.
‡ Data shown are for use of out-of-trial vitamin D supplements at baseline (restricted to ≤800 IU per day from all sources combined, including individual supplements and multivitamins).

major cardiovascular events. A post hoc analysis of the rate of death from cancer suggested a possible benefit with respect to the rate of total deaths from cancer after exclusion of early follow-up data, based on an unadjusted 95% confidence interval that does not include 1.

The results of subgroup analyses raise the possibility of differential effects on cancer incidence according to BMI, with normal-weight participants who received vitamin D having a lower incidence than those who received placebo. However, these analyses should be considered hypothesis-generating, in the context of the negative findings for the primary outcome measures and given that they are not adjusted for multiple comparisons.

Because of its size and long duration (≥5 years), our trial had sufficient power to examine the effect of high-dose vitamin D on the risk of cancer and cardiovascular events. Previous vitamin D trials testing doses of 400 to 1100 IU per day administered with or without calcium have suggested, in aggregate, no significant benefit with respect to the incidence of cancer but a significant benefit with respect to the rate of death from cancer. A 2014 meta-analysis of four such trials[17-20] yielded summary relative risks of 1.00 (95% CI, 0.94 to 1.06) for the incidence of cancer and 0.88 (95% CI, 0.78 to 0.98) for the rate of death from cancer.[9] Another meta-analysis showed similar results.[10] Two trials of high-dose vitamin D have recently been completed. One 4-year trial[21] that tested daily vitamin D (2000 IU) plus calcium (1500 mg) against placebo for cancer prevention in 2303 women in Nebraska showed a suggestive but nonsignificant 30% lower incidence of cancer in association with the active intervention. The 3.3-year Vitamin D Assessment Study (ViDA),[22] which tested monthly vitamin D (100,000 IU) against placebo for prevention of cardiovascular disease in 5110 participants in New Zealand, reported null results for cancer outcomes. However, these trials had shorter durations and fewer deaths from cancer than our trial, as well as few black participants. Also, ViDA used intermittent bolus dosing, which is associated with nonphysiological fluctuations in blood levels of vitamin D.[23]

Data from laboratory studies and studies in animals support mechanisms whereby vitamin D may inhibit carcinogenesis and slow tumor progression, including promotion of cell differentiation,

inhibition of cancer-cell proliferation, and antiinflammatory, immunomodulatory, proapoptotic, and antiangiogenic effects.[1,24] Vitamin D may decrease tumor invasiveness and the propensity to metastasize, leading to a reduced rate of death from cancer.[24] Among patients with cancer, higher 25-hydroxyvitamin D levels at diagnosis or treatment have been linked to longer survival.[9] Observational studies suggest that vitamin D may confer greater protection against death from cancer than against the initial development of clinically evident cancer, albeit with benefits with regard to both end points,[5] with the strongest inverse relationships between 25-hydroxyvitamin D levels and colorectal cancer.[25-27] The power of our trial for analyses of site-specific cancers was limited. In addition, given the long latency for cancer development, extended follow-up is necessary to fully ascertain potential effects.

The observed lack of benefit of vitamin D supplementation for cardiovascular outcomes in our trial is consistent with results of previous trials of vitamin D,[17,20,28-33] even at moderate or high doses.[32] Most recently, in ViDA, the rate of cardiovascular disease was not lower among participants who received monthly administration of high-dose vitamin D than among those who received placebo.[31] Neither our trial nor ViDA[31] showed that vitamin D was associated with a reduced rate of death from any cause; lower-dose vitamin D trials have shown neutral effects or at most modest reductions in this end point.[33-35] However, detection of a decreased rate of death from any cause, if present, may require longer follow-up.

Previous research points to possible mechanisms through which supplementation with vitamin D might reduce the risk of cancer among normal-weight but not overweight or obese participants. Parathyroid hormone appears to be suppressed at lower 25-hydroxyvitamin D levels in overweight and obese persons,[36] which would be consistent with obesity-related hormonal dysregulation leading to less benefit of supplementation. Alternatively, because of volumetric dilution[37] or decreased bioactivity of vitamin D, overweight and obese persons may require higher doses to derive a benefit with respect to cancer, analogous to body-size differences in aspirin dosage requirements.[38] However, in our trial, there was only slight variation in the mean 25-hydroxyvitamin D level in response to the tested dose according to BMI group (Fig. S1B in the Supplementary Appendix). Finally, supplementation with vitamin D is unlikely to affect all mechanistic pathways linking obesity with numerous cancers.[39] These hypothesis-generating issues require further investigation.

The finding of a possible vitamin D–associated benefit with regard to the incidence of cancer among black participants — a group with lower vitamin D requirements for bone health than white persons (lower fracture risk despite lower 25-hydroxyvitamin D levels than white persons)[1] — may imply that the most favorable vitamin D status may vary according to organ system and tissue. We speculate that the possible trial regimen–associated effects on cancer incidence among normal-weight participants and suggestive effects among black participants, which contrast with the null cardiovascular findings in these groups, may be explained by different vitamin D requirements for these outcomes.

In observational studies, the 25-hydroxyvitamin D levels associated with lowest risks tend to be above 30 ng per milliliter (75 nmol per liter) for cancer (at least colorectal cancer)[26] but between 20 and 25 ng per milliliter for cardiovascular disease.[6] Thus, vitamin D requirements for cardiovascular health may have already been met for most participants. Although neither our trial nor ViDA showed a significant cardiovascular benefit of vitamin D among participants with low 25-hydroxyvitamin D levels at baseline, it remains possible that a trial involving persons with extremely low vitamin D levels (i.e., well below the 20 ng per milliliter recommended for bone health[1]) would show stronger effects on risk. However, maintaining participants in a vitamin D–deficient state and circumventing real-world clinical care for 5 years would be neither ethical nor feasible.

Our trial has many strengths, including a large general population sample with racial, ethnic, and geographic diversity; daily vitamin D dosing; high rates of follow-up and adherence to the trial regimen; rigorously adjudicated end points; baseline and follow-up blood samples from many participants; and achieved mean 25-hydroxyvitamin D levels in the targeted range. Ancillary studies addressing treatment effects on diabetes, heart failure, cognition, autoimmune disorders, and other outcomes will inform the overall benefit–risk balance of high-dose supplementation. Our trial also has limitations. The median dura-

tion of follow-up was 5.3 years. The trial tested only one dose of vitamin D. Trials[40] are ongoing to add information regarding other doses, although some are using bolus dosing. A 2-year postintervention follow-up of our cohort is ongoing to capture latency effects and increase statistical power to assess end points.

In summary, daily supplementation with high-dose vitamin D for 5 years among initially healthy adults in the United States did not reduce the incidence of cancer or major cardiovascular events.

The views expressed in this article are those of the authors and do not necessarily represent the views of the Department of Health and Human Services or the National Institutes of Health.

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Review

Role of Vitamin D Metabolism and Activity on Carcinogenesis

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The vitamin D endocrine system regulates a broad variety of independent biological processes, and its deficiency is associated with rickets, bone diseases, diabetes, cardiovascular diseases, and tuberculosis. Cellular and molecular studies have also shown that it is implicated in the suppression of cancer cell invasion, angiogenesis, and metastasis. Sunlight exposure and consequent increased circulating levels of vitamin D are associated with reduced occurrence and a reduced mortality in different histological types of cancer, including those resident in the skin, prostate, breast, colon, ovary, kidney, and bladder. The vitamin D receptor (VDR) as a steroid hormone superfamily of nuclear receptors is highly expressed in epithelial cells at risk for carcinogenesis, providing a direct molecular link by which vitamin D status impacts on carcinogenesis. Because VDR expression is retained in many human tumors, vitamin D status may be an important modulator of cancer progression in persons living with cancer. The aim of this review is to highlight the relationship between vitamin D, VDR, and cancer, summarizing several mechanisms proposed to explain the potential protective effect of vitamin D against the development and progression of cancer.

Key words: Vitamin D; Vitamin D receptor (VDR); Carcinogenesis; Cancer

INTRODUCTION

Laboratory and epidemiological data published over the past several years have contributed to the hypothesis that vitamin D metabolites inhibit cancer development at various tissue sites. In 1937, Peller and Stephenson hypothesized that sunlight exposure reduces the risk of cancer (1), and Apperly demonstrated an association between latitude and cancer mortality in 1941 (2). Four decades later, Garland et al. hypothesized that poor vitamin D status accounts for an elevated risk of colon, breast, and ovarian cancers at higher latitudes in the US (3,4). Schwartz and colleagues hypothesized a similar relationship for prostate cancer (5,6). More recently, Grant demonstrated an inverse correlation between regional type B ultraviolet (UV-B) radiation levels and mortality rates of many cancers, particularly digestive organ cancers, and found that in males approximately 80% of the cancers attributable to low regional solar UV-B were digestive system cancers (7). Mizoue also found an inverse correlation between averaged annual solar radiation levels and mortality from digestive system cancers (i.e., esophagus, stomach, colon, rectum, pancreas, gallbladder, and bile ducts) but not other cancer types in Japan (8).

THE VITAMIN D SYSTEM

The vitamin D system includes a group of lipid-soluble steroids and their respective metabolites. There are two major forms of vitamin D in nature: ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Vitamin D_2 is photochemically synthesized in plants or is acquired by a diet of fortified milk products, while vitamin D_3 is produced in the skin of animals and humans in response to sunlight too, in particular to UV-B radiations of appropriate wavelength: 270–300 nm. In most countries in Europe and in the US, the requirement of vitamin D is given by 90% of the 7-dehydrocholesterol cholesterol synthesis in the skin from solar irradiation, and only about 10% is taken up by the diet (9). The classical synthetic pathway involves 25- and 1-α-hydroxylation of vitamin D_2 and D_3 in the liver and kidney, respectively. First, hydroxylation occurs in the liver, and it is led to generate 25(OH) D_3. 25(OH)D_3 enters the systemic circulation, and it has a

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half-life of 12–19 days. Second, hydroxylation occurs in the kidneys, and it constitutes the most biologically active hormonal form of vitamin D: $1,25(OH)_2D_3$ (calcitriol) (Fig. 1). The serum levels of $25(OH)D_3$ are a reflection of overall vitamin D status in the body. There are two principal enzymes involved in the formation of circulating $1,25(OH)_2D_3$ from dietary absorbed or skin synthesized vitamin D: the hepatic microsomal or mitochondrial vitamin D 25-hydroxylase (CYP27A1) and the renal mitochondrial enzyme 1α-hydroxylase (CYP27B1) for vitamin D and $25(OH)D_3$, respectively (10). These hydroxylases belong to a class of proteins known as cytochrome P450

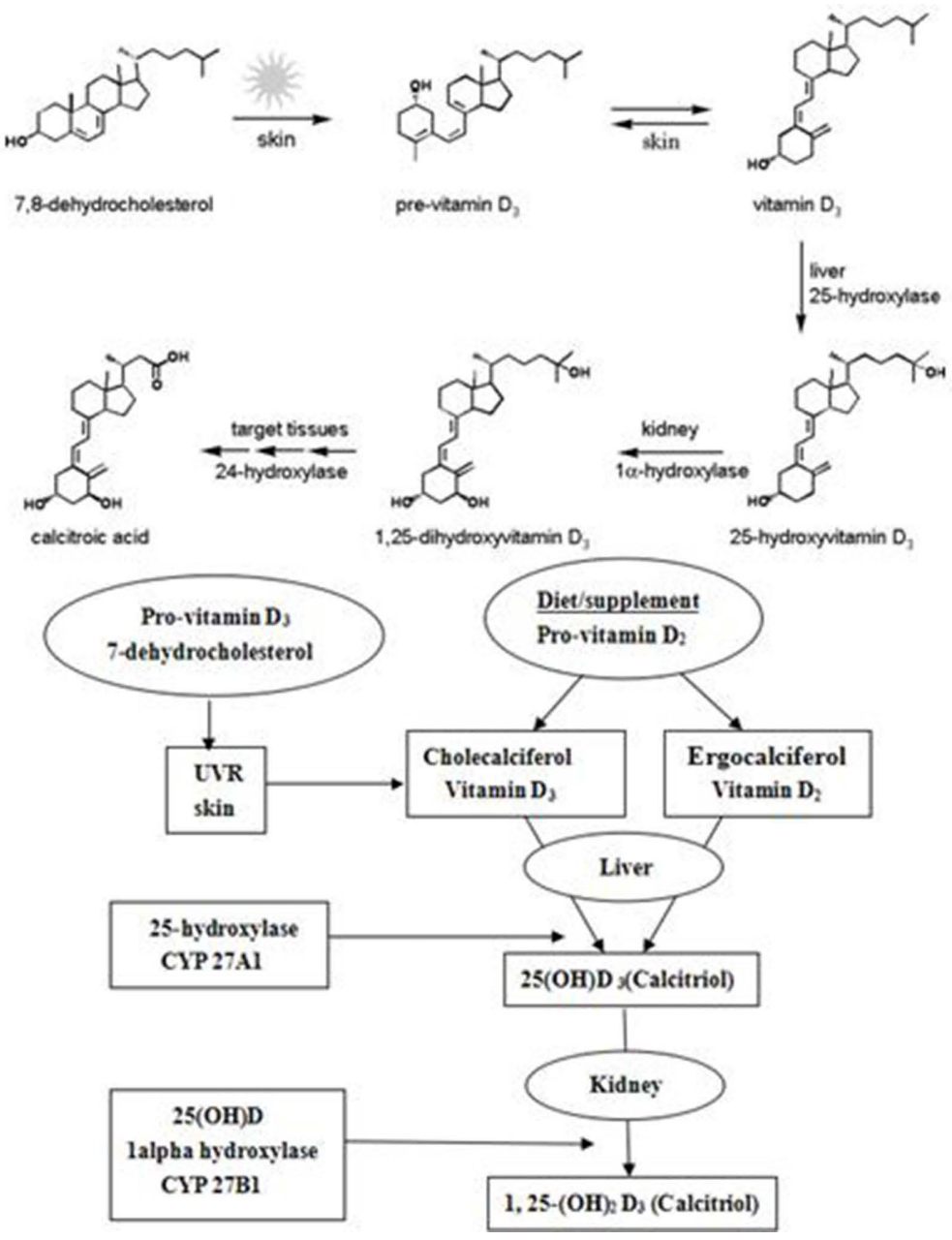

Figure 1. Vitamin D and its metabolites. The vitamin D requirement is from the exposure of skin to sunlight, while a minor portion may be obtained from dietary sources. Upon exposure to ultraviolet B, 7-dehydrocholecalciferol in the skin is photolyzed to form a 9,10-seco-sterol pro-vitamin D_3. Vitamin D_2 (ergocalciferol) or vitamin D_3 (cholecalciferol) made in the skin or ingested in the diet can be stored in and then released from fat cells. The synthetic pathway involves 25- and1-α-hydroxylation of vitamin D_2 and D_3, in the liver and kidney, respectively. First hydroxylation occurs within the liver and lead to the formation of $25(OH)D_3$ or calcidiol; second hydroxylation occurs within the kidneys and constitutes the most biologically active hormonal form of vitamin D: $1,25(OH)_2D_3$ or calcitriol.

mixed function monooxidases. In recent years, extrarenal activity of $25(OH)D_3$-1α-hydroxylase (CYP27B1) has been reported in various cell types including macrophages, keratinocytes, prostates, and colon cancer cells (11,12). It was shown that $1,25(OH)_2D_3$ is produced locally in several tissues. It has been demonstrated that potential vitamin D target tissues (e.g., colon, prostate, breast, lung, pancreas) can synthesize and degrade calcitriol. Local production and degradation of calcitriol have been suggested to represent key factors in several types of human cancer (13–15).

THE VITAMIN D RECEPTOR

The vitamin D receptor (VDR) belongs to the superfamily of transacting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors as well as the retinoid-X receptors and retinoic acid receptors (16). It is an endocrine member of the nuclear receptor superfamily (17) because it is the only nuclear protein that binds the nuclear hormone $1,25(OH)_2D_3$ with high affinity. The human VDR protein is a 427-amino acid peptide that has a DNA-binding domain, a ligand-binding domain, and activating domains. The VDR protein contains two zinc finger motifs that bind to the DNA, while the ligand-binding domain, located at the carboxy l terminus, changes conformation when $1,25(OH)_2D_3$ binds, allowing interaction with transcription factors. Activated VDR forms a heterodimer with the retinoic acid X receptor, which translocates to the nucleus (18,19) and binds to the vitamin D response element in the promoter region of target genes (20). VDR protein is encoded by a large gene (>100 kb) located on chromosome 12q12-14. The VDR gene encompasses two promoter regions, eight protein-coding exons, and six untranslated exons (21). It has an extensive promoter region capable of generating multiple tissue-specific transcripts. It has been demonstrated that VDR requires heterodimerization with auxiliary proteins for effective DNA interaction.

ROLES OF VITAMIN D AND VITAMIN D RECEPTOR ON CARCINOGENESIS

Several levels of evidence support the relationships among vitamin D, VDR, and cancer: (a) solar UV-B irradiance and vitamin D reduce the risk of incidence and death for many types of cancer, (b) a low intake of vitamin D is associated with a increased risk of cancer; (c) high circulating levels of vitamin D are associated with reduced risk of developing cancer; (d) the aggressiveness of a cancer is lower in summer when the production of vitamin D is higher; (e) polymorphisms of VDR genes affect the risk of developing cancer. These relationships are supported by in vitro studies and epidemiologic studies. A lot of in vitro studies have demonstrated that exposure of tumor cells to high concentrations of vitamin D compounds inhibits their proliferation and induce differentiation. Numerous epidemiologic studies have shown the association between factors expected to reduce vitamin D levels (e.g., geography and latitude, history of sun exposure, lifestyle) and the increased rates of cancer, highlighting the protective effects of sunlight and high levels of vitamin D on various types of tumors (2–4,6–9) (Fig. 2).

Colorectal Cancer (CRC)

The ability of $1,25(OH)_2D_3$ to induce differentiation in colon cancer cells was recognized more than 20 years ago (22), and there is substantial evidence supporting an inverse association between circulating $25(OH)D_3$ and CRC risk; meta-analyses and systematic reviews have observed a 50% lower risk of CRC comparing extreme quintiles of $25(OH)D_3$ (23,24). Several mechanisms have been hypothesized to underlie this association, some of which may be shared by pathways associated with the putative functional consequences of CRC susceptibility SNPs proximal to VDR DNA binding sites. In addition, vitamin D signaling occurs through binding of the active form $1,25(OH)_2D_3$ to VDR along specific genomic sequences known as VDREs, which act to activate or repress gene transcription. Several prospective epidemiologic studies, including from this cohort (1), have consistently found an inverse association between higher prediagnostic $25(OH)D_3$ levels and CRC risk. Similar to the results for CRC incidence, higher vitamin D levels have been suggested to be inversely associated with CRC-specific and overall mortality among persons diagnosed with CRC in a small number of studies (25–27). Findings from the Nurses' Health Study and the Health Professionals Follow-up Study have shown an association between either higher prediagnostic $25(OH)D_3$ levels or higher predicted postdiagnosis $25(OH)D_3$ scores and improvement in CRC-specific and overall survival (28). However, one study (29) was limited by its relatively small sample size and the other (30) by its use of predicted, not actual, postdiagnosis vitamin D levels. Another study from Japan has suggested that higher $25(OH)D_3$ levels at surgery are associated with a better survival (31), but it is also limited by small sample size.

Breast Cancer

In 1990, Garland et al. first reported an inverse association between total average annual sunlight energy that strikes the ground and age-adjusted breast cancer mortality in the US (4). Several case-control studies have focused on the association between breast cancer risk and circulating levels of $25(OH)D_3$. Results have consistently revealed an inverse association between $25(OH)D_3$ and breast cancer (32–34). Other studies have examined the effects of vitamin D on mammary carcinogenesis in vitro and in animal models, and the data support a protective role for vitamin D in breast cancer development (35,36). In addition, mice

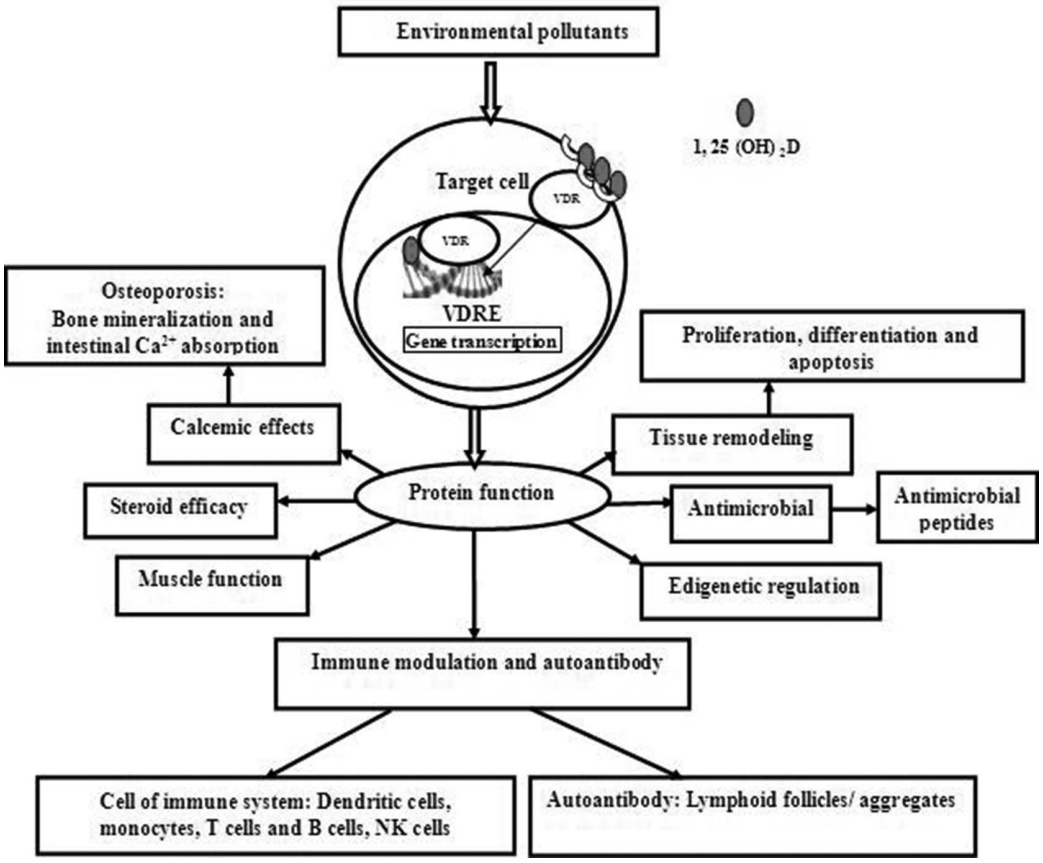

Figure 2. The role of vitamin D/VDR in environmental agent-mediated deregulation. Environmental agents, such as cigarette smoke, particulate matter (less than 10 μm, PM10), ultrafine particles, inhaled oxidants, ozone, and aldehydes activate vitamin D receptor (VDR) and affect different downstream cellular and molecular targets as a result of vitamin D-mediated deregulation. Calcitriol is bound to VDR and vitamin D response elements (VDRE). In conjunction with several transcription factors, this complex led to the transcription of vitamin D-responsive genes. The major cellular and molecular functions affected due to vitamin D/VDR deregulation include calcemic effects, antimicrobial, tissue remodeling, immune modulation and autoantibody production, muscle function, steroid efficacy, epigenetic regulation, immune response, inflammation, and cellular proliferation, differentiation, and apoptosis.

rendered vitamin D deficient exhibit enhanced cancer development (37), as do VDR knockout mice (38). Several mechanisms underlying the inhibitory effects of vitamin D on the growth of breast cancer cells have been proposed. Six case-control studies have examined the relationship between vitamin D intake and breast cancer risk. The largest was an Italian study that included 2,569 cases and 2,588 controls in which a 78-item food frequency questionnaire was used to collect information on dietary sources of vitamin D. Women with the highest vitamin D intake (>190 IU) had a 34% lower risk for breast cancer than those with the lowest vitamin D intake (<60 IU) (39). The odds ratios (ORs) were 0.80 [95% confidence interval (CI) 0.64–0.99] and 0.78 (95% CI 0.66–0.92) among pre- or perimenopausal and postmenopausal women, respectively (40). The strengths of the study are the large dataset and the use of a reproducible and valid food frequency questionnaire (41). The study results were adjusted for many known risk factors for breast cancer. Limitations of the study include the absence of information on sun exposure or serum levels of vitamin D and the use of hospital-based controls. Two other case-control studies also reported a relatively lower breast cancer incidence with greater vitamin D intake (42). A similar finding was reported in the Women's Health Study cohort that included 10,578 premenopausal women and 20,909 postmenopausal women (43). Higher intake of vitamin D was associated with a lower risk for breast cancer in premenopausal women (OR 0.65; 95% CI 0.42–1.00) but not in postmenopausal women (OR 1.30; 95% CI 0.97–1.13) (44). Other studies that included predominantly postmenopausal women either showed a trend toward a lower breast cancer risk with higher vitamin D intake (45,46) or did not show a protective effect of higher vitamin D intake for breast cancer.

Lung Cancer

In vitro and in vivo studies have demonstrated the antiproliferative effects of $1,25(OH)_2D_3$ in lung cancer. Higashimoto et al. reported that $1,25(OH)_2D_3$ inhibited the growth of lung cancer cell lines (47). This effect was mediated by VDR and affected cell cycle regulation in squamous cell carcinoma (SCC) (48). $1,25(OH)_2D_3$ has also been shown to inhibit lung tumor growth and lung metastases in mouse models (49). Owing to the high number of blood vessels in the lungs, circulating tumor cells easily metastasize there and have proven to be difficult to treat with chemotherapy. Nakagawa et al. demonstrated using Lewis lung carcinoma cells: green fluorescent protein (GFP) construct in a murine model that $1,25(OH)_2D_3$ strongly inhibited metastatic growth in the lung of VDR null mice (50). In parallel in vitro experiments using Lewis lung carcinoma cells, it was noted that VEGF mRNA, an indicator of angiogenesis, was suppressed following treatment with $1,25(OH)_2D_3$ at 24 h. The data suggests that $1,25(OH)_2D_3$ directly reduces tumor metastatic growth in lung cancer cells (51). Several studies reported normal tracheobronchial cells have high levels of 1α-hydroxylase (CYP27B1) enzyme that leads to increased local production of $1,25(OH)_2D_3$ and low levels of CYP24A1 that leads to increased breakdown. This is in contrast to lung cancer cells that show higher CYP24A1 expression and low to absent CYP27B1. Reciprocal changes that involve an increase in CYP27B1 mRNA and a decrease in CYP24A1 mRNA may play a pivotal role in maintaining the local tissue level of $1,25(OH)_2D_3$ to be antiproliferative to lung cancer cells (51–53). VDR expression is ubiquitous, and there are data to suggest that higher nuclear VDR expression in lung cancer correlates with improved survival (52). This may relate to increased genomic effects mediated by nuclear VDR on cell cycle-related genes that lead to apoptosis, but this is yet to be confirmed in lung cancer. There are also data to suggest that VDR expression is higher in well-differentiated SCC compared with normal or dysplastic bronchial epithelium (53). This finding is intriguing and worthy of further study to elucidate the relationship between the differentiation status of lung cancer and vitamin D. Chen et al. show a high-level expression of CYP24A1 in subsets of lung cancers and demonstrate an inverse relationship between high CYP24A1 expression and antiproliferative activity of vitamin D (54). Earlier reports regarding increased expression of CYP24A1 in lung adenocarcinoma (55) found that the tumors that had a higher CYP24A1 expression were more poorly differentiated, as well as associated with poor survival. In a parallel in vitro experiment, it was demonstrated that lung cancer cell lines with high CYP24A1 expression had a poorer response to the antiproliferative effects of $1,25(OH)_2D_3$ compared with those with lower levels of CYP24A1 mRNA. Ramnath et al. confirmed that CYP24A1 expression was indeed highly expressed in lung cancer compared with nontumorigenic normal bronchial epithelium (56). Analysis of non-small-cell lung carcinoma (NSCLC) cell cultures revealed time-dependent loss of $1,25(OH)_2D_3$ coincident with the appearance of CYP24A1-generated metabolites. Specific inhibition of CYP24A1 slowed the loss of $1,25(OH)_2D_3$ and increased the $1,25(OH)_2D_3$ half-life. These data suggest that increased CYP24A1 expression in lung tumors restricts $1,25(OH)_2D_3$ antitumor activity.

Prostate Cancer

There is striking geographical variation, such that regional intensity of exposure to solar ultraviolet radiation (UVR) is inversely associated with prostate cancer incidence and mortality in fair-skinned populations (57). Furthermore, inverse associations of cumulative UVR exposure, adult sunbathing, childhood sunburn, and regular holidays in sunny climates with prostate cancer risk have been observed at the individual level (58,59). The effects of UVR on prostate cancer may be mediated by circulating vitamin D levels, the main environmental source of which is sun exposure, which stimulates vitamin D synthesis in the deeper layers of the epidermis. A study based on the Health Professionals Follow-up Study (HPFS) and the Physicians Health Study (PHS) showed that patients with $25(OH)D_3$ levels <40.5 nmol/L were more likely to die from prostate cancer (HR 1.59, 95% CI 1.06–2.39) compared with levels >95.9 nmol/L (60). From both cohorts, prediagnostic serum samples were used. The association was largely explained by the association between low $25(OH)D_3$ levels and cancer of advanced stage and higher Gleason score. The association tended to be stronger when restricting the analyses to patients with samples collected within 5 years of the cancer diagnosis. Similar results were observed in a Norwegian study of prostate cancer patients, based on serum samples collected ±3 months from the date of the cancer diagnosis (61). The risk of cancer death in patients with $25(OH)D_3$ levels >80 nmol/L was 0.16 (95% CI 0.05–0.43) relative to patients with levels <50 nmol/L. A risk reduction was also seen in patients with $25(OH)D_3$ levels 50–79 nmol/L (RR 0.33, 95% CI 0.14–0.77). Mice with prostate epithelial cell-specific deletion of VDR (PEC VDRKO) were generated to study the direct effects of VDR on epithelial cell turnover during castration and in response to testosterone repletion. PEC VDRKO mice exhibit lower rates of apoptosis in response to castration and higher rates of proliferation in response to testosterone administration than control mice. These data show that low vitamin D status and VDR deletion alter cell turnover and hormonal responsiveness in normal prostate tissue changes that likely contribute to an increased susceptibility of VDR null mice to PIN and tumorigenesis.

Skin Carcinogenesis

UV induces various types of DNA damage either photochemically or by UV activation of endogenous photoreceptors that create genotoxic free radicals that modify the DNA molecular structure. The most frequently occurring photolesion in sun-exposed human skin is the cyclobutane pyrimidine dimer (CPD) (62,63) particularly thymine dimers, which are induced primarily by UV-B, and also by UV-A to a lesser extent (64,65). CPDs are produced by the dislocation of double bonds in two adjacent pyrimidines by UV absorption, resulting in a cyclobutane ring conformation linking the two nucleobases as a dimer (66,67). Many studies have shown that $1,25(OH)_2D_3$ reduces thymine dimers in irradiated skin cells in vitro (68) and also in vivo in mouse (69) and human skin (70). Thymine dimers are also reduced in irradiated skin cells in the presence of the low calcemic rapid acting cis-locked nongenomic analogs, $1,25(OH)_2$-lumisterol$_3$(JN) and $1,25(OH)_2$-7-dehydrocholesterol (JM) in vitro (71) and in mouse skin (69) and also by the transcriptionally active hybrid 1-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitamin D_3. Evidence that the vitamin D photoprotective effect on reductions in thymine dimer DNA damage is via the rapid nongenomic pathway is demonstrated with various vitamin D-like compounds. As noted above, studies by our group have shown that the transcriptionally nonactive $1,25(OH)_2$-lumisterol$_3$ protects against UV-induced thymine dimers. Of relevance to the mechanism of action of vitamin D compounds in photoprotection, the coincubation of skin cells with $1,25(OH)_2D_3$ and 25-dehydro-1α-hydroxyvitamin D_3-26, 23S-lactone (TEI-9647), an antagonist of the genomic action of $1,25(OH)_2D_3$, did not alter the protective effects of $1,25(OH)_2D_3$ on thymine dimers. In contrast, coincubation with 1β, 25-dihydroxyvitamin D_3 (HL), an antagonist of the nongenomic pathway, abolished the photoprotective effect of $1,25(OH)_2D_3$ (72,73).

Other Tumors

The pathway of vitamin D seems to be involved in the development of endocrine and neuroendocrine tumors too. Studies by Grant as well as by Freedman et al. on cancer mortality rates in the US and Europe, using latitude or DNA-weighted solar UV-B exposure as surrogate endpoints for photoproduction of vitamin D_3 in the skin, found a highly significant association with the incidence of esophagus, stomach, pancreas, bladder, ovary, and uterus, as well as non-Hodgkin lymphoma (3,6,74–75).

THE EFFECT OF VITAMIN D AND CALCIUM ON CARCINOGENESIS

Studies on tissue-specific expression of the CYP27B1-encoded 25-hydroxyvitamin D-1α-hydroxylase and of the extracellular calcium-sensing receptor (CaR) have led to an understanding of how locally produced $1,25(OH)_2D_3$ and extracellular calcium act jointly as key regulators of cellular proliferation, differentiation, and function. Thus, impairment of antimitogenic, proapoptotic, and prodifferentiating signaling from the $1,25(OH)_2D_3$-activated VDR and from the CaR in vitamin D and calcium insufficiency has been implicated in the pathogenesis of the aforementioned types of cancer. $1,25(OH)_2D_3$ and calcium interact in modulating cell growth in different ways: (a) signaling pathways from the VDR and the CaR converge on the same downstream elements, for example, of the canonical Wnt pathway; (b) high extracellular calcium modulates extrarenal vitamin D metabolism in favor of higher local steady-state concentrations of $1,25(OH)_2D_3$; (c) $1,25(OH)_2D_3$ may upregulate expression of the CaR and thus augment CaR-mediated antiproliferative responses to high extracellular calcium. Grau et al. studied the effect of vitamin D and calcium supplementation on recurrence of colorectal adenomas, who found that calcium supplementation was effective only in patients with normal $25(OH)D_3$ values (76). Conversely, high $25(OH)D_3$ levels were associated with a reduced risk of adenoma recurrence only among subjects receiving calcium supplements. Synergistic actions of calcium and vitamin D are probably the reason why high intake of low-fat dairy products is associated with a reduced risk of breast cancer in premenopausal women. Finally, results from studies in animal models of human autoimmune diseases indicated that calcium supplementation was necessary to optimize the therapeutic effect of vitamin D. Therefore, vitamin D, its analogs, and calcium should be further evaluated in clinical trials in patients with early cancer. In the case of established cancer, it is reasonable to consider that combination therapy will be required and that vitamin D, calcium, or an analog added to other effective therapies will likely increase the benefit of the standard therapy and perhaps reduce some of the side effects.

CONCLUSIONS AND FUTURE PERSPECTIVES

This review highlights the relationship between vitamin D, VDR, calcium, and cancer, summarizing several mechanisms proposed to explain the potential protective effect of vitamin D against the development and progression of cancer. It suggests vitamin D, its analogs, and calcium should be further evaluated in clinical trials in patients with early cancer.

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REVIEW

New insights into vitamin D anticancer properties: focus on miRNA modulation

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Abstract Vitamin D anticancer properties are well known and have been demonstrated in many in vitro and in vivo studies. Mechanistic insights have given an explanation on how vitamin D exerts antineoplastic functions, which are mainly conducted via the canonical vitamin D receptor (VDR)–vitamin D response elements (VDRE) pathway. Numerous findings indicate that dietary components, including vitamin D, could exert chemopreventive effects through alterations of microRNA (miRNA) expression. As miRNAs have important roles in regulating diverse and vital cellular processes, it has been speculated that vitamin D's non-classical effects, including anticancer effects, could be mediated through alterations of miRNA expression level. The current review focuses on up-to-date experimental data on modulation of miRNA expression by vitamin D treatment in cancer, obtained in a cell culture system, animal models and human cohorts. Reported findings in the review show that vitamin D modulates expression of numerous and diverse miRNAs specific for cancer types. Even in its early phases, with many questions remaining to be answered, dissecting the molecular pathways of vitamin D miRNA modulation is an emerging area of science. The complete unraveling of vitamin D molecular mechanisms will emphasize the vitamin D dietary component as a potential chemopreventive agent in cancer and personalized nutrition.

Keywords Vitamin D · miRNA · Cancer

Introduction

Numerous studies have demonstrated the anticancer effects of various bioactive dietary compounds (DiMarco-Crook and Xiao 2015; Chimento et al. 2016; de la Parra et al. 2016), suggesting their potential use as chemopreventive agents. However, the molecular mechanisms linking nutrition and cancer are not fully elucidated. Nutrients can influence numerous cellular processes involved in cancer development and progression by regulation of gene expression through epigenetic mechanisms, such as DNA methylation, histone modifications and non-coding RNAs (Supic et al. 2013, 2016).

Among the nutrients, vitamin D attracts huge scientific interest due to its association with cancer risk and treatment (Deeb et al. 2007; Feldman et al. 2014). This review summarizes the recent findings on the molecular mechanism of vitamin D action, with focus on microRNA regulation and function in cancer.

Vitamin D synthesis, degradation and mechanism of action

Vitamin D is a fat-soluble steroid hormone with a wide spectrum of physiological effects throughout the body

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(Dusso et al. 2005). Vitamin D is present in human skin in its precursor form (7-dehydrocholecalciferol) and converted to vitamin D_3 using the energy of ultraviolet radiation in sunlight. It can also be taken in the diet from food rich in cholecalciferol or ergosterol, vitamin D-fortified dairy products and supplements. These forms of vitamin D are then converted to the active form calcitriol [$1\alpha25(OH)_2D_3$] through two steps of hydroxylation at the 1-alpha and 25-C positions (Dusso et al. 2005). The first step of hydroxylation occurs in the liver, which is mediated by a 25-hydroxylase enzyme (such as CYP2R1, CYP27A1 and CYP2D25), and results in the synthesis of 25-hydroxyvitamin D_3 [$25(OH)D_3$], also known as calcidiol. Calcidiol is the main circulating form of vitamin D, which is transported through the bloodstream to the kidney for the second step of hydroxylation catalyzed by the 1α-hydroxylase (also known as CYP27B1).

In addition to 25- and 1α-hydroxylases, CYP24A1, a 24-hydroxylase enzyme also plays an important role in vitamin D metabolism. This enzyme is expressed in all cells that are responsive to calcitriol and protects the body from its excess (Feldman et al. 2014). CYP24A1 converts both $25(OH)D_3$ and $1\alpha25(OH)_2D_3$ into 24-hydroxylated products, which are molecules with reduced or no apparent biological activity. Besides CYP24A1, the synthesis of calcitriol is also tightly controlled by two hormones, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) (Feldman et al. 2014). PTH stimulates 1α-hydroxylase production and promotes calcitriol synthesis (Bikle 2014). On the contrary, FGF23 induces expression of 24-hydroxylase, but also directly suppresses activity and expression of the 1α-hydroxylase, thus preventing completion of the $1\alpha25(OH)_2D_3$ synthesis (Dusso et al. 2011; Bikle 2014). Increased calcium levels in blood inhibit PTH secretion and consequently suppress 1α-hydroxylase activity, while increased phosphate levels stimulate FGF23 expression (Bikle 2014).

The biological actions of calcitriol are mediated through the vitamin D receptor (VDR), which is a member of the steroid receptor family that acts as a nuclear receptor transcription factor (Fig. 1a). Calcitriol binds to the VDR, dimerizes with the retinoid X receptor (RXR) and forms a heterocomplex that interacts with the vitamin D response elements (VDRE) located in the promoter region of numerous target genes. This results in the recruitment of co-activators or co-repressors that modulate the transcriptional regulation of target genes. Apart from vitamin D-mediated genomic actions, vitamin D can also bind to plasma membrane caveolae-associated VDR, which activates multiple signaling pathways, such as phosphatidylinositol-3′-kinase (PI3K), phospholipase C and protein kinase C (PKC) (Haussler et al. 2011).

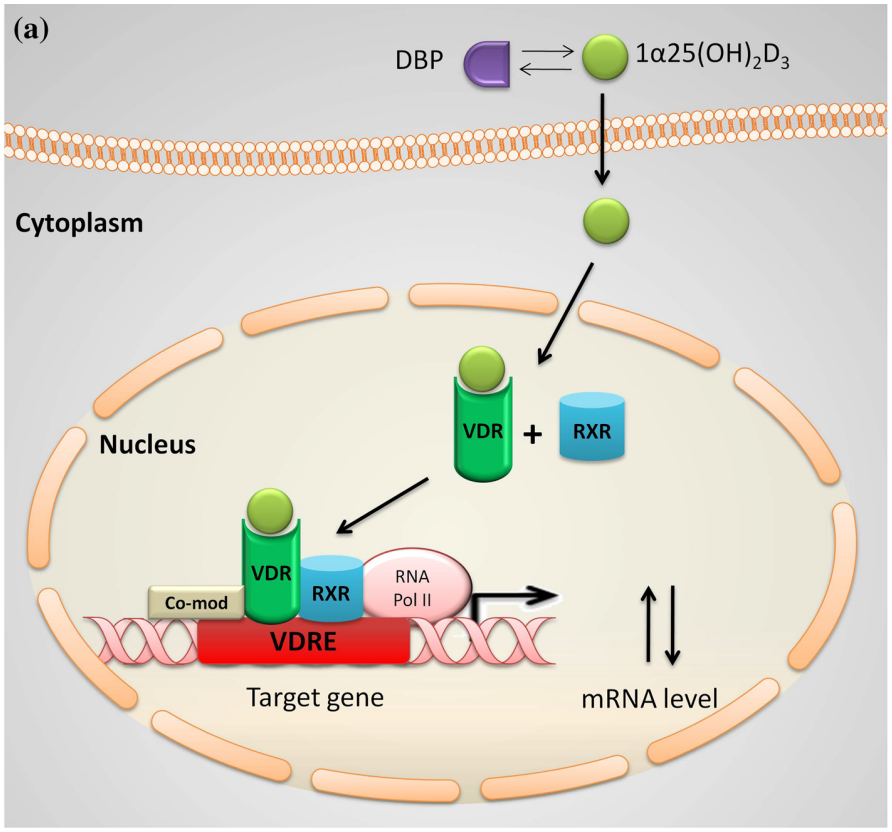
Role of vitamin D in cancer

The anticancer effects of vitamin D were first reported in vitro more than three decades ago (Abe et al. 1981; Colston et al. 1981). At the time it had been shown that growth of malignant melanoma cells was inhibited in the presence of vitamin D (Colston et al. 1981), and that vitamin D induced differentiation of myeloid leukemia cells to macrophages (Abe et al. 1981). Since then, an increasing number of studies have confirmed one of vitamin D's properties to be an anticancer effector in various cancer types (Deeb et al. 2007; Feldman et al. 2014). Also, multiple potentials of vitamin D and its synthetic analogs have been evaluated as an efficient treatment in cancer patients, with minimal risk of side effects, in numerous clinical studies (Feldman et al. 2014). However, knowing all components of the vitamin D anticancer molecular pathway would be of great importance for fully understanding and possible application of vitamin D to cancer prevention and treatment.

Anticancer effects of vitamin D include inducing differentiation and apoptosis, and inhibition of proliferation, angiogenesis, invasion and metastasis (Deeb et al. 2007; Feldman et al. 2014) (Fig. 1b). Specific signaling pathways are regulated by vitamin D in colon, breast and prostate cancers (Feldman et al. 2014). For instance, vitamin D inhibits β-catenin transcriptional activity through repression of the WTN-β catenin signaling pathway which is activated in most colorectal cancers (Larriba et al. 2011, 2013).

Anti-proliferative effects of vitamin D are mainly mediated by increased expression of cyclin-dependent kinase inhibitors p21 (WAF1/CIP1) and p27 (KIP1) leading to G0/G1 cell cycle arrest (Deeb et al. 2007). Inhibition of growth factors, i.e., insulin growth factor 1 (IGF1) and epidermal growth factor (EGF), and inducing the expression of growth factor inhibitors, such as transforming growth factor beta (TGFβ), lead to the inhibition of cancer cell proliferation (Vuolo et al. 2012). Vitamin D acts as an inhibitor of telomerase activity by reducing the expression of telomerase reverse transcriptase (TERT), which also induces apoptosis (Kyo and Inoue 2002; Jiang et al. 2004). Modulation of kinase pathways, such as ERK-MAPK and PI3K has been documented (Deeb et al. 2007). Proliferation of cancer stem-like cells is inhibited by vitamin D through cell cycle arrest (Peng et al. 2016).

Induction of apoptosis upon vitamin D treatment has been demonstrated in different cancer types, such as breast, colon, prostate, melanoma, and glioblastoma. (Hansen et al. 2001). Apoptosis is mainly triggered by suppression of anti-apoptotic genes, i.e., *BCL2* and inducing pro-apoptotic *BAX* and *BAK* (Lamprecht and Lipkin 2003). Caspase pathways are also triggered by vitamin D (Feldman et al. 2014).



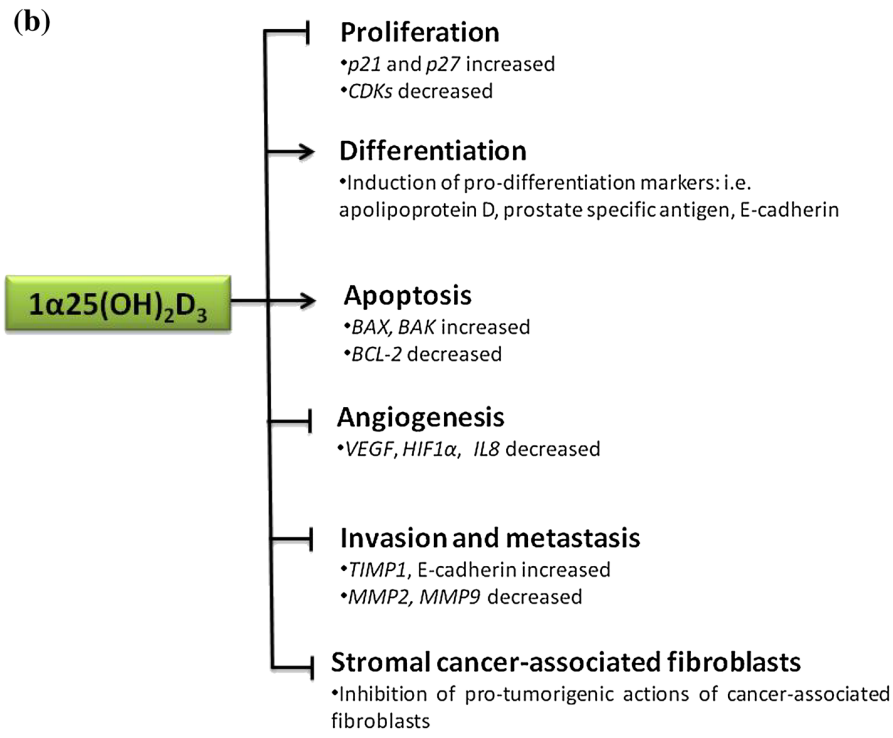


Fig. 1 Genomic action of metabolite active form of vitamin D (calcitriol) and its anticancer properties. **a** Vitamin D ($1\alpha25(OH)_2D_3$) is transported through the bloodstream by vitamin D-binding protein (DBP). Dissociated from the DBP, vitamin D binds to its receptor vitamin D receptor (VDR), which activates binding of the Retinoid X Receptor (RXR). A heterodimer consisting of vitamin D, VDR and RXR interacts with the vitamin D response elements (VDRE) located in the promoter region of the target gene which recruits co-modulators (*co-mod* co-activators and co-repressors). As a result, expression of the target gene will be induced or suppressed. **b** Vitamin D anticancer properties and examples of target genes. *p21 CDKN1A* (*WAF1/CIP1*)—cyclin-dependent kinase inhibitor 1 A; *p27 CDKN1B*—cyclin-dependent kinase inhibitor 1B; *CDKs* cyclin-dependent kinase, *VEGF* vascular endothelial growth factor, *HIF1α* hypoxia-inducible factor 1α *IL8* interleukin 8, *TIMP* tissue inhibitor of metalloproteinases 1, *MMP2, MMP9* matrix metalloproteinase

Stimulation of differentiation in response to vitamin D is demonstrated in various cancer types (Gocek and Studzinski 2009). One of the first examples was vitamin D-induced differentiation of leukemia cells into monocytes by increased expression of p21 (Liu et al. 1996). Vitamin D treatment-induced pro-differentiation markers, such as apolipoprotein D, prostate-specific antigen and E-cadherin (Palmer et al. 2001; Gocek and Studzinski 2009). Pro-differentiation mechanisms which include specific signaling pathways, such as WNT-β catenin, PI3K, NF-kβ are also regulated by vitamin D (Deeb et al. 2007; Gocek and Studzinski 2009).

Invasion and metastasis are mitigated by vitamin D through inhibition of cathepsins and matrix metalloproteinase (MMP), such as MMP2 and MMP9 (Bao et al. 2006b; Chen et al. 2015), increase of tissue inhibitors of metalloproteinase-1 (TIMP-1), and cathepsin inhibitors (Bao et al. 2006b) as well as E-cadherin expression (Lopes et al. 2012).

Vitamin D can inhibit angiogenesis by suppressing expression of vascular endothelial growth factor (VEGF) through hypoxia-inducible factor 1 alpha (HIF1α) inhibition (Mantell et al. 2000; Ben-Shoshan et al. 2007) and interleukin 8 (IL8) (Bao et al. 2006a). In some studies VEGF was found to increase upon vitamin D treatment (Fernandez-Garcia et al. 2005; Garcia-Quiroz et al. 2014), which suggests that vitamin D effects on angiogenesis might depend on tumor and cell type.

Recent evidences show that vitamin D inhibits pro-tumorigenic actions of stromal cancer-associated fibroblasts (CAFs) surrounding the tumour mass, probably through inhibition of NF-kB signaling (Shany et al. 2016). It has been reported that migration of CAFs, derived from colorectal cancer patients, has been inhibited due to vitamin D treatment (Ferrer-Mayorga et al. 2016). Furthermore, vitamin D modulates expression of numerous genes in CAFs, which was associated with longer survival of colorectal cancer patients (Ferrer-Mayorga et al. 2016). Also, vitamin D imposes switching CAFs pro-tumorigenic into more benign phenotype (Ferrer-Mayorga et al. 2016). These new findings expanded the translational importance of using vitamin D as an anticancer agent in treatment of not just tumour mass but also cancer-associated fibroblasts.

MicroRNA and regulation of vitamin D signaling

MicroRNA (miRNA) is a class of small non-coding RNA (ncRNA) with ~18 to 22 nucleotides, which has an important role in post-transcriptional regulation of gene expression and gene silencing. This class of ncRNAs is involved in regulation of numerous key cellular processes, including development, differentiation, cell proliferation, and apoptosis (Kim et al. 2009). Biogenesis of miRNA is a complex multistep, tightly controlled process (Fig. 2). Once synthesized, miRNAs regulate gene expression by directly binding to a complementary sequence in the 3′-untranslated region (3′-UTR) of mRNA, causing either mRNA degradation or translational repression (Kim et al. 2009).

Deregulation of miRNA expression has been reported in numerous diseases, including cancer (Iorio and Croce 2012). It is widely recognized that miRNAs act as an important trigger in cancer initiation and progression (Croce 2009; Iorio and Croce 2012). miRNAs have also been reported to regulate several genes involved in vitamin D pathway, such as *VDR* (Fig. 3a), *CYP24A1, CYP27B1* and *RXRα*. Four miRNAs, *miR-125b, miR-27b, miR-298* and *miR-346*, have been shown to target *VDR* (Mohri et al. 2009; Zhang et al. 2011; Chen et al.

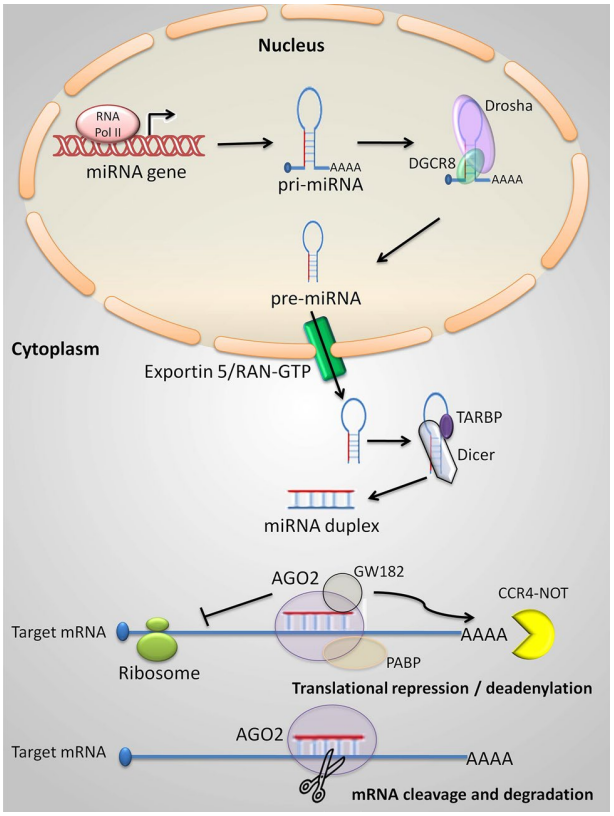


Fig. 2 miRNA biogenesis—canonical pathway. *Nucleus* RNA polymerase II starts the transcription of the miRNA gene. As a result, the primary transcript, pri-miRNA is synthesized, which will be processed by Drosha and Di George Critical Region 8 (DGCR8), which results in the formation of pre-miRNA. Pre-miRNA is transported via Exportin 5/RAN-GTP to the cytoplasm, where it is further processed by Dicer and TARBP (TAR RNA-binding protein). miRNA becomes part of the RISK complex (consisting of AGO2, GW128 and PABP), which in the case of incomplete pairing of miRNA with mRNA, leads to translational repression or deadenylation by CCR4-NOT. Perfect miRNA–mRNA pairing results in mRNA cleavage and degradation

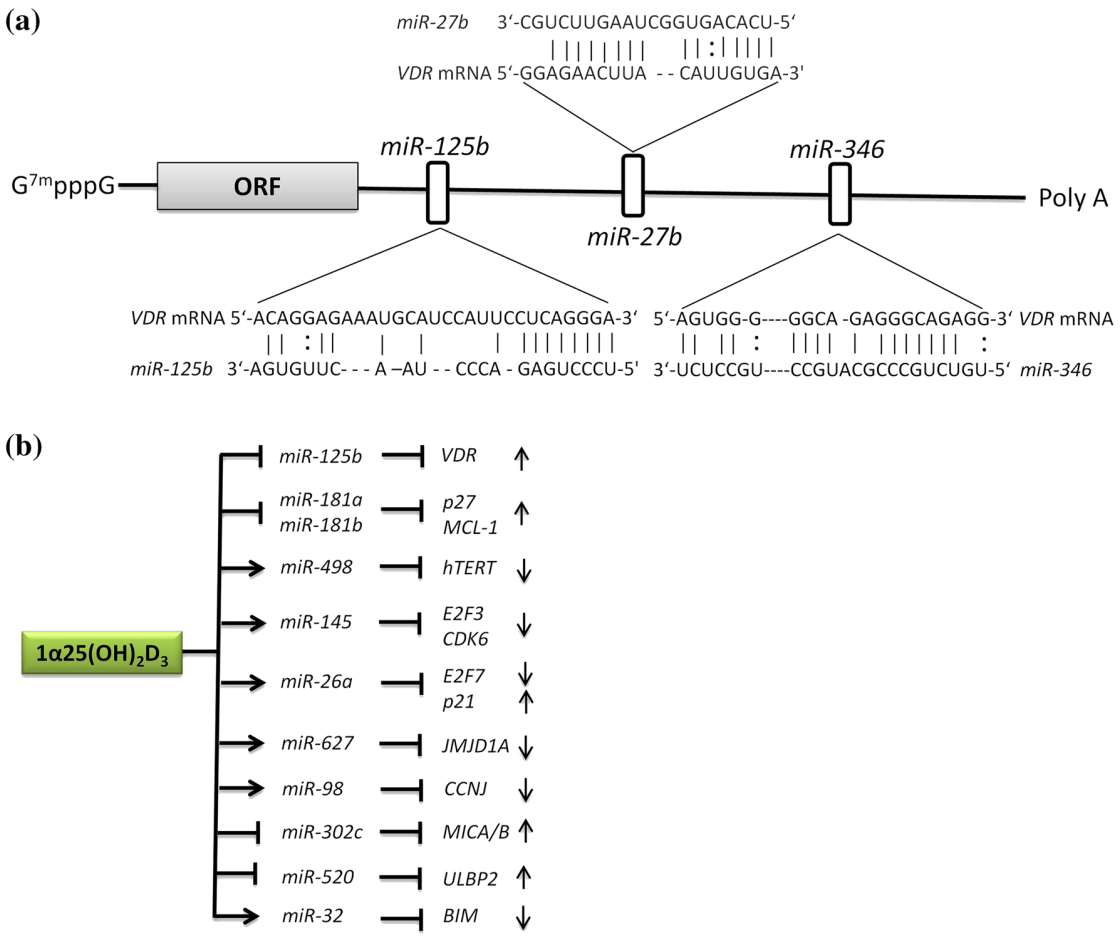


Fig. 3 **a** Schematic presentation of VDR mRNA and predicted target sequence of *miR-125b, miR-27b* and *miR-346*. **b** Examples of miRNAs that are regulated by vitamin D and target genes in different cancer types. ORF—open reading frame; *VDR*, vitamin D receptor; *p27, CDKN1B*—cyclin-dependent kinase inhibitor 1B; *MCL-1*, myeloid cell leukemia 1; *hTERT*, human telomerase reverse transcriptase; *E2F3*, E2F transcription factor 3; *CDK6*, cyclin-dependent kinase 6; *p21, CDKN1A* (*WAF1/CIP1*)—cyclin-dependent kinase inhibitor 1A; *E2F7*, E2F transcription factor 7; *JMJD1A*, Jumonji domain containing 1A; *MICA/B*, MHC class I polypeptide-related sequence A/B; *ULBP2*, UL16-binding protein 2

2014; Li et al. 2015). The first miRNA targeting *VDR* was identified by Mohri et al. (2009), who demonstrated that *miR-125b* directly regulates *VDR* gene expression in the MCF-7 breast cancer cell line and its over-expression can abolish the anti-proliferative effects of vitamin D. Furthermore, *miR-125b*-mediated suppression of *VDR* plays an important role in regulating hair follicle differentiation (Zhang et al. 2011). *miR-27b* was reported to be a regulator of *VDR* gene expression in melanoma, LS-180 colon cancer, PANC1 pancreatic cancer cell lines and human lung fibroblast MRC5 cells (Pan et al. 2009; Essa et al. 2012; Li et al. 2015). Li et al. (2015) verified by luciferase reporter assay that *miR-27b* directly targets *VDR* 3′UTR, which leads to decrease of VDR protein, but not mRNA levels. Pan et al. (2009) also demonstrated that the *miR-298* binding site within the 3′UTR of *VDR* is highly conserved in mice, rats and humans; they verified the direct interaction using a luciferase reporter assay. *miR-346* was found to suppress *VDR* expression during gut mucosal inflammation by direct targeting of *VDR* 3′UTR (Chen et al. 2014).

CYP24A1 has been shown to be regulated by *miR-125b* (Komagata et al. 2009) and a *miR-17~92* cluster (Borkowski et al. 2015). Functional analysis validated direct targeting of *CYP24A1* by *miR-125b* in KGN and MCF-7 cell lines (Komagata et al. 2009). *CYP27B1* expression was found to be directly regulated by *miR-21* in *Mycobacterium leprae*-infected monocytes, which was validated by the luciferase reporter assay (Liu et al. 2012).

It has been shown that *RXRα* is post-transcriptionally regulated by *miR-27a, miR-27b, miR-128-2* and *miR-574-3p* (Ji et al. 2009; Adlakha et al. 2013; Guerit et al. 2013). Rat *RXRα* was directly down-regulated by *miR-27a* and *miR-27b* in activation of hepatic stellate cells (Ji et al.

2009). Direct interaction between *miR-128-2* and *RXRα* has been confirmed in HEK293T cells by the luciferase reporter assay (Adlakha et al. 2013). *miR-574-3p*-mediated suppression of *RXRα* was found to be important in regulating mesenchymal stem cell differentiation to chondrocytes (Guerit et al. 2013).

Vitamin D modulates microRNA expression in cancer

Vitamin D can regulate the transcription of miRNA genes through VDR binding to its sequence motif located in the promoter of target miRNA genes, miRNA maturation through regulating genes involved in miRNA processing (such as Drosha, and Dicer) or miRNA stability (Giangreco and Nonn 2013).

Also, there are suggestions that vitamin D not only increases specific miRNAs, but up-regulates miRNAs expression on the global level, by VDR-dependent chromatin opening and increased pri-miRNA expression (Giangreco and Nonn 2013).

Examples of such regulation of miRNA expression by vitamin D on both transcriptional and post-transcriptional levels in different cancer types are given below (Fig. 3b; Supplementary Tables 1, 2, 3). Cell lines with tissue of origin are listed in the Supplement Table 4.

Ovarian cancer

It has been reported that the active metabolite form of vitamin D suppresses human telomerase reverse transcriptase (*hTERT*) expression and growth of ovarian human cancer cell lines OVCAR3 through *miR-498* induction in a vitamin D dose-dependent manner (Kasiappan et al. 2012). OVCAR3 cells were exposed to different calcitriol concentrations for 24 h, and showed dose-dependent induction of *miR-498* expression. At the lowest concentrations, *miR-498* was first induced, suggesting *miR-498* to be an early response gene to calcitriol treatment (Kasiappan et al. 2012). In the regulatory region of *miR-498* gene, a functional VDRE was identified, which was verified by ChiP assay. Vitamin D treatment induced VDR-RXR and co-activators binding to VDRE of *miR-498* gene. By luciferase reporter assay, *miR-498* direct targeting of *hTERT* 3′-UTR was confirmed. The ability of vitamin D to suppress growth of ovarian cancer and *hTERT* expression was prevented by *miR-498* depletion. Thus, anticancer effects of vitamin D in this ovarian cancer cell line were found to be mediated through transcriptional up-regulation of *miR-498* expression and consequently *hTERT* down-regulation (Kasiappan et al. 2012). In addition, *miR-498*-mediated *hTERT* down-expression is a key event mediating the anti-leptin activity of calcitriol in estrogen-sensitive tumours in women (Kasiappan et al. 2014).

Cervical cancer

In a recent study (Gonzalez-Duarte et al. 2015), vitamin D-sensitive cervical cancer cell lines (HeLa and SiHa) and vitamin D non-responsive C33-A cells were treated with 1 μM calcitriol for 24 and 48 h. Upon calcitriol treatment, the mRNA as well as protein level of *Dicer*, but not *Drosha*, was increased after 24 and 48 h of treatment in the case of SiHa cells, while increased expression was observed only after 48 h in HeLa cells. As C33-A cervical cancer cells do not express the vitamin D receptor, no change in either *Dicer* or *Drosha* mRNA and protein levels were observed (Gonzalez-Duarte et al. 2015). Also, expression analysis in SiHa cells revealed that 16 miRNAs were down-regulated after 24 h of treatment and 15 miRNAs down-regulated after 48h of calcitriol treatment compared with non-treated SiHa cells. Only *miR-3921* was down-regulated at both time points (Gonzalez-Duarte et al. 2015). Numerous miRNAs were up-regulated upon calcitriol treatment after 24 and 48 h, where *miR-22, miR-2963p, miR-29c, miR-342-5p, miR-4455, miR-4462* and *miR-4656* were induced at both time points. One of the induced miRNAs was *miR-498*, also found to be up-regulated in ovarian, breast and endometrial cancer cell lines (Kasiappan et al. 2012), while *miR-22* up-regulation was also confirmed in prostate, colon and bladder cancer cells (Wang et al. 2011; Alvarez-Diaz et al. 2012; Ma et al. 2015). This study showed vitamin D modulation of *Dicer* through the VDRE found in the *Dicer* promoter, which consequently modulated expression of miRNAs (Gonzalez-Duarte et al. 2015).

Breast cancer

Peng et al. (2010) demonstrated calcidiol protective effects against cellular stressors, such as serum starvation, hypoxia, H_2O_2-induced oxidative stress and apoptosis in the epithelial breast cancer cell line MCF-12F. Also, in 24-h low-serum-stressed MCF-12F cells, levels of multiple miRNAs, including *miR-182, miR-200b, miR-200c, miR-26b* and *let-7b* increased, while levels of *miR-18a, miR-106* and *miR-30c* decreased, compared with non-stressed cells. Treatment with calcidiol (250 nM) reversed or inhibited expression of stress-induced miRNAs, which additionally confirmed the protective effects of the main circulating vitamin D form, calcidiol, as well as the important role of miRNAs as mediators of vitamin D biological functions (Peng et al. 2010). Therefore, the possibility of using calcidiol as a natural chemopreventive agent in stress-induced carcinogenesis through maintaining normal miRNA expression level is suggested.

Dose-dependent vitamin D induction of *miR-498* at the transcriptional level was confirmed in breast (MCF-7) and endometrial (Ishikawa) cancer cell lines, implicating *miR-498* regulation in vitamin D's anticancer effects in many vitamin D-sensitive cancers (Kasiappan et al. 2012).

In MCF-7 and MDA-MB-231 breast cancer cell lines, vitamin D treatment resulted in the reduction of *miR-302c* and *miR-520* expression and increased susceptibility of cancer cells to cytotoxic effects of natural killer cells and up-regulated the NKG2D pathway ligands *MICA*/*B* and *ULBP2*, putative targets of *miR-302c* and *miR-520* (Min et al. 2013).

A recent study has shown that VDR negatively regulates expression level of *miR-199a*/*miR-214* cluster (*miR-199a-3p, miR-199a-5p, miR-214*) through modulation of dynamin-3 gene (*Dnm3os*) in breast cancer cell lines (MCF-7, T47D) as well as in murine VDR knock-out (VDRKO) and VDR wildtype (WT-145) mammary tumor cell lines (Alimirah et al. 2016). Vitamin D treatment of T47D cells (50nM, 24h) induced *VDR* and *p21* expression on protein and mRNA level (Alimirah et al. 2016). *miR-214* over-expression attenuated vitamin D signaling in both T47D and MCF-7 cell lines (Alimirah et al. 2016).

Prostate cancer

Several studies examined the association between vitamin D treatment in prostate cancer cell lines and miRNA expression. In LNCaP prostate cancer cells, numerous miRNAs were up-regulated (i.e., *miR-21, miR-22, miR-29a/b, miR-134*) while *miR-17/92* cluster members were down-regulated (*miR-17, miR-18a, miR-20a/b*) after treatment with vitamin D (100nM) and testosterone (5nM) (Wang et al. 2011), indicating additive and/or synergistic effects of vitamin D and testosterone treatment on the expression of miRNAs (Wang et al. 2011). A later mechanistic study demonstrated that *PPARA* (peroxisome proliferator-activated receptor alpha), the predicted target of the *miR-17/92* cluster, was up-regulated, which resulted in increased lipogenesis and altered energy metabolism to the production of neutral lipids (Wang et al. 2013).

In another study (Thorne et al. 2011), RWPE-1 non-malignant prostate epithelial cells, RWPE-2 and P69SV40T human prostate cancer cells were treated with 100 nM calcitriol. The cell cycle was arrested after 24 h of calcitriol treatment. Thorne et al. (2011) reported that VDR induced histone modifications of *p21* (*WAF1*/*CIP1*) gene's promoter, which was followed with increased *miR-106b* expression. *p21* was confirmed as the direct target of *miR-106b*. Vitamin D treatment induced cell cycle arrest due to increased *miR-106b* and concomitant decreased *p21* expression. It has been shown that the *miR-106b* gene is located in the intron of the *MCM7* gene. Vitamin D regulates the *MCM7* gene through VDRE and consequently up-regulates expression of *miR-106b*. Therefore, in prostate cancer cell lines, vitamin D exerts anticancer properties through induction of *MCM7, miR-106b*, and decrease of *p21*, which altogether leads to cell cycle arrest (Thorne et al. 2011).

In primary prostate cells (PrE), PrECa, RWPE-1 and RWPE-2 cell lines, *miR-100* and *miR-125b* were up-regulated after 50 nM vitamin D treatment for 24 h as opposed to non-treated cells. However, this effect was not confirmed in LNCaP, DU145 and PC3 cells (Giangreco et al. 2013). Giangreco et al. (2013) also demonstrated that miRNAs are required mediators of vitamin D-regulated expression of *E2F3* and *PLK1* genes. Vitamin D treatment of PrE and PrE-Ca cells increased *miR-100* and *miR-125b*, while decreasing *E2F3* and *PLK1* expression levels in a VDR-dependent manner. *miR-100* and *miR-125b* were, further, found to have anti-migratory, anti-proliferative and anti-colonigenic properties either in the presence or absence of vitamin D treatment, but depending on the cell type.

Expression of *miR-98* was shown by Ting et al. (2013) to be induced in a VDR-dependent manner, and anticancer properties were significantly promoted by vitamin D treatment in the prostate cancer cell line LNCaP via G2/M cell cycle arrest and cyclin J gene (*CCNJ*) down-regulation. They used the ChiP assay to demonstrate that vitamin D regulates *miR-98* expression directly, at the transcriptional level, by VDR binding to VDRE, identified in the *miR-98* gene promoter. Also, vitamin D indirectly up-regulated *miR-98* post-transcriptionally through suppression of microRNA processing proteins LIN28A and LIN28B. Anti-proliferative effects of vitamin D were decreased due to *miR-98* knockdown. Direct interaction between *miR-98* and *CCNJ* 3′UTR was demonstrated using a luciferase reporter assay. Overall, results of the study suggested *miR-98* to be a key mediator of vitamin D anti-proliferative effects in prostate cancer.

Bladder cancer

So far, only one study has investigated calcitriol regulation of miRNA expression in human bladder cancer cell lines of different tumorigenic and metastatic capacities, 253J (low tumorigenic and non-metastatic) and 253J-BV (highly tumorigenic and metastatic derivative line) (Ma et al. 2015). Both cell lines expressed endogenous VDR as well as CYP24A1 proteins, which were additionally induced after 48 h of treatment with calcitriol (10, 100 and 500 nM) in a dose-dependent manner (Ma et al. 2015). It was demonstrated that numerous miRNAs are differentially modulated upon calcitriol treatment (500 nM) in 253J and 253J-BV bladder cancer cell lines, showing induced expression after 24 and 48 h of treatment. In 253J cell line, *miR-17, let-7a* and *miR-1201* were induced at both time points upon

calcitriol treatment, while numerous miRNAs were found to be up-regulated in 253J-BV, including *miR-22, miR-96* and *miR-125*. Vitamin D was shown to differentially induce miRNAs depending on carcinogen properties of different bladder cancer cell lines. However, there are no mechanistic insights on how vitamin D regulates miRNA expression in bladder cancer cell lines.

Colorectal cancer

In vitro studies conducted on HT-29 and HCT-116 cell lines treated with calcitriol (100 nM) for 24 h demonstrated that *miR-627* was significantly up-regulated (Padi et al. 2013). In the same study, histone demethylase, *JMJD1A* (Jumonji domain containing 1A), was confirmed as the direct target of *miR-627*. Briefly, calcitriol treatment of colorectal cancer cell lines augmented its anticancer properties via *miR-627* up-regulation which consequently led to down-regulation of *JMJD1A*. By decreasing *JMJD1A*, methylation of H3K9 and H3K27 histones was increased, while being reduced in the case of H3K4 histone which suppressed expression of *JMJD1A* target genes, such as *GDF15* (Growth differentiation factor 15) (Padi et al. 2013). The anti-proliferative effects of vitamin D and *JMJD1A* decrease were blocked upon *miR*-627 inhibition. In addition, in the human colon cancer clinical specimens, lower *miR-627* expression was observed in colon cancer tissue compared with normal colon mucosa (Padi et al. 2013). As tumor stages and the presence of nodal metastases were not associated with *miR-627* expression, it was hypothesized that decreased expression of *miR-627* is a feature of the early stages of colorectal cancer formation. Overall, vitamin D anticancer epigenetic activities appear to be mediated through *miR-627* in colorectal cancer cells. The exact molecular mechanism of vitamin D up-regulation of *miR-627* still remains to be elucidated.

Experiments with human colon cancer cells (SW480-ADH and HCT-116) showed time-, dose- and VDR-dependent induction of *miR-22* by calcitriol treatment (Alvarez-Diaz et al. 2012). By inhibiting *miR-22*, anti-proliferative and anti-migratory effects of vitamin D were also inhibited. Furthermore, anti-miR-22 transfection abolished vitamin D down-regulation of target genes *OGN* (osteoglycin), *NELL2* (neural tissue-specific epidermal growth factor-like repeat domain-containing protein), *HNRPH1* (heterogeneous nuclear ribonucleoprotein H1), *RERE* (arginine glutamic acid dipeptide repeats) and *NFAT5* (nuclear factor of activated T cells 5). Reduced expression of *miR-22* in colon cancer tumors was observed, compared with normal tissue (Alvarez-Diaz et al. 2012). Also, an association between the expression of *miR-22* and *VDR* was confirmed (Alvarez-Diaz et al. 2012). Thus, tumor-suppressor *miR-22* was proposed as a mediator in the expression of vitamin D's anticancer properties. How vitamin D regulates *miR-22* expression remains unknown.

Gastric cancer

A recent study by Chang et al. (2015) reported that anticancer effects of vitamin D in gastric cancer cells (SGC-7901 and AGS), treated with 200 nM calcidiol for 48h, are mediated through induction of *miR-145* and consequent down-regulation of its targets *E2F3* and *CDK6*. Interaction between VDR and VDRE upstream of the *miR-145* gene was verified by ChiP assay, highlighting transcriptional regulation of *miR-145* expression by vitamin D. That *miR*-145 directly target *CDK6* and *E2F3* was confirmed by a luciferase reporter assay. Vitamin D anti-proliferative effects in gastric cancer cells were decreased after *miR-145* inhibition. *miR-145* inhibited cell proliferation through *E2F3* down-regulation and downstream cell cycle genes *CDK2* and *CCNA2*. In addition, it was demonstrated that *miR-145* blocks S/G2 transition of gastric cancer cells. Also, down-regulated *miR-145* expression was found in gastric cancer tissue compared with normal tissue and in gastric cell lines compared with normal cells, which indicate that *miR-145* functions as a tumor suppressor (Chang et al. 2015). Together, these results shed new light on miRNA-mediated anti-growth effects of vitamin D in gastric cancer.

Melanoma

According to the literature data, only two studies explored vitamin D miRNA modulation in melanoma. Expression levels of the *VDR* gene as well as several miRNAs were investigated in vitamin D-sensitive (MeWo, SK-Mel28, SM, SK-Mel5) and vitamin D-resistant (SK-Mel25, IGR, Meljuso) melanoma cell lines (Essa et al. 2010). In the vitamin D-sensitive melanoma cell lines MeWo and SK-Mel25, *miR-125b* expression level was inversely associated with the level of *VDR* mRNA, indicating the possible role of *miR-125b* in regulation of VDR expression and vitamin D resistance (Essa et al. 2010). The same study showed that vitamin D sensitivity could be restored using epigenetic drugs, such as histone deacetylase inhibitor and the DNA methyltransferase inhibitor 5-azacytidine (5-Aza) (Essa et al. 2010). *miR-27b* was less expressed in vitamin D-sensitive than -resistant melanoma cell lines (Essa et al. 2012). Combined treatment with vitamin D and 5-Aza significantly reduced the level of *miR-125b* and *miR-27b* while increasing the level of *VDR* mRNA. Essa et al. (2012) also reported that expression levels of *miR-125b* and *miR-27b* in normal human monocytes were not indicative for distinguishing malignant from benign melanocytes.

Lung cancer

In a study conducted by Guan et al. (2013) on lung cancer cell line A549, miRNA *let-7a-2* was up-regulated upon vitamin D treatment (10^{-8}, 10^{-6} mol/L) in a dose-dependent manner. Mechanistic insight demonstrated that the calcitriol–VDR complex up-regulates the expression of *let-7a-2* through interacting with VDRE located in the pre-let-7a-2 promoter, thereby mediating the increased *let-7a-2* expression after calcitriol induction and promoting anti-proliferative effects. Electrophoretic mobility shift and ChiP assays confirmed vitamin D transcriptional regulation of *let-7a-2* expression in vitro and in vivo.

Leukemia

Several studies on leukemia cell lines have dealt with vitamin D treatment and regulation of cellular processes via miRNA alteration. After exposure to low concentrations of calcitriol (0.1–100 nM), decreased levels of *miR-181a* and *miR-181b* were observed in promyeloblastic leukemia cells HL60 and promonocytic leukemia cells U937 (Wang et al. 2009). Down-regulation of *miR-181a* was associated with up-regulation of *p27* mRNA and protein expression, thus inducing G1 cell cycle arrest. Over-expression of *miR-181a* abolished vitamin D-induced *p27* up-regulation, expression of monocytic differentiation markers and stopped G1 cell cycle arrest (Wang et al. 2009). Decreased expression of *miR-181b* was reported in HL60 cells upon vitamin D treatment, accompanied by anti-apoptotic *MCL-1* up-regulation (Zimmerman et al. 2010). It has been reported that vitamin D-induced p53/63 (lyn kinase) activity might modulate *miR-181b* expression, but further mechanistic studies are warranted (Wang et al. 2000; Zimmerman et al. 2010). Combined treatment of HL60 and NB4 cells during monocyte differentiation with vitamin D (100 nM) and phorbol 12-myristate 13-acetate (PMA) (20nM) down-regulated (*miR-181a, miR-181b, miR-130a, miR-135b, miR-146a* and *miR-181d*) (Lutherborrow et al. 2011).

In the study of Duggal et al. (2012), HL60 and U937 cell lines were treated with vitamin D analog doxercalciferol (100 nM) and the rosemary plant-derived antioxidant carnosic acid (10 µM) separately or in combination for 48 h. The findings provided evidence for doxercalciferol-induced monocyte differentiation and cell cycle arrest, which was significantly enhanced with carnosic acid addition. After exposure to either doxercalciferol or carnosic acid or in combination, the *miR-181a* level decreased, followed by *p27* mRNA and protein up-regulation (Duggal et al. 2012). However, expression of *miR-181a* was reduced more strongly in the treatment combining both substances. These results have potential translational significance especially in overcoming the problem of hypercalcemia upon vitamin D treatment of leukemia patients within a clinical setting.

Vitamin D down-regulated the *miR-17-5p/20a/106a* cluster, *miR-125b* and *miR-155*, which was followed with up-regulation of AML1, VDR and CCAAT/enhancer-binding protein (C/EBPβ) (Iosue et al. 2013). In Ago2-depleted HL60 cells, vitamin D-dependent down-regulation of *miR-17-5p/20a/106a, miR-125b* and *miR-155* was impaired. This highlights the requirement of Ago2 for proper vitamin D-induced modulation of miRNAs during the differentiation process (Iosue et al. 2013).

In HL60, NB4 and U937 cell lines treated with vitamin D (100nM), expression of *miR-26a* was increased and followed by c-myc down-regulation (Salvatori et al. 2011). Salvatori et al. (2012) found that *miR-26a* directly targets *E2F7* transcriptional repressor which results in increased *p21* expression and thus G1/S cell cycle arrest. Thus, vitamin D regulation of proliferation and induced differentiation in myeloid leukemia cells is mediated through *miR-26a*.

The expression level of *miR-32* was increased upon calcitriol treatment in human myeloid leukemia cells, HL60 (1nM) and U937 (10nM) as well as in isolated monocytes from healthy individuals (Gocek et al. 2011). Consequently, pro-apoptotic BIM mRNA and protein levels were down-regulated, which suggested *BIM* as a putative *miR-32* target (Gocek et al. 2011). Vitamin D-induced *miR-32* up-regulation was abolished by silencing Drosha and Dicer. Over-expression of *miR-32* promoted vitamin D-induced differentiation of leukemia cells and resulted in decreased *BIM*, thus leading to increased cell survival. Other agents, but not vitamin D, which could inhibit *miR-32*, will be more effective in eradicating leukemia cells.

Recent findings have demonstrated increased susceptibility to natural killer cells (NK92) in human acute myeloid leukemia (Kasumi-1) and K-562 cell lines after treatment with calcitriol for 24h in a dose-dependent manner (Min et al. 2013). Upon vitamin D treatment, down-regulation of *miR-302c* and *miR-520c* was found in Kasumi-1 and K562 cell lines, depending on the dose of vitamin D applied, which indicates a role for *miR-302c* and *miR-520c* as molecular regulators of vitamin D-induced susceptibility to natural killer cells (Min et al. 2013). Functional studies confirmed that *miR-302c* and *miR-520c* serve as negative regulators of NKG2D ligand pathway genes *MICA, MICB* and *ULBP2* by directly interacting and reducing their mRNA and protein levels (Min et al. 2013).

Animal models

The great majority of studies which have investigated vitamin D modulation of miRNA expression have been conducted in maintained cancer cell culture systems. Regarding

miRNA modulation by vitamin D treatment in physiologically normal animal models, one study has been recently published. Namely, the influence of vitamin D treatment on miRNA expression levels was investigated in the *Danio rerio*-zebrafish animal model in vivo (Craig et al. 2014). Upon calcitriol treatment for 7 days after fertilization, 31 miRNAs precursors were differentially expressed (8 down-regulated and 23 up-regulated) in zebrafish (7-day-old post-fertilization larvae in vivo). Functional studies confirmed the role of *miR-125b* in regulating *CYP24A1* gene and protein expression levels in the zebrafish larvae model, which had previously been confirmed in humans (Komagata et al. 2009).

In the study of vitamin D effects on miRNA expression modulation in prostate cancer by Thorne et al. (2011), a mice model was used, namely wildtype C57 BL/6xFBV, treated for 12 and 24 h with calcitriol. They reported increased *miR-106b* expression in prostate tissue followed by p21 (WAF1/CIP1) repression upon vitamin D treatment.

In prostate cancer mice models (TRAMP mice and *wild type* PTEN mice), vitamin D treatment (25 ng/g of mice weight) increased levels of *miR-98* in the blood of both mice models. This highlights the potential use of *miR-98* as a biomarker in prostate cancer and development of a possible vitamin D-based therapy (Ting et al. 2013).

Findings in the ovarian cancer cell line OVCAR3 regarding induced *miR-498* expression levels as mediator upon calcitriol treatment were confirmed in in vivo nu/nu mice models inoculated with *miR-498* OV2008-transfected cells, and treated with vitamin D synthetic analog EB1089 (Kasiappan et al. 2012).

An in vitro study by (Padi et al. 2013) reported that calcitriol exerts anti-proliferative effects by inducing *miR-627* with subsequent down-regulation of *JMJD1A* in colorectal cancer cell lines. These findings were confirmed in the colorectal cancer HT-29 xenograft nude mice model treated with 0.4 µg of calcitriol. Upon calcitriol treatment tumor growth was suppressed in mice, and this was abolished by blocking *miR-627* activity by over-expressing the *JMJD1A* 3′UTR sponge. Additionally, in the mice model stably expressing a *miR-627* tumor xenograft, colon cancer growth was suppressed (Padi et al. 2013). Together, these findings highlight the important role of *miR-627* in promoting anticancer effects of vitamin D in in vitro as well as in vivo models.

Human cohort

The link between vitamin D level and miRNA expression profile has not been extensively studied so far, and studies conducted on a human cohort are limited. The main findings from the few investigations conducted in humans so far are presented here.

In 13 pregnant woman, mRNA and miRNA expression levels in peripheral blood were measured in groups with low (<25.5 ng/ml) and high (>31.7 ng/ml) serum levels of the main vitamin D circulating form calcidiol (Enquobahrie et al. 2011). In total, 305 genes (299 up- and 6 down-regulated) were found differentially expressed between the two groups, mainly genes which are known to have roles in the functioning and development of numerous physiological systems. Ten microRNAs (*miR-589, miR-601, miR-573, miR-138, miR-320d, miR-196a, miR-92b, miR-423-3p, miR-484, miR-93, miR-574-5p*) were down-regulated and *miR-574-5p* was up-regulated in subjects with low calcidiol levels in early pregnancy compared with participants with high calcidiol concentrations. A large number of identified miRNAs target genes were found to be differentially expressed. The study demonstrated that low levels of early pregnancy calcidiol are associated with differences in mRNA and miRNA expression, which could lead to the development of various pathophysiological processes and increased risk for pregnancy complications. At the same time, intrauterine vitamin D deficiency could have subsequent consequences later in childhood and adulthood. However, the study group was small, consisting of only 13 subjects, limiting the generalization of their results, among other concerns in the study (Enquobahrie et al. 2011).

One of the human studies investigated the expression of plasma miRNA in 40 subjects prior to and after 12 months of vitamin D supplementation in high doses (19 subjects given 20,000 IU/week and 21 subjects given 40,000 IU/week), and compared them with a placebo group (37 subjects) (Jorde et al. 2012). Prior to vitamin D supplementation, subject serum levels of calcidiol were positively correlated with plasma *miR-532-3p* expression levels. After 12 months of supplementation, expression levels of *miR-221* were significantly different between subjects and placebo group (Jorde et al. 2012). Although the study used a relatively large group of subjects (in total 77), their results should be taken with caution as findings from the previously conducted pilot study were not reproduced and authors reported an inconsistent association between vitamin D and microRNA levels in plasma (Jorde et al. 2012).

Calcidiol and calcitriol serum and tissue levels were measured in a group of 66 prostate cancer patients treated with 400, 10,000 or 40,000 IU/day for 3–8 weeks prior to prostatectomy (Giangreco et al. 2013). Expression levels of *miR-100, miR-125b, miR-103, miR-331-3p, miR-146a, miR-155, miR-197, miR-106b, miR-141, miR-301a, let-7a* and *let-7b* were down-regulated in prostate cancer tissue compared with normal epithelium. An association between decreased *miR-100* and *miR-125b* expression and *E2F3* increase in prostate tumor was also found. However, after vitamin D treatment, expression levels of *miR-100* and *miR-125b* increased in a vitamin D dose-dependent

manner. Expression levels of *miR-100* and *miR-125b* were positively associated with prostate calcitriol levels. Positive associations were also found between serum calcitriol and calcidiol with *miR-100* and *miR-125b* levels (Giangreco et al. 2013). Overall, this study demonstrated the possibility of using vitamin D supplementation in prostate cancer patients.

Vitamin D levels were measured in a group of 97 acute myeloid leukemia patients in a study by Lee et al. (2014). Here, deficient and insufficient calcidiol levels were associated with worse relapse-free survival (Lee et al. 2014). Although the authors reported that 13 miRNAs were up-regulated and 4 miRNAs were down-regulated in patients with low calcidiol levels (<32 ng/ml), after multiple testing, none of the miRNAs was associated with the level of the main circulating form of vitamin D.

A recently published study by Beckett et al. (2015) found an association between levels of the microRNA *let-7a/8* circulating in serum with vitamin D intake which was dependent on *VDR* gene allele for single nucleotide polymorphisms BsmI (rs1544410) and ApaI (rs7975232). The study involved 200 elderly participants who were surveyed for vitamin D food and supplemental habits. The study demonstrated the importance of considering genotypic variants in vitamin D-related gene-VDR in studies focusing on miRNA expression and vitamin D serum levels. Overall, the findings illustrated the interplay between vitamin D epigenetic modulations and genome variations and highlighted the importance of evaluating human genome variations which could be responsible for differences in responses to vitamin D treatment (Beckett et al. 2015).

Studies with human cohorts are inevitably more complicated compared with in vitro models, particularly bearing in mind crosstalk between different dietary components which could in vitamin D's bioactive form also modulate miRNA expression profiles. In addition, inter-individual genome variability in miRNA and vitamin D-related genes should be considered when interpreting results, as the presence of single nucleotide polymorphisms, for instance, could result in different responses to vitamin D or treatments with other dietary components (Shah et al. 2012).

Conclusion and future perspectives

A growing body of evidence convincingly demonstrates vitamin D as an important cancer chemopreventive and therapeutic agent (Lamprecht and Lipkin 2003; Deeb et al. 2007). Thus, better understanding of the vitamin D molecular pathways is required in different experimental models. So far, little was known about miRNA molecular mediation of the functional effects of vitamin D. From the information presented in this review, it is obvious that vitamin D could have anticancer effects through alteration of miRNA expression in various types of malignancies, such as ovarian, cervical, breast, prostate, bladder, colorectal, gastric, leukemia, melanoma, and lung cancer. Also, miRNAs regulated by vitamin D are specific for certain cancer types. Owing to the limited number of studies which have investigated the role of vitamin D on miRNA expression modulation in cancer, mainly conducted in cell culture systems and animal models, results are inconclusive in terms of a complete elucidation of connections. Further studies are also required with human cohorts.

Numerous studies have reported mainly modulated expression of specific miRNAs upon vitamin D treatment in different cancer types via canonical VDRE regulation. However, there are suggestions that vitamin D can up-regulate the expression of pri-miRNAs on a global level by VDR-dependent chromatin opening (Giangreco and Nonn 2013). Additionally, it is assumed that miRNA expression could be modulated by vitamin D via non-genomic VDR-dependent activation by possible alteration of the miRNA processing machinery or changes in miRNA stability (Giangreco and Nonn 2013). As far as we know, there are still no experimental confirmations of vitamin D modulation of miRNA via non-genomic pathway, thus this assumption remains to be elucidated in the future.

Taken altogether, studies cited in this review have provided new mechanistic insights into vitamin D anticancer effects through miRNA modulation. All findings have potential translational significance. Further studies investigating the role of vitamin D and modulation by dietary components in general of miRNA in cancer and other pathologies are expected in the future.

One of the problems arising in the potential application of vitamin D as a chemoprotective agent is defining the dose which will have optimal biological effects, while not being accompanied by toxic and other side effects. Also, inconsistent results obtained in different types of cancer cell lines and in vivo models, including humans should be examined further. Thus, using vitamin D as a therapeutic agent with our current state of knowledge is still a matter of considerable controversy. At the same time, in in vitro studies it is easy to control the inclusion of dietary components and, therefore, to determine the precise mechanisms by which miRNA expression is altered. However, extrapolating results from in vitro studies to humans could be problematic, as it is unlikely that only one dietary component is having an effect, with apparent associations being the result of synergistic or antagonistic effects of dietary components.

Elucidating the molecular mechanisms of vitamin D modulation of miRNA will contribute to a better understanding of the potential use of vitamin D for therapeutic and preventive purposes in cancer management. Studies of vitamin D modulatory effects on miRNAs expression are

expected to gain more attention in the future. Also, apart from vitamin D, numerous bioactive dietary components are currently under investigation as potential modulators of miRNA molecular signatures in different cancer types. Knowing the details of molecular functions of vitamin D and other dietary components will help in developing the concept of personalized nutrition, where miRNAs could serve as biomarkers and molecular targets which could be modulated by nutritional interventions in health and disease.

The evidence for miRNA regulation by vitamin D treatment discussed in this review gives rising hope for opening a new area of translational biomedical science for the development of novel vitamin D- and miRNA-based therapeutics and improved treatment for an increasing number of cancer patients worldwide.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Magnesium status and supplementation influence vitamin D status and metabolism: results from a randomized trial

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ABSTRACT

Background: Previous in vitro and in vivo studies indicate that enzymes that synthesize and metabolize vitamin D are magnesium dependent. Recent observational studies found that magnesium intake significantly interacted with vitamin D in relation to vitamin D status and risk of mortality. According to NHANES, 79% of US adults do not meet their Recommended Dietary Allowance of magnesium.

Objectives: The aim of this study was to test the hypothesis that magnesium supplementation differentially affects vitamin D metabolism dependent on baseline 25-hydroxyvitamin D [25(OH)D] concentration.

Methods: The study included 180 participants aged 40–85 y and is a National Cancer Institute independently funded ancillary study, nested within the Personalized Prevention of Colorectal Cancer Trial (PPCCT), which enrolled 250 participants. The PPCCT is a double-blind 2 × 2 factorial randomized controlled trial conducted in the Vanderbilt University Medical Center. Doses for both magnesium and placebo were customized based on baseline dietary intakes. Subjects were randomly assigned to treatments using a permuted-block randomization algorithm. Changes in plasma 25-hydroxyvitamin D_3 [$25(OH)D_3$], 25-hydroxyvitamin D_2 [$25(OH)D_2$], 1,25-dihydroxyvitamin D_3, 1,25-dihydroxyvitamin D_2, and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] were measured by liquid chromatography–mass spectrometry.

Results: The relations between magnesium treatment and plasma concentrations of $25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$ were significantly different dependent on the baseline concentrations of 25(OH)D, and significant interactions persisted after Bonferroni corrections. Magnesium supplementation increased the $25(OH)D_3$ concentration when baseline 25(OH)D concentrations were close to 30 ng/mL, but decreased it when baseline 25(OH)D was higher (from ∼30 to 50 ng/mL). Magnesium treatment significantly affected $24,25(OH)_2D_3$ concentration when baseline 25(OH)D concentration was 50 ng/mL but not 30 ng/mL. On the other hand, magnesium treatment increased $25(OH)D_2$ as baseline 25(OH)D increased.

Conclusion: Our findings suggest that optimal magnesium status may be important for optimizing 25(OH)D status. This trial was registered at clinicaltrials.gov as NCT03265483. *Am J Clin Nutr* 2018;108:1249–1258.

Keywords: magnesium, vitamin D metabolism, interaction, calcium-to-magnesium ratio, randomized clinical trial

INTRODUCTION

Epidemiologic studies and randomized trials have generated inconsistent findings on the role of vitamin D in bone fractures (1) and extraskeletal chronic diseases (2), such as colorectal adenoma recurrence (3), colorectal cancer incidence (4), total cancer incidence (5, 6), and cardiovascular disease (CVD) (7). Large-scale randomized trials testing vitamin D supplementation with cancer and CVD as primary outcomes are ongoing (2, 8). One striking observation is that a large portion of the interperson heterogeneity in circulating 25-hydroxyvitamin D [25(OH)D] concentrations is unexplained (9).

The 2015 Dietary Guidelines Advisory Committee determined that magnesium is underconsumed relative to the Estimated Average Requirement and is one of the shortfall nutrients in the US population (10). According to the NHANES, 79%

of US adults do not meet their Recommended Dietary Allowance of magnesium (11). For patients with "Mg-dependent vitamin-D-resistant rickets" (12), characterized by reduced 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] and impaired parathyroid response (13), intramuscular infusion with ≤600,000 IU vitamin D alone did not lead to any improvements in biochemical measures of vitamin D deficiency. However, magnesium supplementation did substantially reverse the resistance to vitamin D treatment (12–14). Furthermore, we reported from observational studies in the general US population that magnesium intake significantly interacted with vitamin D intake in affecting vitamin D status, and also interacted with circulating 25(OH)D in the risk of CVD mortality and possibly colorectal cancer mortality (15). The potential interaction between magnesium and vitamin D was supported by 2 subsequent studies, including a Finnish cohort study (16) and a study using a mouse model (17).

Previous studies indicate that magnesium status affects concentrations of cytochrome P450 (CYP) enzymes (18). Cytochrome P450 enzymes include not only the vitamin D–activating enzymes [i.e., 25-hydroxylase (e.g., CYP2R1) and 1α-hydroxylase (i.e., CYP27B1)] but also vitamin D–deactivating enzymes [i.e., 24-hydroxylase (i.e., CYP24A1 and CYP3A4)]. 25-Hydroxylase synthesizes 25(OH)D from vitamin D_3 or vitamin D_2 in the liver, and then 1α-hydroxylase synthesizes active 1,25(OH)$_2$D from 25(OH)D in the kidney. 24-Hydroxylase metabolizes both 25(OH)D and 1,25(OH)$_2$D to inactive forms: 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D, respectively. Finally, CYP3A4 (19) degrades 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D (20) (**Figure 1**). Both in vitro and in vivo studies have shown that 1α-hydroxylase and 24-hydroxylase are magnesium dependent (21, 22).

Based on these observations, we hypothesize that magnesium supplementation interacts with baseline circulating 25(OH)D concentrations in affecting biomarkers of vitamin D synthesis and metabolism. In other words, we hypothesize that magnesium supplementation has different relations with vitamin D synthesis and metabolism dependent on the baseline circulating 25(OH)D concentration. To test this hypothesis, we conducted an ancillary study within the Personalized Prevention of Colorectal Cancer Trial (PPCCT), which is a double-blind, placebo-controlled, randomized controlled trial (23) testing the association of magnesium supplementation with colorectal carcinogenesis among 250 participants.

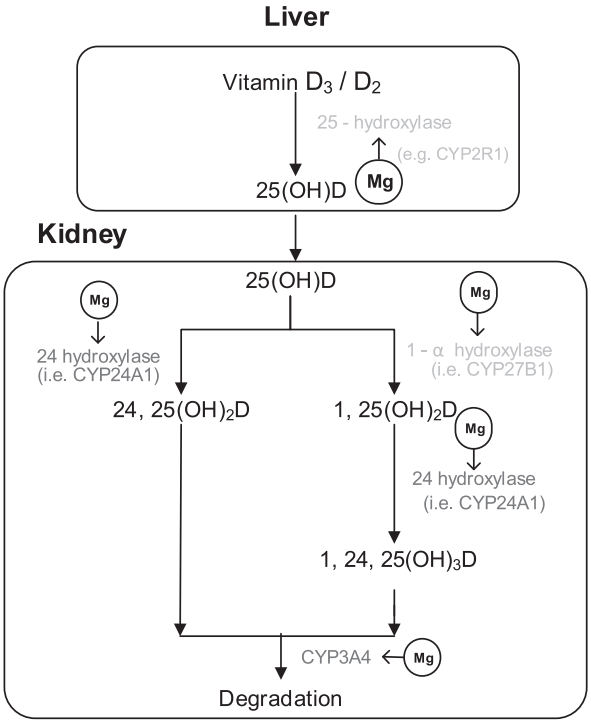

FIGURE 1 Magnesium and vitamin D metabolism. Dark gray indicates deactivating enzymes, and light gray indicates activating enzymes. CYP, cytochrome P450; 25(OH)D, 25-hydroxyvitamin D; 1,25(OH)$_2$D, 1,25-dihydroxycholecalciferol; 24,25(OH)$_2$D, 24,25-dihydroxyvitamin D; 1,24,25(OH)$_3$D, 1,24,25-trihydroxyvitamin D.

METHODS

Participants and randomization

This is a US National Cancer Institute (NCI)–funded ancillary study (registered at clinicaltrials.gov as NCT03265483) nested in the parent study, the PPCCT (registered at clinicaltrials.gov as NCT01105169). The PPCCT is a double-blind 2 × 2 factorial randomized controlled trial (23) conducted at the Vanderbilt University Medical Center, Nashville, TN. The Vanderbilt Survey Research Shared Resource enrolled the participants. A modified R program was used to generate the randomization schedule by Chang Yu, one of the principal investigators of the PPCCT. The randomization procedure used randomized blocks of 2 or 4 to allocate subjects in a 1:1 ratio to 2 treatment arms—magnesium treatment or placebo—within 3 strata defined by the transient receptor potential cation channel, subfamily M, member 7 (*TRPM7*) genotype: GG, GA, and AA. Eligible subjects were enrolled sequentially and were assigned sequentially to receive magnesium treatment or placebo according to the

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Abbreviations used: CVD, cardiovascular disease; CYP, cytochrome P450; eGFR, estimated glomerular filtration rate; LCMS, liquid chromatography–mass spectrometry; NCI, National Cancer Institute; NIST, National Institute of Standards and Technology; PPCCT, Personalized Prevention of Colorectal Cancer Trial; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; *TRPM7*, transient receptor potential cation channel, subfamily M, member 7; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 1,25(OH)$_2$D$_2$, 1,25-dihydroxyvitamin D$_2$; 1,25(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$; 24,25(OH)$_2$D$_3$, 24,25-dihydroxyvitamin D$_3$; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D$_2$, 25-hydroxyvitamin D$_2$; 25(OH)D$_3$, 25-hydroxyvitamin D$_3$.

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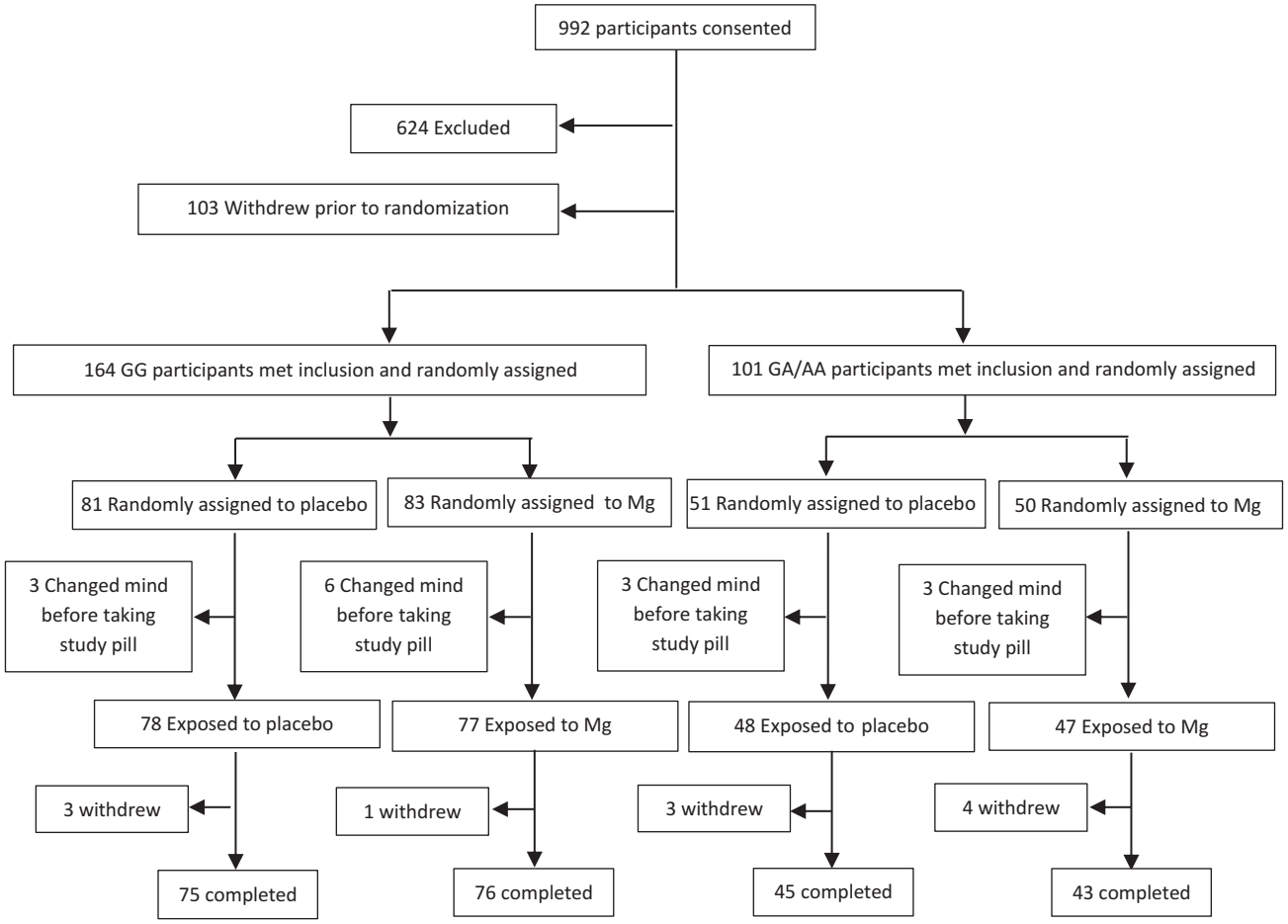

FIGURE 2 Flow diagram of trial recruitment and retention. AA, GA, GG, *TRPM7* genotypes. *TRPM7*, transient receptor potential cation channel, subfamily M, member 7.

randomization schedule. Participants, study investigators, and staff were all blinded to the assigned interventions. The blinding was implemented through the Vanderbilt Investigational Drug Service. A research pharmacist at the Drug Service maintained the randomization schedule and was the only person who was aware of the actual interventions. A total of 265 participants were randomly assigned and allocated to either the magnesium treatment or placebo arm. Of these, 15 withdrew their consent before taking the magnesium treatment or placebo. Thus, 250 participants were randomly assigned and started the treatments. Among them, 239 completed the trial, with the other 11 participants finishing only part of the study before withdrawing (see **Figure 2**). We recruited the first participant on 21 March 2011 and completed recruitment on 27 January 2016, because we had fulfilled the primary recruitment aim for the PPCCT.

The primary aim of the PPCCT was to examine the effects of magnesium supplementation and magnesium-*TRPM7* genotype interaction on the expression of biomarkers (i.e., TRPM7, mixed lineage kinase domain-like pseudokinase (MLKL), Ki67:Bax, Ki67:terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), cyclooxygenase-2) in colorectal mucosa. The secondary outcomes in the PPCCT included serum magnesium, body magnesium status, C-reactive protein, 25(OH)D, and urinary excretion of prostaglandin E2 metabolite. In the PPCCT, we proposed to measure total 25(OH)D using an ELISA-based approach. However, following our novel finding of magnesium–vitamin D interaction from an observational study published in 2013 (15), we submitted a separate grant application to the NCI for an ancillary study. We proposed to measure 5 vitamin D metabolites in the ancillary study. The analysis of this ancillary study is reported herein.

Participants, aged 40–85 y, were recruited from Vanderbilt patient sources as follows: *1*) 236 individuals with adenomas or hyperplastic polyps diagnosed from 1998 to 2014 or *2*) 14-polyp free individuals with high risk of colorectal cancer. All participants had a calcium intake of $\geq$700 and $<$2000 mg/d, and their calcium-to-magnesium intake ratio was $>$2.6, measured using two 24-h dietary recalls. A list of exclusion criteria was applied: a history of colectomy, inflammatory bowel disease, any organ transplantation, cancer other than nonmelanoma skin cancer, gastric bypass, chronic renal diseases (glomerular filtration rate $<$50 mL $\cdot$ min^{-1} $\cdot$ 1.73 m^{-2}), hepatic cirrhosis, chronic ischemic heart disease, diarrhea, type 1 diabetes, and pituitary dwarfism; current use of lithium carbonate therapy, blood anticoagulant drugs, digoxin, and licorice; and without contact information and informed consent.

For the current study, funded by an independent NCI project, 180 participants who had completed the PPCCT study by

October 2015 were selected. This included 90 women and 90 men (87 participants in the treatment arm and 93 in the placebo arm). The parent PPCCT was still blinded for primary outcomes; thus, an unblinded independent statistician outside of the study team conducted all the statistical analyses for the current report.

Interventions and precision-based dosing strategy

Two 24-h dietary recalls were performed for all participants at the baseline of the PPCCT. Based on their baseline intakes of calcium and magnesium as well as their calcium-to-magnesium intake ratio, each participant was assigned to a customized dose of magnesium supplementation that would reduce the calcium-to-magnesium intake ratio to ∼2.3, as suggested by several previous studies (23–27). The mean recorded intake from 24-h recalls was used to estimate the baseline intakes of calcium and magnesium, and the calcium-to-magnesium ratio. Placebos of microcrystalline cellulose were made to appear identical to magnesium capsules. The capsules, which were made of gelatin, were filled by the Vanderbilt Investigational Pharmacy personnel following USP 797 conditions according to the compounding instructions. The intervention period was designed to be 12 wk. The Vanderbilt Clinical Pharmacist in the Investigational Drug Service dispensed the capsules.

There were 4 additional 24-h dietary recalls conducted for all participants during the intervention period, with 2 taking place during weeks 1–6 and the other 2 taking place during weeks 6–12. Participants were scheduled for 3 clinic visits (weeks 1, 6, and 12). Information on the participant's use of medications and nutritional supplements, and other information on health and diet, was collected at each clinic visit. Blood samples were collected and processed at each clinic visit. Anthropometric measurements (weight, height, and waist and hip circumferences) were measured at least twice at each clinic visit.

Vitamin D metabolite assay and kidney function

Blood was collected from a forearm vein at each clinic visit after participants had fasted for ≥8 h. Both serum and plasma were rapidly cooled and frozen at −80°C before biochemistry analysis. In order to minimize potential errors caused by batch effects, samples were randomly organized into sets that included ≥1 pair of pre- and postsamples from a participant in the treatment arm and ≥1 pair of pre-and postsamples from the placebo arm. The samples were shipped on dry ice overnight to AAF's laboratory. This assay of vitamin D metabolites is validated by participation in quality-assurance programs organized by DEQAS (the Vitamin D External Quality Assessment Scheme) and the National Institute of Standards and Technology (NIST). In the current study, plasma samples were used as described previously (28). To control for batch-to-batch variability, samples for each set were analyzed in the same laboratory run. A pool of quality-control samples was added to each batch of samples to be assayed. Laboratory staff were blinded to the samples' status (in treatment or placebo arms or quality control) to eliminate bias.

1,25-Dihydroxyvitamin D_2 [$1,25(OH)_2D_2$] and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] were extracted from plasma using an ALPCO (Laboratory Equipment Supplier in Salem, New Hampshire) immunoextraction kit following the manufacturer's protocols. In brief, 550 μL plasma was centrifuged in a 1.5-mL microcentrifuge tube at 13,500 × *g* for 10 min. Next, 500 μL of the clear plasma supernatant was transferred to an ImmunoTube with 10 μL internal standard solution [$1,25(OH)_2D_3$-d3 at 10 ng/mL in methanol]. The ImmunoTube was then capped, placed on a rotator, and mix-rotated (rotated end over end) at room temperature for 1 h. After the rotation mixing, the tube was placed in a 13- × 75-mm test tube and centrifuged at 600 × *g* for 1 min, followed by removal of the cover and the outlet of the tubes, before being further centrifuged at 600 × *g* for 2 min to remove the liquid. The ImmunoTube was then washed 3 times with 500 μL WASHSOL, followed by elution of the analytes with 250 μL ELUREAG. The eluant was collected and dried under a nitrogen flow. The derivatization reaction was carried out by adding 50 μL 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) solution (0.5 mg/mL in anhydrous acetonitrile) to the dried sample and mixing it manually on a vortex at 2000 rpm for 10 s. The vial was then capped and incubated at room temperature for 1 h. The reaction was quenched by adding 50 μL deionized water, and the resulting mixture was transferred to HPLC inserts and subjected to liquid chromatography–mass spectrometry (LCMS) analysis.

25-Hydroxyvitamin D_2 [$25(OH)D_2$], 25-hydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] were extracted from plasma by liquid-liquid extraction. In brief, 100 μL plasma was mixed gently with 100 μL internal standard solution 25-hydroxyvitamin D_3-d_6 at 260 ng/mL in methanol], followed by incubation at room temperature for 15 min. The mixture was then extracted with 1 mL hexanes by mixing on a vortex at 1750 rpm for 5 min, followed by centrifugation at 1037 × *g* for 5 min to separate the 2 layers. The upper layer was dried completely under a nitrogen flow and treated with PTAD as described above, followed by LCMS analysis. To the dried sample we added 50 μL PTAD solution, which was manually mixed on a vortex at 2000 rpm for 10 s. The vial was then capped and incubated at room temperature for 1 h. The reaction was quenched by adding 50 μL deionized water, and the resulting mixture was transferred to HPLC inserts and subjected to LCMS analysis.

LCMS was performed using a model Accela ultra HPLC system coupled with a Q Exactive Orbitrap Mass Spectrometer and a CTC PAL autosampler (all from Thermo Fisher). Aliquots of 25 μL of the above mixture for 25(OH)D and $24,25(OH)_2D_3$ and 40 μL for $1,25(OH)_2D$ were injected into an Agilent SB C18 column (50 × 2.1 mm, 1.8 μm; Agilent) with a precolumn filter (0.2 μm; Thermo Fisher). Gradient elution was performed at a flow rate of 300 μL/min with the use of 0.1% formic acid in H_2O (A) and 0.1% formic acid in acetonitrile (B) as follows: 0- to 10.0-min linear gradient from 60% A to 20% A; hold at the same ratio for 1 min; then go back to the first line condition and equilibrate for 5 min. The total HPLC time including equilibration was 15 min. Mass analysis was performed in positive electrospray target SIM mode, under the following conditions: (+) electrospray ionization (ESI) spray voltage 4.5 kV, capillary transfer temperature 350°C, heated electrospray ionization (HESI) heater temperature 350°C, sheath gas flow rate

30 units, auxiliary gas 5 units, in-source collision induced dissociation (CID) 5 eV. Quantitation of all analytes was performed with Xcalibur software within 5 ppm of the calculated exact masses {$[M + H]^+$: $25(OH)D_2$ = 570.36920; $25(OH)D_3$ = 558.36902; $25(OH)D_3$-d_6 = 564.40668; $24,25(OH)_2D_3$ = 574.36393}, and the detection limit for these analytes was 2–22 pg/mL {$[M + H]^+$: $1,25(OH)_2D_3$ = 574.36393; $1,25(OH)_2D_3$-d_3 = 577.38276; $1,25(OH)_2D_2$ = 586.39393}; the detection limit for these analytes was 1 pg/mL. Consistent with previous studies (29), we found that 96.7% of participants had undetectable concentrations of $1,25(OH)_2D_2$. The CVs for intrabatch variation were 5.58, 7.24, 7.74, and 9.38 for $1,25(OH)_2D_3$, $25(OH)D_2$, $25(OH)D_3$, and $24,25(OH)_2D_3$, respectively. The corresponding CVs for interbatch variations were 5.57, 7.34, 5.31, and 2.72, respectively. The concentrations of $25(OH)D_3$ were 26.8 and 28.7 ng/mL, respectively, when the NIST-assigned values were 24.1 and 28.3 ng/mL.

Serum creatinine was measured by a kinetic alkaline picrate method with the use of a Cobas Mira Plus clinical autoanalyzer and a kit from Randox Laboratories; CVs were <6%. An estimated glomerular filtration rate (eGFR) based on serum creatinine was obtained by using the modified 4-variable Modification of Diet in Renal Disease study equation (30).

Statistical analyses

Based on 3 earlier clinical studies (12–14), we estimated that we needed only 12 individuals/arm to have 80% power to detect the magnesium–vitamin D interaction. However, the earlier clinical studies were conducted in those with severe magnesium deficiency, whereas our randomized trial was conducted in those at risk of magnesium deficiency. To be conservative, we also conducted a power estimation based on the NHANES data used in our previous report. Because NHANES is conducted in the general US population, very low cutoffs were selected to define low intakes of vitamin D and magnesium to estimate the power. We understand that our power estimation could not take into account the effect of sun exposure on 25(OH)D.

Previous data from NHANES (15) have shown that in subjects with low daily vitamin D intake (≤40 IU), serum 25(OH)D was 20.0 ± 9.2 ng/mL (mean ± SD) with a high daily magnesium intake (>420 mg) compared with 17.9 ± 8.8 ng/mL with a low daily magnesium intake (≤225 mg), whereas in subjects with high vitamin D intake (>1000 IU), serum 25(OH)D was 27.3 ± 7.8 ng/mL with high magnesium intake compared with 18.7 ± 15.7 ng/mL with low magnesium intake. Assuming an SD of 8 ng/mL for serum 25(OH)D, 90 subjects in the magnesium treatment and 90 in the placebo group will give us 83.2% power to detect a difference of 7 ng/mL in the effect of magnesium supplement intake between subjects with a low baseline 25(OH)D concentration and those with a high baseline 25(OH)D concentration, with a 2-sided type I error rate of 0.05 based on a *t* test.

Summary statistics for continuous variables (mean ± SD, median, and IQR) and categorical variables (count and percentage) were reported for the 2 randomly assigned arms. The Wilcoxon rank sum test was conducted to evaluate whether pretreatment values were different between the 2 arms

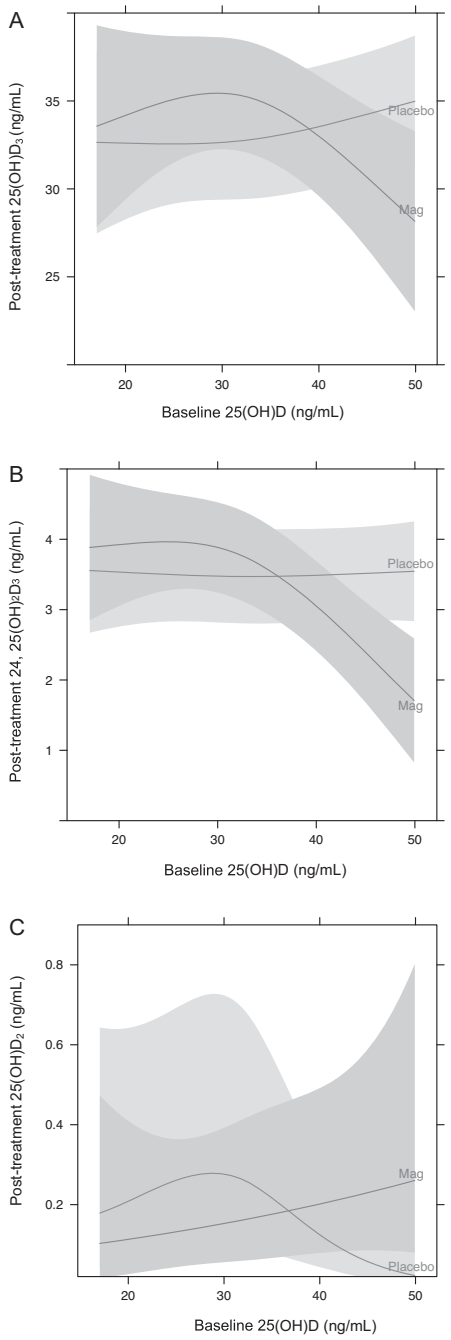

FIGURE 3 Post-treatment plasma vitamin D metabolite concentrations calculated by a linear model. Concentrations were adjusted for age, sex, baseline BMI, eGFR, total 25(OH)D, and blood collection season among 87 participants randomly assigned to the magnesium treatment arm and 93 participants randomly assigned to the placebo arm. The solid curves represent point estimates and the gray regions represent 95% CIs. (A) Adjusted to $25(OH)D_3$ = 31.37 ng/mL, age = 60 y, sex = female, BMI (kg/m^2) = 29.1, baseline GFR = 78 · min^{-1} · 1.73 m^{-2}, blood sample collection season = summer. (B) Adjusted to $25(OH)D_3$ = 3.095 ng/mL, age = 60 y, sex = female, BMI = 29.1, baseline GFR = 78 · min^{-1} · 1.73 m^{-2}, blood sample collection season = summer. (C) Adjusted to $25(OH)D_2$ = 0.485 ng/mL, age = 60 y, sex = female, BMI = 29.1, baseline GFR = 78 · min^{-1} · 1.73 m^{-2}, blood sample collection season = summer. eGFR, estimated glomerular filtration rate; GFR, glomerular filtration rate; Mag, magnesium; 25(OH)D, 25-hydroxyvitamin D; $25(OH)D_2$, 25-hydroxyvitamin D_2; $25(OH)D_3$, 25-hydroxyvitamin D_3; $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3.

(magnesium treatment or placebo) for continuous variables. Pearson chi-square tests were conducted to compare categorical variables between treatment arms. Ordinary linear regression models were fitted to examine the association of magnesium treatment with vitamin D metabolites, adjusting for age, sex, baseline eGFR, BMI, 25(OH)D, and other vitamin D metabolites and baseline sample collection season. We assumed a linear relation for baseline vitamin D metabolite and a smooth relation for other continuous variables using restricted cubic regression splines with 3 knots (knot locations were chosen at 5%, 50%, and 95% of sample quantiles). The interaction between treatment and baseline 25(OH)D was also included in the models. Log-transformation was conducted to appropriately fit the model in the analysis of $25(OH)D_2$. The fold-change of $25(OH)D_2$ is presented in **Figure 3** and **Table 1**. We conducted an analysis with additional adjustment for smoking and drinking status but found that the results did not alter appreciably, and so present models without these adjustments. The data analysis used R 3.3.0 software (https://www.r-project.org/).

RESULTS

In the parent study (PPCCT), 250 participants were allocated to either the magnesium treatment or placebo arm and began the treatment. After the treatments had begun, 11 of the 250 participants withdrew from the trial. Self-reported adverse events were responsible for 6 of the withdrawals, 4 of which were in the treatment arm and 2 were in the placebo arm. To note, the other 5 withdrew due to lack of time or interest.

The participants in the magnesium treatment arm did not significantly differ from those in the placebo arm with regard to averages or distributions for age, sex, BMI, eGFR, *TRPM7* genotype, smoking status, alcohol drinking status, physical activity status, education achievement, race, season when the baseline blood sample was collected, or vitamin D metabolites concentrations, including 25(OH)D, $25(OH)D_3$, $25(OH)D_2$, $1,25(OH)_2D_3$, and $24,25(OH)_2D_3$. The baseline calcium-to-magnesium intake ratios were comparable between the placebo and magnesium treatment arms, although the baseline magnesium intake was higher in the treatment arm (**Table 2**). However, age, sex, BMI, and eGFR were still adjusted for in the subsequent analyses due to their important impact on vitamin D status. The mean daily dose of personalized magnesium supplementation was 205.52 mg, with a range from 77.25 to 389.55 mg. Compliance with the treatment regimen was very high for both the placebo and treatment arms (mean ± SD values based on capsule counts were 97.3% ± 4.4% and 97.5% ± 3.9%, respectively; $P = 0.83$ for difference between the arms). The mean ± SD calcium-to-magnesium ratios for the treatment and placebo arms after administration of magnesium and placebo supplementation were 2.27 ± 0.13 and 3.84 ± 1.43, respectively ($P < 0.001$ for difference between the arms), based on the two 24-h dietary recalls performed at baseline, and remained stable at 2.13 ± 0.68 and 3.50 ± 1.31, respectively ($P < 0.001$ for difference between the arms), based on the four 24-h dietary recalls conducted over the 12-wk period of the trial.

The relations of magnesium treatment with the plasma concentrations of $25(OH)D_3$ ($P = 0.001$ for interaction), $25(OH)D_2$ ($P = 0.009$ for interaction), and $24,25(OH)_2D_3$ ($P < 0.0001$ for interaction) were significantly different based on the baseline plasma concentrations of 25(OH)D. The interactions were statistically significant after Bonferroni corrections. However, magnesium treatment did not interact significantly with baseline 25(OH)D in changing $1,25(OH)_2D_3$ concentration ($P = 0.25$ for interaction; see **Supplemental Figure 1**).

When the baseline 25(OH)D was higher, at ~30–50 ng/mL, magnesium treatment reduced $25(OH)D_3$ (Figure 3A). At a baseline 25(OH)D concentration of 50 ng/mL, magnesium treatment caused a significantly reduced $25(OH)D_3$ concentration compared with the placebo arm, with an estimated mean (95% CI) difference of −6.87 (−11.30, −2.45) (Table 1). At a baseline 25(OH)D of 30 ng/mg, but not 20 ng/mg, magnesium treatment led to a significantly elevated concentration of $25(OH)D_3$ compared with the placebo arm, with an estimated mean (95% CI) difference of 2.79 (0.25, 5.34) (Table 1). The association of magnesium treatment with $24,25(OH)_2D_3$ was similar to the pattern observed for $25(OH)D_3$ only when baseline 25(OH)D was higher, from 30 to 50 ng/mL (Figure 3B). A significant decrease in $24,25(OH)_2D_3$ was observed with supplementation at 50 ng/mL, but there were no signs of a treatment effect at the other concentrations (Table 1). Thus, there was no consistent evidence that magnesium supplementation increased vitamin D metabolite concentrations [i.e., $25(OH)D_3$ and $24,25(OH)_2D_3$] at lower concentrations. On the other hand, magnesium treatment increased concentrations of $25(OH)D_2$ as the baseline 25(OH)D continuously increased (Figure 3C). Magnesium treatment led to a significant 8.39-fold (95% CI: 2.38-, 29.63-fold) increase in $25(OH)D_2$ compared with placebo at a baseline 25(OH)D concentration of 50 ng/mL (Table 1).

DISCUSSION

We found that magnesium supplementation interacted with baseline plasma concentrations of 25(OH)D in affecting the concentrations of $25(OH)D_3$, $25(OH)D_2$, and $24,25(OH)_2D_3$. We found that magnesium supplementation reduced $25(OH)D_3$ and $24,25(OH)_2D_3$ when 25(OH)D concentrations were >30 ng/mL, particularly at 50 ng/mL. These findings are novel. However, there was no consistent evidence that magnesium supplementation increased vitamin D metabolite concentrations [i.e., $25(OH)D_3$ and $24,25(OH)_2D_3$] at lower concentrations. Magnesium supplementation increased $25(OH)D_3$ when 25(OH)D concentrations were at 30 ng/mL, but not 20 ng/mL, whereas magnesium supplementation did not increase $24,25(OH)_2D_3$. Also, we found that the pattern of the magnesium association was different with $25(OH)D_2$ and $25(OH)D_3$.

Both in vitro and in vivo studies have indicated that magnesium deficiency affects 1α-hydroxylase (i.e., CYP27B1) and 24-hydroxylase (i.e., CYP24A1), which synthesize and metabolize 25(OH)D and $1,25(OH)_2D$, respectively (21, 22). Magnesium deficiency, which leads to reduced $1,25(OH)_2D$ and impaired parathyroid hormone response (13), has been implicated in "Mg-dependent vitamin-D-resistant rickets" (12). However, magnesium supplementation substantially reversed the resistance to vitamin D treatment (12–15). These earlier studies were case reports or small, nonrandomized, placebo-controlled clinical studies conducted in patients with severe clinical magnesium deficiency. The current study was conducted in individuals almost without clinical symptoms of magnesium deficiency (i.e.,

TABLE 1
Relation between magnesium treatment and vitamin D metabolism, by baseline 25(OH)D concentrations[1]

Baseline 25(OH)D	Group, ng/mL		Difference (treatment − placebo) (95% CI)	*P*
	Magnesium	Placebo		
$25(OH)D_3$, ng/mL				
20	30.95 ± 2.18	29.37 ± 2.08	1.58 (−2.28, 5.44)	0.42
30	32.20 ± 1.56	29.41 ± 1.61	2.79 (0.25, 5.34)	0.03
40	29.76 ± 1.84	30.31 ± 1.80	−0.54 (−3.26, 2.18)	0.69
50	24.89 ± 2.74	31.76 ± 2.00	−6.87 (−11.30, −2.45)	0.002
$24,25(OH)_2D_3$, ng/mL				
20	4.35 ± 0.41	3.96 ± 0.39	0.39 (−0.39, 1.17)	0.33
30	4.31 ± 0.32	3.90 ± 0.32	0.41 (−0.10, 0.91)	0.11
40	3.47 ± 0.32	3.91 ± 0.32	−0.44 (−0.99, 0.11)	0.12
50	2.12 ± 0.44	3.97 ± 0.36	−1.85 (−2.70, −0.99)	<0.0001
$25(OH)D_2$, ng/mL				
20	−2.22 ± 0.57	−1.65 ± 0.55	0.56 (0.19, 1.69)[2]	0.30
30	−1.94 ± 0.45	−1.38 ± 0.46	0.57 (0.27, 1.18)[2]	0.13
40	−1.68 ± 0.44	−2.13 ± 0.45	1.57 (0.72, 3.41)[2]	0.25
50	−1.43 ± 0.55	−3.56 ± 0.57	8.39 (2.38, 29.63)[2]	0.001

[1] Values are means ± SEMs unless otherwise indicated. Values are based on our multiple linear regression model, adjusting for age = 60 y, sex = male, BMI (kg/m^2) = 30, baseline eGFR = $7 \cdot min^{-1} \cdot 1.73\ m^{-2}$, blood sample collection season = summer, baseline $25(OH)D_3$ = 30 ng/mL or $24,25(OH)_2D_3$ = 4 ng/mL or $25(OH)D_2$ = 0.5 ng/mL among 87 participants randomly assigned to the magnesium treatment arm and 93 participants randomly assigned to the placebo arm. The *P* values for the interactions between magnesium intake with baseline 25(OH)D in changing $25(OH)D_3$, $24,25(OH)_2D_3$, and $25(OH)D_2$ were 0.001, <0.0001, and 0.009, respectively. Total 25(OH)D, $25(OH)D_3$: 1 ng/mL = 2.4959 nmol/L; $25(OH)D_2$: 1 ng/mL = 2.4233 nmol/L. $24,25(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D; $25(OH)D_2$, 25-hydroxyvitamin D_2; $25(OH)D_3$, 25-hydroxyvitamin D_3.

[2] Log-transformation was conducted to appropriately fit the model in the analysis of $25(OH)D_2$. The fold-change of $25(OH)D_2$ is presented.

only 1 participant had serum magnesium <1.7 mg/dL). In this population, we found that magnesium supplementation did not interact with baseline plasma 25(OH)D in affecting plasma $1,25(OH)_2D_3$ concentration. However, we found that magnesium supplementation significantly changed plasma $25(OH)D_3$ concentration depending on the patient's baseline plasma 25(OH)D concentration. These findings are supported by a study conducted in the US general population (15). In that study, we reported that magnesium intake significantly interacted with the intake of vitamin D in relation to risk of both vitamin D deficiency and insufficiency in NHANES 2001–2006 (15). However, this previous observational study was a cross-sectional study, and the $25(OH)D_2$ and $24,25(OH)_2D_3$ metabolites were not measured.

Thus, the findings from the current study provide the first evidence in humans that magnesium supplementation reduces $25(OH)D_3$ and $24,25(OH)_2D_3$ when 25(OH)D is higher but may increase $25(OH)D_3$ when 25(OH)D is lower. The precise molecular mechanism is not clear. One possible explanation is that magnesium supplementation affects both vitamin D–activating enzymes (i.e., CYP27B1 and CYP2R1) and vitamin D–deactivating enzymes [i.e., CYP24A1 and CYP3A4 (Figure 1)]. When baseline 25(OH)D is <30 ng/mL, the activity of CYP3A4 on vitamin D degradation is limited; thus, the relation of magnesium supplementation is primarily with vitamin D synthesis enzymes. When baseline 25(OH)D concentrations are >30 ng/mL, CYP3A4 activity starts to elevate and the activity is further enhanced by magnesium supplementation, which leads to a significant reduction in concentrations of $24,25(OH)_2D_3$. In addition, the reduction in $24,25(OH)_2D_3$ seems stronger than the reduction in $25(OH)D_3$, indicating that the reduction in $25(OH)D_3$ could be secondary to the $24,25(OH)_2D_3$ reduction. Our observation about plasma $25(OH)D_3$ is also consistent with previous reports. We found from the NHANES III cohort study that the longitudinal inverse associations between circulating 25(OH)D and total mortality, particularly due to CVD, were modified by magnesium intake (15). Following our findings, a borderline significant interaction between magnesium intake and circulating 25(OH)D in relation to total mortality was observed in a Finnish cohort study conducted in a population with low circulating 25(OH)D status and high magnesium intakes (16); and another study showed that magnesium treatment modified the association of vitamin D analogs with vascular calcification in mice with experimental chronic kidney disease (17). The most recent Institute of Medicine Report on Dietary Reference Intakes of Calcium and Vitamin D mentioned that several cohort studies found a U-shaped relation between plasma 25(OH)D concentration and risk of incident CVD (7). For example, in the Framingham Offspring Study, although a significant inverse association was found between plasma 25(OH)D and incident CVD, there was no additional reduction in risk once plasma 25(OH)D concentrations increased beyond 30 ng/mL (7, 31). This finding has been supported by 2 subsequent meta-analyses of cohort studies (8, 32, 33). Similarly, a reverse J-shaped association was found between plasma 25(OH)D and CVD mortality in a recent large cohort study in 247,574 participants (34); and concentrations of 25(OH)D at 28 ng/mL were associated with the lowest risk of CVD mortality. However, none of those studies examined the potential interaction between vitamin D status and magnesium status in relation to CVD risk. Because the 2015 Dietary Guidelines Advisory Committee determined that magnesium is underconsumed in the US population (10, 11), future studies are necessary to further

TABLE 2
Descriptive characteristics of 180 participants at baseline[1]

	Magnesium treatment ($n = 87$)	Placebo ($n = 93$)	P
Age, y	60.4 ± 8.3	61.7 ± 8.3	0.30[2]
Male sex, %	52	48	0.66[3]
BMI, kg/m^2	29.4 ± 6.0	30.3 ± 6.5	0.30[2]
eGFR	81.0 ± 14.0	78.0 ± 15.0	0.16[2]
TRPM7 genotype GG, %	65	68	0.64[3]
Smoking status, %			0.21[3]
Never	46	59	
Ever	44	33	
Current	10	8	
Drinking status, %			0.25[3]
Never	44	32	
Ever	17	20	
Current	39	48	
Physically active ≥2 d/wk, %	84	78	0.33[3]
Education less than college, %	10	9	0.91[3]
White race, %	98	99	0.52[3]
Family history of colorectal cancer, %	14	11	0.53[3]
Daily nutrients intake, mg/d			
Total calcium	1327 ± 332	1236 ± 364	0.06[2]
Total magnesium	366 ± 97	333 ± 96	0.01[2]
Calcium-to-magnesium intake ratio	3.7 ± 0.9	3.9 ± 1.6	0.86[2]
Season, %			0.82[3]
Spring	16	20	
Summer	41	35	
Fall	26	27	
Winter	16	17	
Aspirin use, %	30	24	0.34[3]
NSAID use, %	20	18	0.83[3]
Plasma 25(OH)D, ng/mL	33.4 ± 10.2	32.0 ± 12.7	0.53[2]
Plasma 25(OH)D_3, ng/mL	32.3 ± 10.4	30.1 ± 11.3	0.20[2]
Plasma 25(OH)D_2, ng/mL	1.12 ± 2.31	2.84 ± 10.89	0.32[2]
Plasma 1,25(OH)$_2D_3$, pg/mL	81.4 ± 50.9	84.2 ± 51.3	0.70[2]
Plasma 24,25(OH)$_2D_3$, ng/mL	4.66 ± 3.71	3.82 ± 2.78	0.24[2]

[1]Continuous variables are means ± SDs; categorical variables are percentages. eGFR, estimated glomerular filtration rate; NSAID, nonsteroidal anti-inflammatory drug; *TRPM7*, transient receptor potential cation channel, subfamily M, member 7; 1,25(OH)$_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin D_3; 25(OH)D, 25-hydroxyvitamin D; 25(OH)D_2, 25-hydroxyvitamin D_2; 25(OH)D_3, 25-hydroxyvitamin D_3.
[2]Wilcoxon test.
[3]Pearson chi-square test.

understand the clinical relevance of the finding from the current study.

In this study, we found that magnesium supplementation increased plasma concentrations of 25(OH)D_2. Previous studies indicate that vitamin D–specific CYP enzymes (i.e., CYP2R1, CYP27B1, and CYP24A1) are unable to differentiate vitamin D_2 from vitamin D_3 (20). On the other hand, nonvitamin D-specific enzymes (i.e., CYP3A4) may degrade 24-vitamin D_2 more efficiently than 24-vitamin D_3 in the intestine, and this may provide an explanation for the lower toxicity of vitamin D_2 compared with vitamin D_3 compounds (20). Our findings indicate that magnesium supplementation may not only accelerate the metabolism and degradation of 25(OH)D_3 but also shift CYP3A4 to selectively degrade vitamin D_3 over vitamin D_2 when plasma 25(OH)D is high. Thus, our findings provide the first evidence that adequate magnesium status could potentially prevent vitamin D–related adverse events. Hypomagnesemia is often concurrent with hypocalcemia in humans (18). A number of previous clinical trials conducted in adults consistently indicated that high calcium supplementation increases urinary excretion of magnesium (35–38), whereas magnesium homeostasis is mainly regulated by kidney reabsorption. Thus, individuals with high calcium-to-magnesium intake ratios in their habitual diet are at high risk of magnesium deficiency. In several epidemiologic studies, calcium-to-magnesium intake ratios between 1.7 and 2.6 were reported to be critical for calcium and magnesium intakes to be protective against colorectal cancer, mortality due to CVD, and total mortality (23–27). In the US general adult population, >76% had calcium-to-magnesium intake ratios ≥2.6 based on the NHANES 2009–2010 data. In the current randomized trial, all participants at baseline had a calcium-to-magnesium intake ratio ≥2.6. A precision-based dosing strategy of magnesium supplementation was used to reduce the calcium-to-magnesium ratios in the diet to ∼2.3. Thus, it is not clear if magnesium supplementation among those with calcium-to-magnesium ratios <2.6 would show similar changes in vitamin D metabolites, or if other magnesium dosing strategies would have the same vitamin D association.

The current study has several strengths, including the randomized, placebo-controlled design. Furthermore, a precision-based design was utilized. Thus, all the background intakes of magnesium and calcium from both diet and supplements were measured 2 times before and 4 times during the treatment, and a personalized dosing strategy of magnesium supplementation was provided to each individual. We found that the calcium-to-magnesium ratios remained stable. In addition, we had a high compliance with the study medication, and the dropout rate was very low. The study does, however, have some weaknesses. The primary concern is that this is an independent ancillary study. Thus, our study may not be powerful enough to detect the interactions. We did find that 3 of the interactions were statistically significant and remained significant after Bonferroni corrections; however, we cannot eliminate the possibility that we did not have sufficient statistical power to detect the interaction between magnesium supplementation and baseline 25(OH)D on 1,25(OH)$_2D_3$. The other concern is that there were only 2 participants with baseline 25(OH)D <12 ng/mL. Thus, we did not have the power to test how magnesium supplementation affects vitamin D synthesis and metabolism among those with overt vitamin D deficiency at baseline. However, the results did not change after removing these 2 individuals with overt vitamin D deficiency at baseline. Thus, our study only provides evidence of how magnesium supplementation affects the vitamin D status and metabolism among those without overt vitamin D deficiency. We did not measure magnesium concentrations in 24-h urine samples at baseline. This may have led to an underestimation in the measurement of magnesium intake amounts. Also, our findings might be explained by the risk of bias from "regression to the mean." However, we found clearly different patterns in the changes of concentrations of 25(OH)D_3, 24,25(OH)$_2D_3$, and 25(OH)D_2 after administration of the magnesium treatment compared with placebo. Also, we adjusted for baseline 25(OH)D_3. In addition, we found that the

correlation between baseline blood $25(OH)D_3$ and personalized dose of magnesium was minimal (−0.003 for all participants, −0.009 in the treatment group, and 0.004 in the placebo group). The standardization to NIST was reasonable for $25(OH)D_3$. However, we did not have data for $24,25(OH)_2D_3$. Thus, interpretation of the $24,25(OH)_2D_3$ results should be done with caution. However, we found the effects for magnesium supplementation on $25(OH)D_3$ and $24,25(OH)_2D_3$ were similar when the baseline 25(OH)D was >30 ng/mL. In this ancillary study, to increase the sample size and efficiency, we included participants who completed the trial but not those who enrolled and withdrew. Thus, the analyses were not carried out on an intention-to-treat sample. We cannot eliminate the possibility that the significant effect of magnesium supplementation on $25(OH)D_3$ at 30 ng/mL might be due to chance. Finally, the baseline intake amount of magnesium was significantly higher in the magnesium treatment arm than in the placebo arm, although the baseline calcium-to-magnesium ratio intake did not differ significantly by treatment arm. In the parent study, 236 of the enrolled participants had been previously diagnosed with colorectal adenomas or hyperplastic polyps. Although their polyps and adenomas were removed when they participated in the trial, cautious interpretation of our results is warranted, particularly regarding generalization of our findings.

In summary, among individuals with calcium-to-magnesium intake ratios ≥2.6, who account for >76% of the US general adult population, magnesium supplementation increases $25(OH)D_3$ but not $24,25(OH)_2D_3$ when baseline 25(OH)D concentrations are <30 ng/mL, but decreases the concentrations of both in a dose-response manner when baseline 25(OH)D concentrations are higher (from 30 to 50 ng/mL). On the other hand, magnesium treatment increases $25(OH)D_2$ as baseline 25(OH)D concentrations increase. Our findings suggest that optimal magnesium status may be important for optimizing 25(OH)D status. Further dosing studies are warranted in appropriate animal models.

The authors' responsibilities were as follows—QD and MJS: contributed to the hypothesis development and to the manuscript preparation; QD, MJS, CY, and DLS: contributed to the study design; HN and XZ: were responsible for the data analysis; QD, XL, and AAF: contributed to the assay of the vitamin D metabolites; XZ, JEM, YS, XL, AAF, RBC, AR, HN, LF, HM, RMN, DLS, CY, and MJS: contributed to the data interpretation and manuscript revision; and all authors: read and approved the final manuscript. None of the authors had a conflict of interest.

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Vitamin D3 supplementation, low-risk prostate cancer, and health disparities.

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Abstract

Vitamin D promotes the differentiation of prostate cancer cells, raising the possibility that vitamin D deficiency over time may contribute to the progression from subclinical prostate cancer to clinical disease. Since low-risk prostate cancers are monitored over time in an effort to determine which progress into clinically important, more aggressive cancers, they provide an excellent model in which to study, over an extended period of time, the effects of enhancing vitamin D status and related changes in tumor progression. This is particularly relevant to African-American men, who exhibit a high prevalence of vitamin D deficiency as well as higher incidence of prostate cancer and higher mortality rates from prostate cancer than Caucasians. Our research team has

recently completed an open-label clinical trial aimed at assessing the safety and potential efficacy of vitamin D3 supplementation at 4000 international units (IU) per day for one year in subjects diagnosed with early stage, low-risk prostate cancer. The results of this clinical study suggest that supplementation with vitamin D3 at 4000IU per day may benefit patients with early stage, low-risk prostate cancer on active surveillance, because of the improved outcome (a decreased number of positive cores at repeat biopsy) in more than half of the subjects enrolled in the trial. We also observed that, after one year of supplementation, there was no difference in circulating levels of vitamin D between African-American and Caucasian subjects who completed the study. These clinical results also suggest that robust and sustained vitamin D3 supplementation can reduce prostatecancer-related health disparities in African-American men and that these health disparities are at least in part the result of widespread hypovitaminosis D within the African-American population. This article is part of a Special Issue entitled 'Vitamin D Workshop'.

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Vitamin D exposure and Risk of Breast Cancer: a meta-analysis

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The relationship between vitamin D and breast cancer is still controversial. The present meta-analysis examines the effects of the 25(OH)D, 1,25(OH)2D and vitamin D intake on breast cancer risk. For this purpose, a PubMed, Scopus and Web of Science-databases search was conducted including all papers published with the keywords "breast cancer" and "vitamin D" with at least one reported relative risk (RR) or odds ratio (OR). In total sixty eight studies published between 1998 and 2018 were analyzed. Information about type of study, hormonal receptors and menopausal status was retrieved. Pooled OR or RR were estimated by weighting individual OR/RR by the inverse of their variance Our study showed a protective effect between 25 (OH) D and breast cancer in both cohort studies (RR = 0.85, 95%CI:0.74–0.98) and case-control studies (OR = 0.65, 95%CI: 0.56–0.76). However, analyzing by menopausal status, the protective vitamin D – breast cancer association persisted only in the premenopausal group (OR = 0.67, 95%CI: 0.49–0.92) when restricting the analysis to nested case-control studies. No significant association was found for vitamin D intake or 1,25(OH)2D. Conclusion: This systematic review suggests a protective relationship between circulating vitamin D (measured as 25(OH) D) and breast cancer development in premenopausal women.

Breast cancer is an important public health problem in developed countries as it is one of the most common cancers, being the most if only the female population is considered[1]. The incidence is decreasing every year, which is partly due to early detection programs[2].

In the last decades, cellular *in vitro* experiments and *in vivo* models have evaluated the role of vitamin D in the development of breast cancer, finding a protective anticancer role of 1,25(OH)D3[3]. It has been demonstrated that treating breast cancer cells with 1,25(OH)D3 induces two beneficial effects: an anti-proliferative effect[4] and a pro-apoptotic effect[5,6]. The former is linked to the suppression of growth stimulatory signals and the potentiation of growth inhibitory signals, whilst the second one is explained by the bcl-2 family proteins. The interaction between vitamin D and its receptors induces an increase in the expression of pro-apoptotic family member (bax and bak protein) and simultaneously a decrease of anti-apoptotic (bcl-2/bcl-XL)[6]. In addition, the breast tissue contains the 1-α-hydroxylase, allowing for the generation of the active vitamin D metabolite (1,25 dihydroxyvitamin D) from the circulating precursor (25 hydroxyvitamin D). As vitamin D receptors are found in the breast[6], an autocrine role of vitamin D has been suggested[7].

Despite this biological background, literature shows inconsistent results[8–16] (Table 1). Several additional observational studies have appeared since the last meta-analysis publication (including articles until 2013). The main purpose of the present meta-analysis is to update the relationship between vitamin D exposure and breast cancer risk by adding the studies published more recently. Thus sixty-eight observational studies: thirty of these were case-control, twenty-one were nested case-control and the remaining were cohort studies.

Methods

Search strategy. Firstly, the following inclusion criteria were defined: we looked for cohort or case-control studies performed in humans, which reported, at least, one relative risk (RR) or odds ratio (OR) with confidence interval at 95%. (95% CI)

We began our search in Pub-Med, Scopus and Web of Science database using "breast cancer" and "vitamin D" as keywords, finding 2313 articles. After having read the title and abstract, 2123 articles that did not meet the above criteria were eliminated. Next, we carried out a more exhaustive and complete reading, which allowed us to reject another additional 69 articles (Fig. 1). Finally, sixty eight studies meeting our inclusion criteria were

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Source	Type of vitamin D	Number of included studies	Type of included studies	RR (95%IC)
Bauer SR *et al.* (2013)	25(OH)D	9	Cohort & nested case-control studies	0.9 (0.97–1.00)
Chen P *et al.* (2010)	25(OH)D	21	Case control, cohort, & cross-sectional studies	0.55 (0.38–0.80)
	Intake of vitamin D			0.91 (0.85–0.97)
	1,25(OH)2D			0.99 (0.68–1.44)
Chen P *et al.* (2013)	25(OH)D	21	Nested case-control & retrospective studies	0.86 (0.75–1.00)
			Population based case control studies	0.35 (0.24–0.52)
			Hospital based case-control studies	0.08 (0.08–0.33)
Gandini S *et al.* (2011)	25(OH)D	10	Case-control	0.83 (0.79–0.87)
			Nested case-control & cohort studies	0.97 (0.92–1.03)
Gissel T *et al.* (2008)	Intake of vitamin D	6	Cross sectional, Case-control, cohort & r&omized-control trials	0.98 (0.93–1.03)
Kim Y and Je Y. (2014)	Intake of vitamin D	24	Cohort & nested case-control studies	0.95 (0.88–1.01)
	25(OH)D			0.92 (0.83–1.02)
Wang D *et al.* (2013)	25(OH)D	14	Cohort & nested case-control studies	0.84 (0.75–0.95)
Mohr SB *et al.* (2011)	25(OH)D	11	All	0.61 (0.47–0.80)
			Case-control studies	0.87 (0.77–0.99)
			Nested case-control studies	0.41 (0.31–0.56)
Yin L *et al.* (2010)	25(OH)D	9	All	0.73 (0.60–0.88)
			Nested case-control	0.92 (0.82–1.04)
			Case- control	0.59 (0.48–0.73)

Table 1. RR of breast cancer and vitamin D in previous meta-analysis.

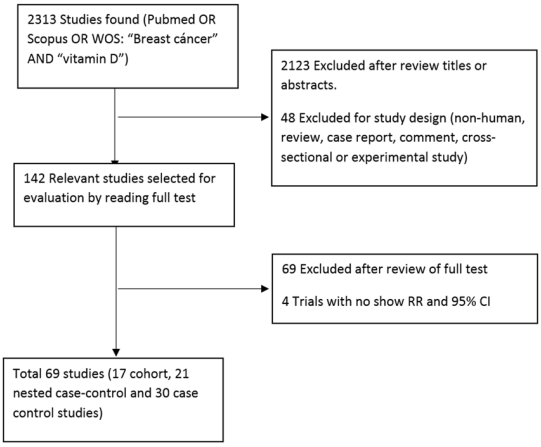

Figure 1. Flowchart which describes the methodology of selection of the articles.

identified: fifty one case-control[10,17–65] and seventeen cohort studies[65–81]. Tables 2 and 3 summarize the main characteristics of the included articles.

Data extraction. The following step was to create a database to gather all relevant information extracted from each article: year of publication, author, journal, follow up, country, sample size, exposure levels, units of measure, data for the creation of the contingency table and RR/OR with 95% CI; as well as a section to assess the quality of the study using the STROBE scale[82].

Statistical analysis. Statistical analysis was performed separately for cohort and case-control studies. In the case control studies a sensitivity analysis was also carried-out including only nested case-control studies. We performed separate analyses for any type of vitamin D exposure reported in at least three studies: 25(OH)D, dietary intake of vitamin D, 1,25(OH)2D and vitamin D supplements.

The ways that doses or levels of vitamin D were reported in each individual article were not standardized across studies (for instance, some papers reported vitamin D levels in quartiles; others in tertiles, and so on), making it difficult to extract them in an analyzable form. Therefore, in order to provide a consistent criterion of comparability, we selected the OR or RR reported for the highest category compared to the lowest one.

Regarding the type of breast cancer, we analyzed all invasive breast cancers together, and breast cancer stratified according to the cancer estrogen receptor status and woman's menopausal status. Pooled OR or RR were

Nested Case-Control	Country	Exposition	Group	OR 95% CI	No. of participants	Age at baseline[a]	Follow-up period	Upper vs lower cut off levels	Adjusted by Time of blood draw
Almquist M *et al.*(2010)[£,¥,$,φ]	Sweden	25(OH)D3	All	0.99 (0.72–1.36)	1524	57 years	1991–2006	≥106 vs ≤70 ng/mL	Yes
		25(OH) D3 + D2	All	1.01 (0.73–1.40)				≥107 vs ≤71 ng/mL	
		25(OH)D3	PRE	1.58 (0.77–3.25)				≥106 vs ≤70 ng/mL	
			POST	0.88 (0.60–1.28)				≥107 vs ≤71 ng/mL	
		25(OH) D3 + D2	PRE	1.74 (0.84–3.60)				≥106 vs ≤70 ng/mL	
			POST	0.88 (0.60–1.29)				≥107 vs ≤71 ng/mL	
Amir E *et al.* (2012)[£]	Canada	25(OH)D	All	0.86 (0.62–1.21)	1087	53.6 years	1992–1997	≥34.4 vs <12 ng/mL	No
Bertone-Johnson ER *et al.* (2005)[£,¥,§]	USA	25(OH)D	All	0.73 (0.49–1.07)	1425	52.7 cases 57.1 controls	1989–1996	≥48 vs <20 ng/mL	No
		1,25(OH)D	All	0.76 [0.52–1.11]				≥38.2 vs <28.5 ng/mL	
Chlebowski RT *et al.* (2008)[€,£,§,‡,$]	USA	25(OH)D	POST	0.82 (0.60–1.12)	2134	50–79 years	1995–2002	≥27.04 vs <12.96 ng/mL	Yes
Deschasaux M *et al.* (2016)[£,¥,‡,φ]	France	25(OH)D	All	0.98 (0.60–1.61)	699	49.3 cases 49.1 controls	1994–2007	≥23.5 vs <11.4 ng/mL	Yes
Eliassen AH *et al.* (2011)[£,¥]	USA	25(OH)D	All	1.20 (0.88–1.63)	1827	45 cases 44.9 controls	1996–2007	≥30.6vs <18.4 ng/mL	No
			ER+	1.21 (0.84–1.75)					
			ER−	1.31 (0.63–2.74)					
Eliassen AH *et al.*(2016)[£,¥]	USA	25(OH)D	All	0.84 (0.58–1.21)	3012	56.7 cases 56.8 controls	1989–2010	≥32.7 ng/ml vs <17.5	No
			ER+	0.89 (0.74–1.08)				≥30 ng/ml vs <30	
			ER−	0.87 (0.63–1.20)					
Engel P *et al.* (2010)[€,£, ¥, ‡]	France	25(OH)D	All	0.73 (0.55–0.96)	1908	56.9 years	1995–2005	>27 vs <19.8 ng/ml	Yes
			PRE	0.37 (0.12–1.15)					
			POST	0.80 (0.60–1.07)					
Freedman M *et al.* (2008)[€,£,¥¥,§]	USA	25(OH)D	POST	1.04 (0.72–1.51)	2010	55–74 years	1993–2005	33.7 vs 18.3 ng/mL	Yes
Hiatt RA *et al.* (1998)[¥,φ]	USA	1,25(OH)2D	All	1.00 (0.20–3.40)	192	>55 years	1980–1991	≥51 vs <32 pg/ml	No
Kim Y *et al.* (2014)[£,¥,$]	USA	25(OH)D	White	0.13 (0.03–0.71)	1414	68.5 cases 68.4 controls	2001–2006	>0 vs 0 ng/mL	Yes
			African-american	1.35 (0.65–2.78)					
			Hawaian	1.35 (0.23–7.69)					
			Japanese	1.04 (0.51–2.13)					
			Latino	1.11 (0.51–2.44)					
Kühn T *et al.* (2013)[£,¥,‡,φ]	Europe	25(OH)D	All	1.07 (0.85–1.36)	2782	50.7 years	1992–2006	>63 vs ≤39.3nmol/L	No
			ER+	0.97 (0.67–1.38)					
			ER−	0.97 (0.66–1.42)					
McCullough ML *et al.* (2009)[£,¥,$]	USA	25(OH)D	All	1.09 (0.70–1.68)	1032	69.5 cases 69.4 controls	1998–2005	>76.2vs <36.7 nmol/ml	Yes
			ER+	1.15 (0.80–1.65)				>64.2 vs <45.9 nmol/ml	
			ER−	0.95 (0.43–2.06)					
Mohr SB *et al.* (2013)[$]	USA	25(OH)D	All	0.84(0.56–1.25)	1200	39.6 years	1994–2009	≥35.2 vs ≤14.9 ng/mL	No
Neuhouser ML *et al.* (2012)[£,‡]	USA	25(OH)D	POST	0.94 (0.70–1.28)	2160	50–79 years	1994–2005	≥25.96vs ≤14.68 ng/mL	No
Rejnmark L *et al.* (2009)[#]	Denmark	25(OH)D	All	0.52 (0.32–0.85)	562	58 years	2003–2007	>33.6 vs <24 ng/mL	No
			PRE	0.38 (0.15–0.97)					
			POST	0.71 (0.38–1.30)					
Scarmo S *et al.* (2013)[£,¥,$]	USA&Sweden	25(OH)D	All	0.94 (0.76–1.16)	4525	34–69 years	1985–2007 1995–2010	N.A. (Quintiles)	No
			PRE	0.67 (0.48–0.92)					
			POST	1.21 (0.92–1.58)					
Shirazi L *et al.* (2016)[€,£, ¥,§]	Sweden	25(OH)D3	All	0.97 (0.75–1.25)	1520	46–73 years	1991–1996/2006	≥98nmol/L vs ≤76nmol/L	Yes
Wang J *et al.* (2014)[£,¥]	USA	25(OH)D	All	0.95 (0.67–1.36)	1168	45 years		>= 5.59 vs <3.76nmol/L	No
Case-Control	**Country**	**Exposition**	**Group**	**OR 95% CI**	**No. of participants**	**Age at baseline**	**Follow-up period**	**Upper cut off levels**	

Continued

Nested Case-Control	Country	Exposition	Group	OR 95% CI	No. of participants	Age at baseline[a]	Follow-up period	Upper vs lower cut off levels	Adjusted by Time of blood draw
Abbas S *et al.* (2009)[€,¥,φ]	Germany	25(OH)D	PRE	0.45 (0.29–0.70)	884	42.1 cases 41.6 controls	1992–1995	≥60 vs <30nmol/L	Yes
			ER+	0.56 (0.31–1.00)					
			ER−	0.40 (0.20–0,81)					
Abbas S *et al.* (2008)[£,¥,§]	Germany	25 (OH)D	POST	0.31 (0.24–0.42)	2759	63.6 cases 63.5 controls	2001–2005	>=75 vs <30nmol/L	Yes
Alipour S *et al.* (2014)[€,¥]	Iran	25 (OH)D	All	0.33 (0.12–0.91)	500	44.2 cases 43.2 controls	N.A.	>35 ng/ml vs <12.5 ng/ml	No
Bilinski K *et al.* (2012) [€,φ]	Australia	25(OH)D	All	0.43 (0.23–0.77)	1066	55.4 cases 55.5 controls	2008–2011	≥75nmol/L vs <25nmol/mL	Yes
			<50years	0.29 [0.08–1]					
			≥50 years	0.45 [0.23–0.71]					
Chen P *et al.* (2013)[€,¥,§]	China	25(OH)D	All	0.11 (0.07–1.17)	1173	53.0 cases 55.3 controls	2005–2008	>17.9 ng/ml vs <10.4 ng/ml	Yes
Colagar AH *et al.* (2015)[#]	Iran	25(OH)D	All	0.26 (0.13–0.50)	261	48.7 cases 47.0 controls	2009–2013	≥16 vs <9 ng/mL	No
Crew KD *et al.* (2009) [€,£,¥,§,‡,$]	USA	25(OH)D	All	0.56 (0.41–0.78)	2101	58.6 cases 56.1 controls	1996–1997	≥40 vs <20 ng/mL	Yes
			PRE	0.83 [0.36–1.30]					
			POST	0.46 [0.09–0.83]					
Fedirko V *et al.* (2012)[£,¥¥,§,‡,φ]	Mexico	25(OH)D3	All	0.53 (0.36–0.78)	2074	53.1 cases 51.3 controls	2004–2007	>25 vs ≤20 ng/mL	Yes
			PRE	0.40 (0.30–0.81)					
			POST	0.55 (0.33–0.90)					
Jamshidinaein Y *et al.* (2016)[£,§,φ,$]	Iran	25(OH)D	All	0.26 (0.12–0.59)	270	50.4 cases 50.0 controls	2013–2014	≥29.5 vs <10.30 ng/ml	Yes
			PRE	0.25 (0.09–0.69)					
			POST	0.42(0.15–1.17)					
Janowsky EC *et al.* (1999)[€]	USA	1,25(OH)2D	All	0.31 (0.17–0.59)	331	NA	1990–1991	≤34.6 vs>63.6pmol/ml	Yes
Lowe LC *et al.* (2005)[€]	UK	25(OH)D	All	0.17 (0.07–0.43)	358	58.0 cases 58.0 controls	1998–2003	≥150 vs ≤50 nM	Yes
Oliveira-Sediyama CM *et al.*(2016)[‡]	Brazil	25(OH)D	All	0.34 (0.16–0.71)	378	54.0 cases 47.5 controls	NA	≥20vs <20 ng/mL	No
Park S *et al.* (2015)[€,£, ¥,§]	Korea	25(OH)D	All	0.82 (0.75–0.90)	20767	50.7 cases 49.7 controls	2006–2012	≥20 vs <20 ng/mL	Yes
			PRE	0.84 (0.74–0.96)					
			POST	0.82 (0.73–0.93					
Sofi NY *et al.* (2016)[#]	India	25(OH)D	All	0.40 (0.14–1.11)	200	45.0 cases 46.0 controls	2014–2015	≥20 ng/mL vs <20 ng/mL	No
Sofi NY *et al.* (2018)[#]	India	25(OH)D	All	0.42 (0.20–0.83)	400	45.0 cases 47.0 controls	2015–2017	≥20 ng/mL vs <20 ng/mL	No
Yao S *et al.* (2011)[€,£,¥]	USA	25(OH)D	All	0.37 (0.27–0.51)	1153	NA	2003–2008	≥30 vs <20 ng/mL	Yes
			PRE	0.57 (0.34–0.93)					
			POST	0.29 (0.19–0.45)					
Yousef FM *et al.* (2013)[€,£,φ]	Saudi Arabia	25(OH)D	All	0.16 (0.07–0.42)	240	18–75 years	2009	≥20 vs <10 ng/mL	No
Ordoñez-Mena JM *et al.* (2016)[€,£,‡,φ]	Europe	25(OH)D	POST	0.73 (0.22–2.43)	252	>=60 years	1992–2000	>50 vs <30 nmol/L	Yes
Cohort	**Country**	**Exposition**	**Group**	**RR 95% CI**	**Cases (No. of participants)**	**Age at baseline**	**Follow-up period**	**Upper cut off levels**	
Skaaby T *et al.* (2014)[£,‡,φ]	Denmark	25(OH)D	All	1.1 (0.7–1.71)	159 (5606)	18–71 years	1993–2008	N.A. (Quartiles)	Yes
O´Brien KM (2017) et al[€,£, ¥,§,‡,φ,$]	USA	25(OH)D	All	0.79 (0.63–0.98)	1600 (3422)	35–74 years	2003–2009	>38 vs <24.6 ng/mL	Yes
Ordonez-Mena JM *et al.* (2013)[€,£,‡,φ]	Germany	25(OH)D	All	1.08 (0.72–1.6)	137 (5261)	50–74 years	2000–2002	<30 vs >55 nmol/L*	No
Palmer JR *et al.* (2016)[€,£, ¥,§]	USA (African American Women)	25(OH)D	All	0.81 (0.68–0.96)	1454 (2856)	21–69 years	2012–2017	≥49 vs <21 ng	No

Continued

Nested Case-Control	Country	Exposition	Group	OR 95% CI	No. of participants	Age at baseline[a]	Follow-up period	Upper vs lower cut off levels	Adjusted by Time of blood draw
Ordonez-Mena JM *et al.* (2016)[€,£,ǂ,φ]	Germany	25(OH)D	POST	1.35 (0.38–2.27)	63 (4990)	63 years	2000–2002	>50 vs <30nmol/L	Yes
Ordonez-Mena JM *et al.* (2016)[€,£,ǂ,φ]	Norway	25(OH)D	POST	2.63 (0.82–8.33)	89 (2471)	62 years	1994–1995	>50 vs <30nmol/L	Yes

Table 2. Studies included in our meta-analyses of blood 25-hydroxyvitamin D and breast cancer risk. [a]Mean or range of age. Adjusted by: [€]age; [£]BMI; [¥]reproductive factors (menopausal status, age at menopause, age at menarche, parity, etc); [$]hormone therapy; [ǂ]physical activity; [φ]educative or socioeconomic variables; [$]race or sun exposure. [#]Unadjusted. Abbreviations: CI = confidence interval; POST = postmenopausal; PRE = premenopausal; OR = odds ratio; NA: Not available.

estimated by weighting individual OR/RR by the inverse of their variance. OR or RR heterogeneity was measured using Q and I^2 statistics[83]. A fixed-effect model was preferred if the Q statistic was higher than 0.1 or I^2 lower than 25%, indicating no relevant heterogeneity; a random-effect model was otherwise chosen[84]. The presence of small-study bias was explored with Rosenthal model and with Egger test[85]; due to the low sensitivity of Egger test, the cut-off was set at $p = 0.1$. Funnel plots[86] were applied to detect publication bias.

An analysis of influence was performed via the re-estimation of pooled OR/RR by removing one study at a time. Studies that, when removed, strongly changed the OR/RR would be considered as highly influential. Results are displayed as forest plots showing OR/RR and their 95% confidence intervals for each individual study and for the pooled result. Cumulative meta-analyses were carried out to deem the stability of the OR/RR estimates. In order to do that, all studies considered were arranged from oldest to neweest. Then an OR/RR estimate was obtained for the two eldest studies; another for the three eldest, and so on, adding a study each time. Results are reported as forest plots.

All the statistical analyses were carried out with the package Stata 14/SE (Stata Corporation, College Station, TX, US).

Results

Relationship between 25(OH) D and breast cancer. Twenty-nine case control studies were analyzed to study the relationship between 25 (OH) D and breast cancer[10,19–22,25,27,29–35,38,42,44–46,48,49,51,55,56,58–63] obtaining a pooled OR of 0.65 (95%CI: 0.56–0.76) (Fig. 2a, Table 4). This value was calculated using the random effects model because of the high heterogeneity ($I^2 = 77.76\%$) of the fixed-effect. Although Egger test cannot rule out a small-study effect ($p = 0.001$), no study shows a relevant influence. The funnel plot shows asymmetry (Supplementary Fig. 1a), indicating either publication bias or heterogeneity that cannot be explained by a random-effect meta-analysis. Rosenthal model shows that 1194 negative studies would be needed to lose statistical significance. In order to further clarify the heterogeneous result, we carried out a sensitivity analysis including only nested case-control studies[21,22,25,31–34,42,45,46,51,55,56,59] reaching a pooled OR = 0.92 (95%CI: 0.83–1.01) (Fig. 2b) with $I^2 = 15.87\%$, Q-based p value = 0.22 and a very symmetrical-looking funnel plot (Supplementary Fig. 1b).

Four cohort studies[75,78–80] provided results on 25(OH)D and breast cancer relationship, from which we obtained a pooled RR of 0.85 (95% CI:0.74–0.98).

We also analyzed the relationship between 25(OH) D and breast cancer, stratifying results by hormonal receptors (ER+/ER−) and menopausal status (postmenopausal or premenopausal). Regarding hormonal receptors (Table 4), we have found only one cohort study[80] and five case-control studies[19,32,33,42,45]. In both cases (ER+ and ER− tumors) statistical significance was not reached. With respect to menopausal status (Table 4), we obtained a protective effect in both groups: nineteen case-control studies targeted postmenopausal women[18,21,28,30,34–36,38,41,47,49,51,55,60,81] with a pooled OR of 0.74 (95%CI: 0.59–0.93), and nine focused on premenopausal[21,30,34,35,38,49,51,55,60] obtaining a pooled OR of 0.63 (95%CI: 0.49–0.80) (Fig. 3a). When the sensitivity analysis was carried out including only nested case-control studies, the protective vitamin D – breast cancer association persisted only in the premenopausal group (Fig. 3b, Supplementary Table 1). On the other hand three cohorts studies analyzed separately postmenopausal women[79,81] without reaching statistical significance (OR = 1.15 (0.59–2.23)).

Relationship between 1,25(OH)2D and breast cancer. Three case-control studies[25,37,39] examined the relationship between circulating 1,25(OH)2D and breast cancer; significant association was not found either in the whole analysis (pooled OR = 0.61 (0.33–1.16)) or in postmenopausal women (combined OR = 1.28 IC 95%: 0.98–1.67)[36,37].

Relationship between dietary vitamin D and breast cancer. We found eight case-control studies[24,38,40,50,52,53,57,64] on the relationship between dietary vitamin D and breast cancer with a pooled OR of 0.91 (95%CI: 0.72–1.17) (Table 4, Supplementary Fig. 2a). In addition, by combining five cohort studies[66,68,70–72] we obtained a RR of 1.00 (95% CI 0.93–1.07) (Table 4, Supplementary Fig. 2b).

When stratifying by menopausal status, four case-control[38,40,53,64] and five cohort studies[66,73,74,76,77] assessed the risk of breast cancer in postmenopausal women. The pooled OR for case-control studies was 0.78 (95%CI: 0.68–0.90) and the pooled RR for cohort studies was 0.95 (95%CI: 0.83–1.09) (Table 4). In both analyses, Egger

Case-Control	Country	Exposition	Group	OR (95% CI)	No. of participants	Age at baseline	Follow-up period	Upper vs lower cut off levels
Abbas S *et al.* (2007)[€,£,¥]	Germany	Dietary Vitamin D	PRE	0.50 (0.26–0.96)	944	41.7 cases 41.6 controls	1992–1995	≥200 vs <80 IU/day
Anderson LN *et al.* (2010)[€,¥,ǂ,φ]	Canada	Total vitamin D intake	All	0.99 (0.78–1.26)	6572	56 years	2002–2003	≥15 vs <2.5 mg/day
		Dietary Vitamin D		1.13 (0.88–1.45)				≥10 vs <2.5 mg/day
		Vitamin D supplement		0.76 (0.59–0.98)				≥10 vs 0 mg/day
Anderson LN *et al.* (2011)[€]	Canada	Vitamin D supplement	All	0.80 (0.60–1.08)	3616	56 years	2002–2003	>400 vs 0 IU/day
		Total Vitamin D intake		0.87 (0.71–1.06)				≥600 vs <200 IU/day
Bidgoli SA *et al.* (2014)[#]	Iran	Vitamin D supplement	PRE	0.89 (0.84–0.95)	176	36.5 cases 34.2 controls	2010–2012	Yes vs No
Jamshidinaein Y *et al.* (2016)[€,£,¥,§,φ]	Iran	Dietary vitamin D	All	0.38 (0.18–0.83)	270	50.4 cases 50 controls	2013–2014	NA (Quartile)
		Dietary vitamin D	PRE	0.39 (0.15–1.00)				
		Dietary vitamin D	POST	0.40 (0.15–1.12)				
		Total vitamin D intake	All	0.52 (0.25–1.14)				
		Total vitamin D intake	PRE	0.36 (0.13–1.06)				
		Total vitamin D intake	POST	0.70 (0.27–1.82)				
Kawase T *et al.* (2010)[£,¥,§,ǂ]	Japan	Dietary Vitamin D	All	0.76 (0.63–0.90)	5409	20–79	2001–2005	>6.66 vs <2 mg/day
			PRE	0.65 (0.50–0.86)				
			POST	0.83 (0.64–1.07)				
Lee MS *et al.* (2011)[€,£,¥,φ]	Taiwan	Dietary Vitamin D	All	0.57 (0.28–1.19)	400	52.5 cases 48.9 controls	2004–2005	>5 vs <2 mg/day
		Dietary Vitamin D	PRE	0.38 (0.14–0.98)				
		Dietary Vitamin D	POST	0.60 (0.20–1.69)				
		Total vitamin D intake	All	0.52 (0.25–1.07)				NA (Quartile)
		Total vitamin D intake	PRE	0.47 (0.18–1.23)				
		Total vitamin D intake	POST	0.68 (0.23–1.27)				
Levi F *et al.*(2001)[€,£,¥,φ]	Switzerland	Vitamin D supplement	All	1.43 (0.90–2.26)	731	23–74	1993–1999	≥2.7 vs <1.4 mg/day
Leung *et al.*(2016)[€]	China	Vitamin D supplement	All	0.78 (0.63–0.98)	323612	>18	2000–2011	≤15 DDD
Potischman N *et al.* (1999)[€,¥,§,φ]	USA	Dietary Vitamin D	All	0.98 (0.80–1.20)	2019	20–44	1990–1992	≥400 vs <0 IU
Rollison DE *et al.* (2012)[€,£,¥,§,ǂ]	USA	Dietary Vitamin D	All	1.35 (1.15–1.60)	4839	24–79	1999–2004	7.71 vs <3.06 mg/day
		Vitamin D supplement	All	0.79 (0.65–0.96)		24–79 years	1999–2004	0 vs>10 mg/day
Rossi M *et al.* (2009)[€,£,¥,§,φ]	Italy	Dietary Vitamin D	All	0.76 (0.58–1.00)	5157	55 years cases 56 controls	1991–1994	>3.57 vs ≤3.57 mg
			PRE	0.80 (0.64–0.99)				
			POST	0.78 (0.66–0.92)				
Salarabadi A *et al.* (2015)[#]	Iran	Vitamin D supplement	PRE	0.53 (0.14–1.96)	152	NA	2012–2014	Yes vs No
Cohort	**Country**	**Exposition**	**Group**	**RR (95% CI)**	**Cases/Total**	**Age at baseline**	**Follow-up period**	**Upper cut off levels**
John EM *et al.* (1999)[€,£,¥,ǂ,φ]	USA	Dietary vitamin D	All	0.85 (0.59–1.24)	190/5009	25–74	1971–1992	≥200 vs <100 IU/day
		Vitamin D supplement	All	0.89 (0.60–1.32)		25–74	1971–1993	Daily vs never
		Total vitamin D intake	All	0.86 (0.61–1.2)		25–74	1971–1994	≥200 or daily suppl vs <100 IU/day without daily suppl

Continued

Case-Control	Country	Exposition	Group	OR (95% CI)	No. of participants	Age at baseline	Follow-up period	Upper vs lower cut off levels
Shin MH *et al.* (2002)[€,£,¥,‡]	USA	Total vitamin D intake	PRE	0.89 (0.68–1.15)	3482/88 691	46.7	1980–1996	>500 vs ≤150 IU/day
			POST	0.93 (0.8–1.08)				
		Dietary Vitamin D	PRE	0.84 (0.59–1.18)				
			POST	0.86 (0.7–1.05)				
Lin J *et al.* (2007)[€,£,¥,§,‡]	USA	Total vitamin D intake	PRE	0.65 (0.42–1)	1019/31487	55 (≥45)	1993–2003	≥548 vs <162 IU/d
			POST	1.30 (0.97–1.73)				
		Dietary vitamin D	PRE	1.02 (0.69–1.53)				≥319 vs <142 IU/d
			POST	1.22 (0.95–1.55)				
		Vitamin D supplement	PRE	0.76 (0.5–1.17)				≥400 vs 0 IU/d
			POST	0.87 (0.68–1.12)				
Robien K *et al.* (2007)[€,£,¥,§,φ]	EEUU	Vitamin D supplement	POST	0.89 (0.74–1.08)	2440/34321	61 (55–69)	1986–2004	≥800 IU/d vs No
		Dietary Vitamin D	POST	0.55 (0.24–1.22)				≥800 vs <400 IU/d
		Total vitamin D intake	POST	0.89 (0.77–1.03)				≥800 vs <400 IU/d
Kuper H *et al.* (2009)[€,£,¥,§,‡]	Sweden	Dietary vitamin D	All	0.90 (0.80–1.1)	848/41889	30–49	1991–2003	N.A. (Quartile)
Cadeau C *et al.* (2015) [€,£,¥,§,‡]	France	Vitamin D supplement	All	1.10 (0.92–1.31)	2482/57403	40–65	1995–2008	Current vs never
			ER+	1.23 (1–1.51)		40–65	1995–2008	Current vs never
			ER−	0.93 (0.55–1.55)		40–65	1995–2008	Current vs never
Abbas S *et al.* (2013)[€,¥,§,‡,φ]	Europe	Dietary vitamin D	All	1.04 (0.94–1.14)	7760/319985	50.2	1992–2005	≥5.46 vs <1.85 mg/day
			PRE	1.07 (0.87–1.32)				≥5.46 vs <1.85 mg/day
			POST	1.02 (0.9–1.16)				≥5.46 vs <1.85 mg/day
McCullough ML *et al.* (2005)[€,¥,§,‡,φ]	USA	Total vitamin D intake	POST	0.94 (0.8–1.1)	2855/68567	50–74	1992–2001	>700 vs ≤100 IU/day
		Dietary vitamin D	POST	0.87 (0.75–1)				>300 vs ≤100 IU/day
Edvarsen K *et al.* (2011) [€,£,¥,§]	Norway	Dietary vitamin D	All	1.07 (0.87–1.32)	948/41811	40–70	1997–2007	12.31 vs <3.99 mg/day
Frazier *et al.* (2004)[€,£,¥,§]	USA	Dietary vitamin D	All	0.92 (0.66–1.27)	838/47355	34–51	1989–1998	591 vs 159.6 IU/day
Engel P et al. (2011)[€,¥,§,‡]	France	Total vitamin D intake	All	0.94 (0.86–1.03)	2871/67721	41.8–72	1990–2008	>113 vs <80 IU/day
			PRE	1.03 (0.85–1.25)				
			POST	0.92 (0.86–1.03)				
Nested Case-Control	**Country**	**Exposition**	**Group**	**OR (95% CI)**	**No. of participants**	**Age at baseline**	**Follow-up period**	**Upper vs lower cut off levels**
Simard A *et al.* (1991)[#]	Canada	Dietary Vitamin D	All	2.79 (0.85–9.15)	430	40–59	1981–1983	>200 vs <50 IU/day
Kim Y *et al.* (2014)[£,¥,‡]	USA	Vitamin D supplement	White	1.29 (0.75–2.23)	1414	67.8	2001–2010	>=16 ng/mL vs <16 ng/mL
			African-american	0.29 (0.12–0.70)				
			Hawaian	0.46 (0.16–1.34)				
			Japanese	1.32 (0.90–1.93)				
			Latino	0.85(0.46–1.56)				
			PRE	1.03 (0.85–1.25)				
			POST	0.92 (0.86–1.03)				

Table 3. Studies included in our meta-analyses of dietary or supplements vitamin D and breast cancer risk. [a]*Mean or range of age*. Adjusted by: [€]age; [£]BMI; [¥]reproductive factors (menopausal status, age at menopause, age at menarche, parity, etc); [§]hormone therapy; [‡]physical activity; [φ]educative or socioeconomic variables; [$]race or sun exposure. [#]Unadjusted. *Abbreviations: CI = confidence interval; POST = postmenopausal; PRE = premenopausal;* OR = odds ratio*; NA: Not available*.

test rejected the possibility of small study bias (p = 0.536 in case-control studies and p = 0.68 in cohort studies). On the other hand, five case-control studies[17,38,40,53,63] and three cohort studies[66,73,77] targeted premenopausal women; the pooled OR was 0.65 (95%CI: 0.52–0.82) for case-control studies and the RR for cohort studies was 1.01 (95% CI: 0.86–1.18) (Table 4).

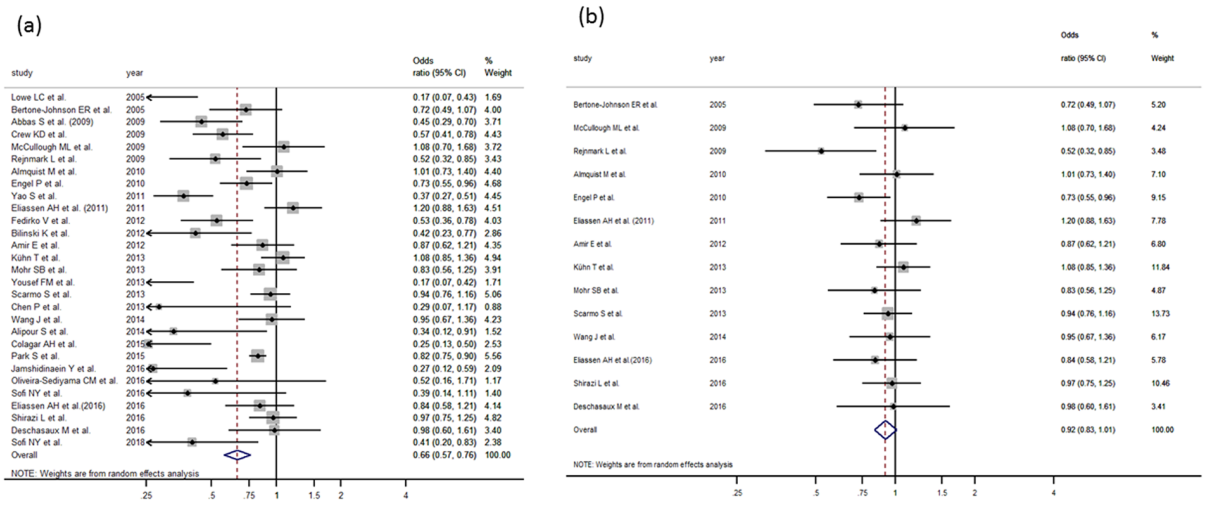

Figure 2. (**a**) Forest plot for the relationship between 25(OH)D and breast cancer in case control studies. (**b**) Forest plot for the relationship between 25(OH)D and breast cancer in nested case control studies.

Exposition	Group (Number of studies)	Type of study	OR/RR (95% CI)	I^2
25(OH)D	All (n = 29)	Case-control	0.65 (0.56–0.76)	40.87%
	All (n = 4)	Cohort	0.85 (0.74–0.98)	3.56%
	ER+ (n = 5)	Case-control	0.98 (0.85–1.13)	0%
	ER– (n = 5)	Case-control	0.86 (0.64–1.15)	15.60%
	Postmenopausal (n = 19)	Case-control	0.74 (0.59–0.93)	13.16%
	Postmenopausal (n = 3)	Cohort	1.15 (0.59–2.23)	8%
	Premenopausal (n = 9)	Case-control	0.63 (0.49–0.80)	8.37%
Dietary vitamin D	All (n = 8)	Case-control	0.91 (0.72–1.17)	30.73%
	All (n = 5)	Cohort	1.00 (0.93–1.07)	0%
	Postmenopausal (n = 4)	Case-control	0.78 (0.68–0.90)	0%
	Postmenopausal (n = 5)	Cohort	0.95 (0.83–1.09)	19.13%
	Premenopausal (n = 5)	Case-Control	0.65 (0.52–0.82)	0%
	Premenopausal (n = 3)	Cohort	1.01 (0.86–1.18)	0%
Vitamin D supplements	All (n = 5)	Case-control	0.78 (0.63–0.98)	25.94%
	All (n = 2)	Cohort	1.06 (0.90–1.25)	0%
Total Vitamin D intake (dietary + supplements)	All (n = 4)	Case-control	0.84 (0.68–1.05)	18.65%
	All (n = 2)	Cohort	0.93 (0.86–1.02)	0%
	Postmenopausal (n = 5)	Cohort	0.94 (0.87–1.02)	17.64%
	Premenopausal (n = 3)	Cohort	0.90 (0.72–1.12)	10.83%

Table 4. Results from the meta-analysis.

Relationship between supplements of vitamin D and breast cancer. We identified five case-control studies[23,24,43,52,65] and two cohort studies[67,71] that had evaluated the association between supplements of vitamin D and breast cancer risk. The pooled OR and RR were 0.78 (95% CI: 0.63–0.98) and 1.06(95% IC: 0.90–1.25) respectively (Table 4). Regarding menopausal status, Kim *et al.*[41] published a study on five different populations of postmenopausal women; when combining all five results, we found no significant association (OR: 0.82 95%CI: 0.49–1.35).In addition, we found two case-control studies[26,54] focused on premenopausal women obtaining a weak protection (pooled OR 0.89 95%CI (0.84–0.95)).

Relationship between total vitamin D intake (dietary and supplements) and breast cancer. Finally, we found two cohort studies[69,71] and four case control studies[23,24,38,64] on vitamin D intake (dietary plus supplements) and breast cancer risk, providing no separate results on dietary/supplemented vitamin D origin. We obtained a combined RR = 0.93 (95% CI: 0.86–1.02) for cohort studies, and a combined OR = 0.84 (95% CI: 0.68–1.05) for case-control studies. Five cohort studies[69,73,74,76,77] provided results on postmenopausal women (RR = 0.94 95% CI: 0.87–1.00) and three cohort studies[69,73,77] on about premenopausal women (RR = 0.90 95% CI: 0.72–1.12) (Table 4). Only two case-control studies provided results according menopausal status[38,64] without being significant in both groups.

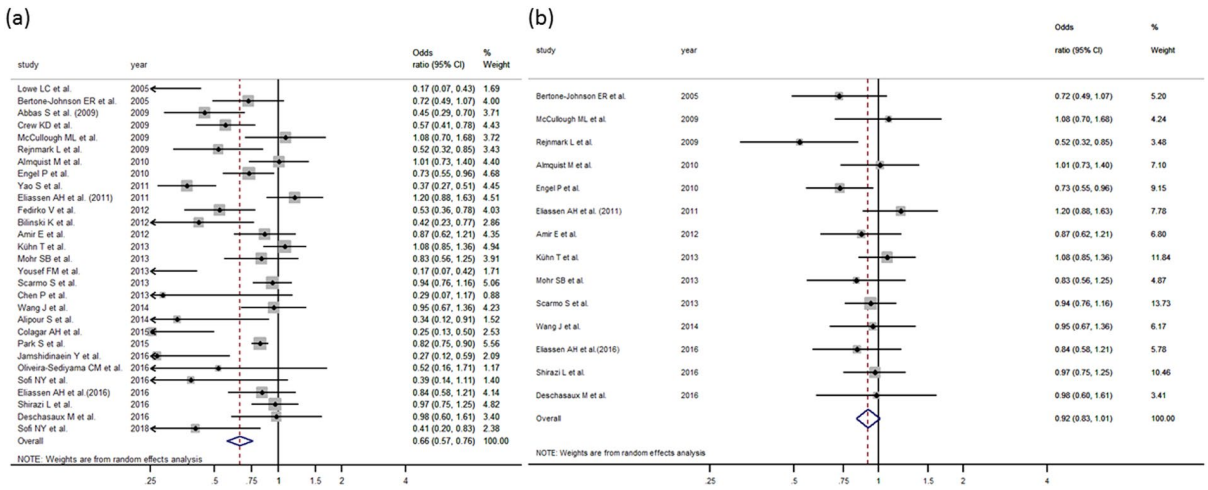

Figure 3. (**a**) Forest plot for the relationship between 25(OH)D and premenopausal breast cancer in case control studies. (**b**) Forest plot for the relationship between 25(OH)D and premenopausal breast cancer in nested case control studies.

Discussion

According to our results, 25(OH)D levels were associated with smaller risk of breast cancer in both case-control and cohort studies; these results were consistent on premenopausal women for case-control studies but could not be analyzed for cohort studies. Results for the relationships between breast cancer and dietary vitamin D or between breast cancer and vitamin D supplements, however, showed a protective association only in case-control studies.

In relation to the influence of vitamin D on breast cancer development prospective (cohort and nested case-control) and case control studies tend to show discrepant results: case-control studies usually show a protective effect while prospective studies rarely find it[87]. This discrepancy might be the result of several factors: Firstly, it is well known that prospective studies are less prone to be affected by both information and reverse-causation bias. Secondly, several authors highlight the season when the vitamin D measurement was made as a potential limitation of case-control studies. Eliassen *et al.*[33] in a nested case-control study found an inverse association between serum 25(OH) D levels and breast cancer limited only to summer measures. It can be assumed that people with low vitamin D levels in summer would also have low levels year-round; therefore, vitamin D levels in summer would be more adequate for analyzing vitamin D – breast cancer relationship than vitamin D levels in any other moment of the year.

When stratifying by menopausal status, our meta-analysis shows a consistent protective effect of 25(OH) D in both case-control and nested case-control studies, but only in premenopausal women. There are different explanations for the influence of menopausal status in the relationship between vitamin D and breast cancer. One of them may be related to the joint relationship between vitamin D and insulin-like growth factors (IGFs). IGF-I is a mitogenic and antiapoptotic peptide that can stimulate the proliferation of breast epithelial cells, increasing the risk of neoplastic transformation[88,89]. The active vitamin D metabolite is able to block the mitogenic effects of IGF-I, leading to a decrease in proliferation and an increase in apoptosis[90]. As there is a physiological decline of the IGF with aging[91], the interaction between IGF pathways and vitamin D is likely to be stronger for premenopausal than for postmenopausal women, leading to greater risk reduction in premenopausal breast cancer[73,92]. Finally, high levels of vitamin D may reduce progesterone and estradiol, providing a potential mechanism for reducing breast cancer risk in young women[93].

Previous meta-analyses of prospective studies showed contradictory results. Kim *et al.*[13] (who included 24 studies, 14 of those having measured serum 25(OH)D) found a slightly stronger inverse association among premenopausal than among postmenopausal women but without significant differences, whereas in the meta-analysis of Bauer *et al.*[8] (nine studies included) the inverse association was only observed in postmenopausal women. In our meta-analysis, new prospective studies[31,33,41,56,58,59,67,78–81,94] not included in previous reviews, were added and this fact may explain the differences in the results.

Concerning hormonal receptors (ER+/ER−), the relationship with breast cancer remains controversial. On the one hand, a decreased risk in ER+ would be expected, since it seems that sensitivity to 1,25(OH)2D is generally reported as being higher in breast cancer cells that express the estrogen receptor than in those that do not[93,95]. It has been demonstrated that treating breast cancer cells ER+ with 1,25(OH)D3 induces a cell cycle shutdown in GO/G1[3,96]. On the other hand, two-thirds of triple negative tumors express VDR[97] and it has been demonstrated that VDR expression is inversely associated with more aggressive breast cancer[98]. In consonance with previous epidemiological studies[32,33,42,45], our study does not reach significant differences when the analysis was performed separately in ER+ or ER− subgroups. However, other studies found a decreased risk of ER− breast cancer regarding the serum levels of 25 (OH) D[18,60].

No relationship is found between the level of circulating 1,25(OH)2D and breast cancer. This result is consistent with previous studies[9], while Janowsky *et al.*[39] found an inverse association. Several authors consider that 1,25(OH)2D is not a good indicator of vitamin D status: First, 1,25(OH)2D's half-life is only 4–6 h, whereas 25(OH)D's half-life is 3 weeks; second, 1,25(OH)2D is influenced by many factors[10], for instance, it can be elevated in patients with vitamin D deficiency as a result of hyperparathyroidism[12,99]; finally, as 1,25(OH)2D is metabolized by 1-α -hydroxylase in breast tissue, plasma levels may not adequately represent breast tissue levels[12,100].

We do not find a relationship between vitamin D intake and breast cancer in the overall analysis. In contrast, when stratifying by menopausal status, a protective effect is observed in case-control studies in both premenopausal and postmenopausal women, whereas this association is not present in cohort studies. On the other hand, when analyzing the influence of vitamin D supplements on breast cancer risk, we find a borderline protective effect.

In the relationship between vitamin D intake (dietary and/or supplements) and breast cancer, most observational studies showed non-significant differences; only two articles[17,53] found a protective association. In a previous meta-analysis[13], this association was not significant for either vitamin D intake or supplements.

A probable explanation for the lack of association observed in the analysis of dietary intake or supplements compared to the 25(OH)D levels may be that the main source of vitamin D is sunlight rather than food or supplements.

In addition, the French E3N Cohort Study[12] reported that high vitamin D intake is associated with lower breast cancer risk in regions with high ultraviolet solar radiance. These results suggested that the total amount of vitamin D needed to reach a protective effect on breast cancer is too high to be achieved in regions with low ultraviolet radiance. Under these circumstances, as the vitamin D intake has to be higher than the usually recommended, it could eventually lead to side effects such as hypercalcemia, constipation or muscle weakness.

Our study has some limitations; firstly each article uses different cutoff points according to serum levels of vitamin D. To analyze it we restricted our analysis to the comparison between the highest vs. lowest category of exposure. This analysis strategy does not allow for a dose-response analysis. Moreover, we carried out a sensitivity analysis excluding one study at a time, showing that no single study substantially affected the pooled RR/OR. Secondly, there is huge variability in the literature on the type of vitamin D studied, which makes it difficult to perform the analysis. In addition, levels of vitamin D depend on the season, so it would be advisable to take all samples at the same time, or at least refer to when they were collected[75]. Thirdly, case-control studies are more prone to methodological issues, such as recall and selection biases, which limits the strength and quality of evidence. However, about half of the case-control studies included in our meta-analysis are nested in cohort studies, which minimizes the possibility of introducing biases. Finally, breast cancer is a heterogeneous disease and it is possible that vitamin D only affects certain breast cancer subtypes. However, this aspect has been scarcely studied in primary articles, so we have not been able to analyze it in the present meta-analysis.

Despite these limitations, our study also has several strengths; first, we have gathered all the observational studies published in the last twenty years. In addition, we have focused the analysis on different types of vitamin D exposure (diet, supplements and blood-levels of 25(OH) D and 1,25(OH)2D) whereas other meta-analyses are only focused on 25(OH)D levels[9,10,16,99] or vitamin D intake[12]. This strategy allows us to obtain a more detailed analysis of the relationship between vitamin D and breast cancer.

In conclusion, our meta-analysis supports the hypothesis that high serum levels of 25(OH) vitamin D has a protective effect on breast cancer risk in premenopausal women; we cannot draw the same conclusion regarding vitamin D intake or supplements of vitamin D since the number of studies are still limited and publication biases cannot be excluded.

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Author Contributions

N.E., T.D.S. and I.G.A. contributed substantially to the conception, design and acquisition of data. N.E. and T.D.S.: wrote the main manuscript text. N.E. and C.P. prepared figures. T.D.S., I.G.A. and J.L. contributed to the analysis and interpretation of the data. N.E. and T.D.S., I.G.A., C.P. and J.L. contributed to devising the draft of the article and all of the other authors revised it critically. All authors participated in revising the manuscript and in the final approval of the version to be published.

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RESEARCH ARTICLE

Breast cancer risk markedly lower with serum 25-hydroxyvitamin D concentrations ≥60 vs <20 ng/ml (150 vs 50 nmol/L): Pooled analysis of two randomized trials and a prospective cohort

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Abstract

Background

While numerous epidemiologic studies have found an association between higher serum 25-hydroxyvitamin D [25(OH)D] concentrations and lower breast cancer risk, few have assessed this association for concentrations >40 ng/ml.

Objective

To investigate the relationship between 25(OH)D concentration and breast cancer risk across a broad range of 25(OH)D concentrations among women aged 55 years and older.

Methods

Analyses used pooled data from two randomized clinical trials (N = 1129, N = 2196) and a prospective cohort (N = 1713) to examine a broad range of 25(OH)D concentrations. The outcome was diagnosis of breast cancer during the observation periods (median: 4.0 years). Three analyses were conducted: 1) Incidence rates were compared according to 25(OH)D concentration from <20 to ≥60 ng/ml (<50 to ≥150 nmol/L), 2) Kaplan-Meier plots were developed and 3) multivariate Cox regression was used to examine the association between 25(OH)D and breast cancer risk using multiple 25(OH)D measurements.

Results

Within the pooled cohort (N = 5038), 77 women were diagnosed with breast cancer (age-adjusted incidence: 512 cases per 100,000 person-years). Results were similar for the three

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analyses. First, comparing incidence rates, there was an 82% lower incidence rate of breast cancer for women with 25(OH)D concentrations ≥60 vs <20 ng/ml (Rate Ratio = 0.18, $P = 0.006$). Second, Kaplan-Meier curves for concentrations of <20, 20–39, 40–59 and ≥60 ng/ml were significantly different ($P = 0.02$), with the highest proportion breast cancer-free in the ≥60 ng/ml group (99.3%) and the lowest proportion breast cancer-free in the <20 ng/ml group (96.8%). The proportion with breast cancer was 78% lower for ≥60 vs <20 ng/ml ($P = 0.02$). Third, multivariate Cox regression revealed that women with 25(OH)D concentrations ≥60 ng/ml had an 80% lower risk of breast cancer than women with concentrations <20 ng/ml (HR = 0.20, $P = 0.03$), adjusting for age, BMI, smoking status, calcium supplement intake, and study of origin.

Conclusions

Higher 25(OH)D concentrations were associated with a dose-response decrease in breast cancer risk with concentrations ≥60 ng/ml being most protective.

Introduction

Breast cancer is the most common non-skin cancer in women [1]. More than 252,000 new cases of female breast cancer and 40,600 deaths were projected to occur in 2017 in the United States [1]. While more early detection and improvements in treatment have reduced the mortality rate, there has been no reduction in the incidence of breast cancer in the past 20 years [2]. Identifying and implementing effective primary prevention strategies could reduce breast cancer incidence rates.

Epidemiologic studies by Gorham et al. [3,4] and Garland et al. [5] were the first to propose that vitamin D prevents breast cancer. Since then, the mechanisms by which vitamin D might prevent the development and growth of breast cancer have been well documented [6] and numerous epidemiologic studies have found an association between higher serum 25-hydroxyvitamin D [25(OH)D] concentrations, the physiological measure of vitamin D status, and a lower risk of breast cancer [7–18]. However, few studies have assessed this association in concentrations >40 ng/ml [7,8].

The objective of this analysis was to investigate the relationship between 25(OH)D concentration and breast cancer risk across a broad range of 25(OH)D concentrations among women aged 55 years and older. Data from two randomized clinical trials (RCT) and a prospective cohort study were pooled: the 2007 Lappe et al. cohort (RCT, median 25(OH)D = 31 ng/ml, N = 1129) [19,20], the 2017 Lappe et al. cohort (RCT, median 25(OH)D = 36 ng/ml, N = 2196) [21], and the GrassrootsHealth cohort (prospective cohort study, median 25(OH)D = 49 ng/ml, N = 1713) [20]. This pooled cohort provided a larger sample size for improved statistical power and allowed for analysis across a broad range of 25(OH)D concentrations that would otherwise not have been possible due to the lack of a sufficient number of participants with 25(OH)D concentrations higher than 40 ng/ml.

Materials and methods

Women in the 2007 Lappe et al. cohort (hereafter termed 2007 Lappe cohort) participated in a four year, population-based, double-blind, placebo-controlled trial of vitamin D and calcium supplementation in a 9-county area in Eastern Nebraska. Participants were randomly assigned

to: 1) calcium plus vitamin D3 (1400–1500 mg/day of calcium plus 1100 IU/day of vitamin D3), 2) calcium (calcium as mentioned previously plus vitamin D placebo), or 3) control (calcium and vitamin D placebos). This trial was registered at clinicaltrials.gov as NCT00352170.

In another study, women in the 2017 Lappe et al. cohort (hereafter termed 2017 Lappe cohort) participated in a four year, population-based, double-blind, placebo-controlled trial of vitamin D and calcium supplementation in a 31-county area in Eastern Nebraska. Participants were randomly assigned to: 1) intervention (1500 mg/day of calcium and 2000 IU/day of vitamin D3) or 2) control (calcium and vitamin D placebos). This trial was registered at clinicaltrials.gov as NCT01052051.

For both Lappe cohorts, inclusion criteria included women aged 55 years or older who were free of known cancer at enrollment and within the prior 10 years. As described previously [19–22], supplement intake by bottle weight and health status were assessed at 6-month intervals. Medical records were examined to confirm reports of cancer diagnosis and ascertain diagnosis date. Participants who did not complete at least two health assessments were excluded from this study because of lack of prospective data. Serum 25(OH)D concentrations were measured at enrollment and annually thereafter using radioimmunoassay (IDS Radioimmunoassay (RIA) kit, Fountain Hills, AZ for the 2007 cohort and Liaison® Analyzer, Diasorin, Stillwater, MN for the 2017 cohort) at the Creighton University Osteoporosis Research Center Laboratory (Omaha, NE). The intra-assay coefficient of variation was 5% for IDS RIA and 5% for Liaison®. Additionally, the Creighton Laboratory participates in the Vitamin D External Quality Assessment Scheme (DEQAS) with findings on test samples regularly close to the international mean. Detailed descriptions of the Lappe trials and results of other outcomes can be found elsewhere [19–22]. All participants provided written informed consent and the studies were approved by the Creighton University Institutional Review Board (Omaha, NE).

Women in the GrassrootsHealth cohort participated in a prospective population-based cohort study run by a non-profit public health research organization. Voluntary participants, who reside in 57 countries worldwide (91% in the United States or Canada) submitted home blood spot 25(OH)D test kits and completed online health questionnaires. There were no exclusion criteria nor any requirements related to 25(OH)D concentration or supplement intake dose. Participants included both genders and a wide range of ages; however, only female participants aged 55 years or older who were free of known cancer at enrollment and within the prior 10 years who completed at least two health assessments were included in this pooled analysis to match the inclusion criteria of the Lappe cohorts. As described previously [20], cancer diagnosis dates and cancer type were reported as were average daily calcium supplement intake, age, smoking status, height, and weight. Serum 25(OH)D concentrations were determined by analysis of dried blood spot test kits using liquid chromatography-mass spectroscopy (LC-MS/MS) by ZRT Laboratory (Beaverton, OR) or Purity Laboratory (Lake Oswego, OR). The intra-assay coefficient of variation was 9% for ZRT and 5% for Purity. Additionally, the ZRT and Purity assays have been validated against the DEQAS LC-MS/MS consensus group (R^2 values of 0.998 and 0.994 respectively). LC-MS/MS with dried blood spot cards has been validated against the radioimmunoassay method (R^2 value of 0.91 and a slope not different from 1.0) [23]. All participants provided informed consent and the study was approved by the Western Institutional Review Board (Olympia, WA).

Overall, this analysis included 1129 women from the 2007 Lappe cohort (median follow-up time, 4.0 years), 2196 women from the 2017 Lappe cohort (median follow-up time, 4.0 years), and 1713 women from the GrassrootsHealth cohort (median follow-up time, 1.9 years) (pooled cohort N = 5038; median follow-up time, 4.0 years).

Statistical methods

Demographic characteristics were summarized and comparisons between cohorts were performed using Kruskal-Wallis tests for age, BMI, calcium supplement intake (study and non-study combined for Lappe cohorts), and serum 25(OH)D. The chi-square test was used for smoking status. While data was collected for all types of cancer diagnoses, the outcome of interest for this current study was the diagnosis of breast cancer (invasive or in situ) during the observation periods. Age-adjusted breast cancer incidence rates were calculated using direct standardization to the 2010 US population [24].

Three analyses were conducted to investigate the relationship between 25(OH)D concentration and breast cancer. First, breast cancer incidence rates and their 95% confidence intervals (95% CI) were calculated for successive 20 ng/ml strata of serum 25(OH)D concentration from <20 to ≥60 ng/ml using a moving average method [25–27] to assess incidence trends across the range of 25(OH)D. A rate ratio (incidence density ratio) for <20 vs ≥60 was calculated to compare incidence rates.

Second, Kaplan-Meier curves comparing the proportion of breast cancer-free participants by 25(OH)D group were developed to estimate breast cancer-free survival over time and account for varying lengths of follow-up. Four a priori categories of 25(OH)D were used: <20 ng/ml, 20–39 ng/ml, 40–59 ng/ml, and ≥60 ng/ml. The 20 ng/ml cut point is from the National Academy of Medicine (NAM, formerly Institute of Medicine) recommendation for bone health [28], the 40 ng/ml cut point is from articles recommending this concentration for the prevention of cancer [29–33], and the 60 ng/ml cut point is from the Lowe et al. study showing reduced breast cancer risk above this concentration [7] and is the top end of the range recommended by a consortium of scientists and physicians to prevent many diseases including breast cancer [29]. Participants were allowed to move between strata of 25(OH)D according to changes in 25(OH)D concentration over the course of the observation periods.

Third, multivariate Cox regression was used to quantify the association between serum 25(OH)D and the risk of breast cancer after adjusting for the following breast cancer risk factors: age, BMI, smoking status, and calcium supplement intake. Indicator variables for study of origin were included to adjust for differences in study methods and demographics. Serum 25(OH)D concentration was assessed as a categorical variable (<20 ng/ml, 20–39 ng/ml, 40–59 ng/ml, and ≥60 ng/ml), as was calcium supplement intake (<1000 mg/day vs ≥1000 mg/day) based on the NAM recommendation for bone health [28]. Serum 25(OH)D concentration and calcium supplement intake changed during the course of the studies for most participants; therefore, these variables were entered as time varying covariates (multiple values were used for each participant to allow for changes in status over time). Age and BMI at baseline were entered as continuous variables and smoking status at baseline was entered as a categorical variable for "current smoker" (yes/no). Since breast cancers diagnosed in the first year were likely present but undiagnosed at study entry, multivariate Cox regression was repeated including only participants free of breast cancer at one year. Additionally, restricted cubic splines with three knots in default locations were used to assess the nature of the association between 25(OH)D (as a continuous variable) and cancer risk, including possible increased risk in the upper serum concentrations. Analyses and graphics were done with the R software (www.r-project.org).

Results

Baseline demographic characteristics of the pooled and individual cohorts are shown in Table 1. The GrassrootsHealth cohort had a lower median age, BMI, and calcium supplement intake and a lower proportion of participants who were current smokers than either Lappe

Table 1. Characteristics of the pooled, 2007 Lappe, 2017 Lappe, and GrassrootsHealth cohorts.

	Pooled cohort (N = 5038)	2007 Lappe cohort (N = 1129)	2017 Lappe cohort (N = 2196)	GrassrootsHealth cohort (N = 1713)	*P*-value[a]
Age (years): median (IQR[b])	63 (59–69)	66 (60–71)	63 (59–69)	61 (57–66)	<0.0001
BMI: median (IQR[b])	27 (23–32)	28 (25–32)	29 (25–33)	24 (21–28)	<0.0001
Smoking status: N (%)					<0.0001
Current smoker	272 (5%)	104 (9%)	130 (6%)	38 (2%)	
Never or former smoker	4765 (95%)	1025 (91%)	2066 (94%)	1674 (98%)	
Calcium supplement intake: median (IQR[b])	600 (91–1271)	1176 (483–1616)	825 (373–1448)	100 (0–600)	<0.0001
Serum 25(OH)D (ng/ml): median (IQR[b])					
Baseline	34 (27–43)	28 (23–34)	33 (26–39)	43 (33–58)	<0.0001
Most recent[c]:	38 (29–50)	31 (24–39)	36 (29–46)	49 (37–64)	<0.0001

[a]Statistical comparison of characteristics between the 2007 Lappe, 2017 Lappe, and GrassrootsHealth cohorts. Age, BMI, calcium supplement intake, and serum 25(OH)D concentration were compared using Kruskal-Wallis tests. Smoking status was compared using chi-square test. All risk factors were significantly different ($P<0.0001$) between cohorts and were included in the multivariate Cox regression model to account for these differences.

[b]IQR, interquartile range.

[c]Most recent measurement prior to end of observation (or diagnosis for cases).

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cohort. The 2007 Lappe cohort had the lowest baseline median serum 25(OH)D concentration (28 ng/ml) and the GrassrootsHealth cohort had the highest (43 ng/ml).

During the observation periods, 77 women in the pooled cohort were diagnosed with breast cancer (19 from the 2007 Lappe cohort, 44 from the 2017 Lappe cohort, and 14 from the GrassrootsHealth cohort). The age-adjusted incidence rate of breast cancer was 512 cases per 100,000 person-years in the pooled cohort (458 cases per 100,000 person-years in the 2007 Lappe cohort, 619 cases per 100,000 person-years in the 2017 Lappe cohort, and 337 cases per 100,000 person-years in the GrassrootsHealth cohort).

Within the pooled cohort, results were similar for the three analyses used to investigate the relationship between 25(OH)D concentration and breast cancer (incidence rate comparison, Kaplan-Meier plot, and multivariate Cox regression). First, breast cancer incidence rates according to 25(OH)D group are shown in Fig 1. Rates were lower with higher serum 25(OH)D categories (Fig 1). Comparing incidence rates, there was an 82% lower incidence rate of breast cancer for ≥60 ng/ml vs <20 ng/ml (Rate Ratio = 0.18, 95% CI: 0.04–0.62, $P = 0.006$).

Second, Kaplan-Meier curves comparing the proportion of breast cancer-free participants by 25(OH)D group are shown in Fig 2. These curves were significantly different ($P = 0.02$), with the highest proportion breast cancer-free at 4 years in the ≥60 ng/ml group (99.3%) and the lowest proportion breast cancer-free in the <20 ng/ml group (96.8%). The proportion with breast cancer was 78% lower for ≥60 ng/ml vs <20 ng/ml ($P = 0.02$) in the Kaplan-Meier analysis.

Third, the results of multivariate Cox regression are shown in Table 2 and Fig 3. Women with 25(OH)D concentrations ≥60 ng/ml had an 80% lower risk of breast cancer compared to women with concentrations <20 ng/ml (HR = 0.20, $P = 0.03$), adjusting for age, BMI, smoking status, calcium supplement intake, and study of origin (Table 2). The dose-response decrease in breast cancer risk for women with 25(OH)D concentrations of 20–39 ng/ml and 40–59 ng/ml vs <20 ng/ml are shown in Table 2. Age, BMI, smoking status, calcium supplement intake, and study of origin were not significant predictors of breast cancer risk in this pooled cohort. Among women free of breast cancer at one year (N = 4406), those with 25(OH)D concentrations ≥60 ng/ml had a 93% lower risk of breast cancer compared to women with concentrations <20 ng/ml (HR = 0.07, $P = 0.02$). Spline regression with 25(OH)D as a continuous

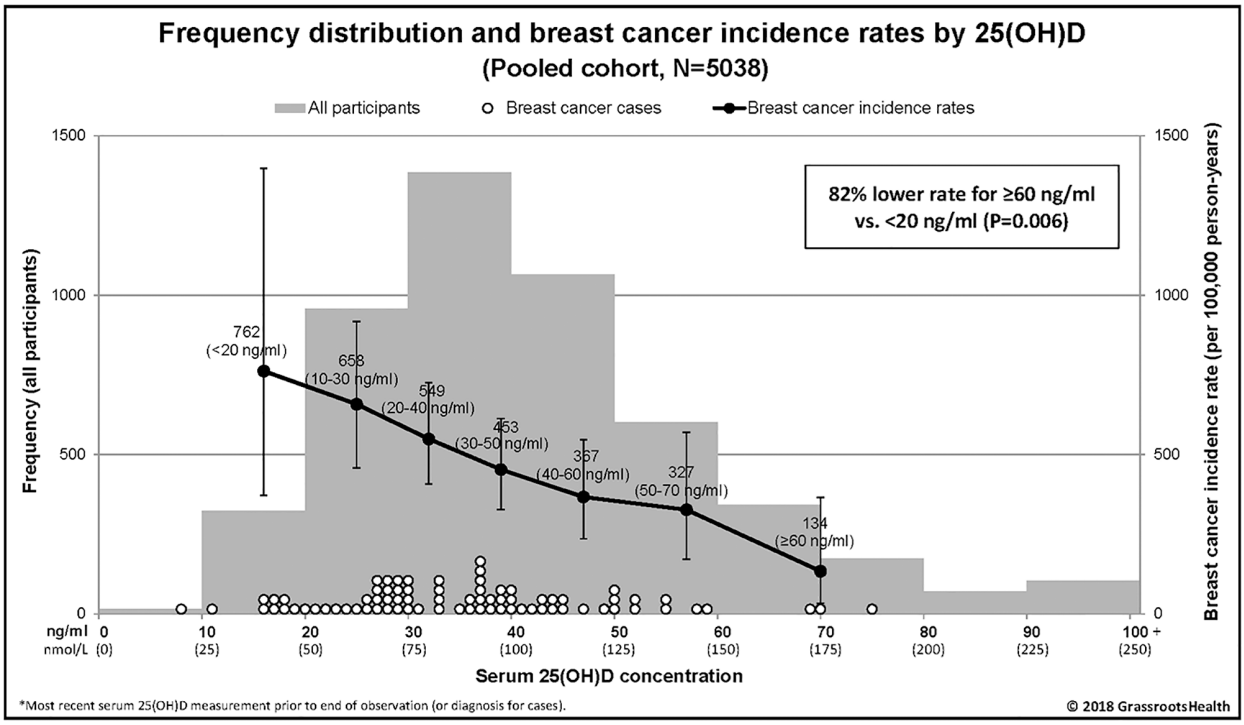

Fig 1. Frequency distribution and breast cancer incidence rates by 25(OH)D concentration, pooled cohort (N = 5038). The bars represent the number of participants by groupings of 10 ng/ml (left y-axis), white dots represent the 25(OH)D concentration for each breast cancer case, black dots represent breast cancer incidence rates per 100,000 person-years for each 25(OH)D group (plotted at the median value for each group: 16, 25, 32, 39, 47, 57, and 70 ng/ml) (right y-axis). Vertical error bars represent the 95% confidence intervals.

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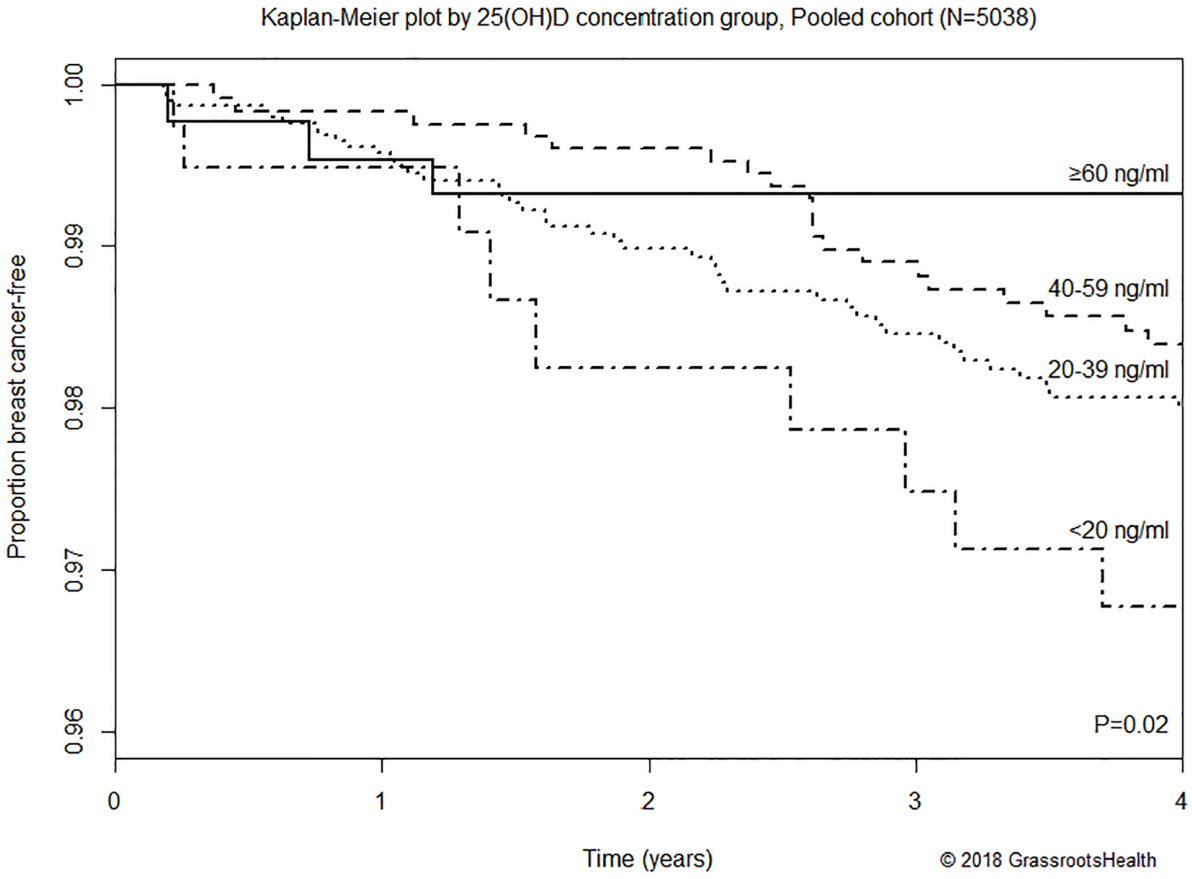
variable revealed consistently lower risk of breast cancer with higher 25(OH)D concentration, with no evidence of increased risk in the higher 25(OH)D concentrations (Fig 3).

Sensitivity analyses were conducted using 25(OH)D concentration quartiles, baseline 25(OH)D concentration only, excluding non-US residents in the GrassrootsHealth cohort, and for each individual cohort. All revealed lower risk of breast cancer with higher 25(OH)D concentration.

Discussion

In this pooled cohort, 25(OH)D concentration was significantly inversely associated with breast cancer risk. All three analyses showed that women with 25(OH)D concentrations ≥60 ng/ml had significantly lower risk of breast cancer (~80%) compared to women with concentrations <20 ng/ml. There was a consistent decrease in breast cancer risk as 25(OH)D concentrations increased, with no evidence of increased risk in higher concentrations. Using a pooled cohort allowed for analysis across a wider range of serum 25(OH)D concentrations than any of the cohorts alone. While a novel approach, similar inclusion criteria were used for all three cohorts and analyses were adjusted for study of origin and breast cancer risk factors to account for differences in methodology and demographics.

The findings from this analysis support the previously reported inverse association between 25(OH)D and risk of breast cancer [7–18]. Another study assessed breast cancer risk across a broad 25(OH)D concentration range with similar findings [7]. In that hospital-based case control study, Lowe et al. found that women with 25(OH)D concentrations >60 ng/ml had an



Participants at risk:

<20 ng/ml	390	285	231	260	262
20-39 ng/ml	3034	2510	2033	1819	1706
30-59 ng/ml	1181	1198	1318	1231	1121
≥60 ng/ml	433	425	382	367	315
Total	5038	4418	3964	3677	3404

Fig 2. Kaplan-Meier plot comparing the proportion of breast cancer-free participants by 25(OH)D concentration, pooled cohort (N = 5038). Participants were allowed to move between strata of 25(OH)D according to changes in 25(OH)D concentration over the course of the observation periods. Four-year cumulative breast cancer-free proportion was 99.3% among participants with 25(OH)D concentrations ≥60 ng/ml compared to 96.8% for those with 25(OH)D concentrations <20 ng/ml (the proportion with breast cancer was 78% lower for ≥60 ng/ml vs <20 ng/ml, $P = 0.02$).

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83% lower risk of breast cancer than women with concentrations <20 ng/ml ($P<0.001$) [7]. The present study replicated these findings in a much larger, population-based study, thus increasing generalizability, and it's prospective design enabled use of 25(OH)D values before diagnosis to distinguish between cause and effect.

The Women's Health Initiative (WHI) trial did not find an association between assigned vitamin D treatment group and breast cancer risk [34]; however, low dosage (400 IU/day) and poor compliance (~50%) likely contributed to the lack of effect. A subsequent re-analysis of the WHI data showed a significant reduction in breast cancer risk among women not taking a vitamin D or calcium supplement before enrollment [35]. A few other nested case-control studies have found no effect [36–38]. Those studies used a single 25(OH)D measurement at enrollment to predict cancer risk over a long follow-up period. That study design does not accommodate changes in vitamin D status over time and diminishes the predictive value of

Table 2. Association between serum 25(OH)D and risk of breast cancer, pooled cohort (N = 5038).

	Hazard ratio (95% CI), adjusted for study of origin	*P*-value	Hazard ratio (95% CI), adjusted for study of origin and other covariates[a]	*P*-value
Serum 25(OH)D				
<20 ng/ml (<50 nmol/L)	Reference		Reference	
20–39 ng/ml (50–99 nmol/L)	0.61 (0.30,1.26)	0.19	0.55 (0.26,1.16)	0.12
40–59 ng/ml (100–149 nmol/L)	0.52 (0.24,1.16)	0.11	0.48 (0.20,1.14)	0.10
≥60 ng/ml (≥150 nmol/L)	**0.21 (0.05,0.85)**	**0.03**	**0.20 (0.05,0.82)**	**0.03**
P-value for trend		**0.03**		**0.04**

Bold values signify significant hazard ratios.

[a]Age, BMI, smoking status, and calcium supplement intake.

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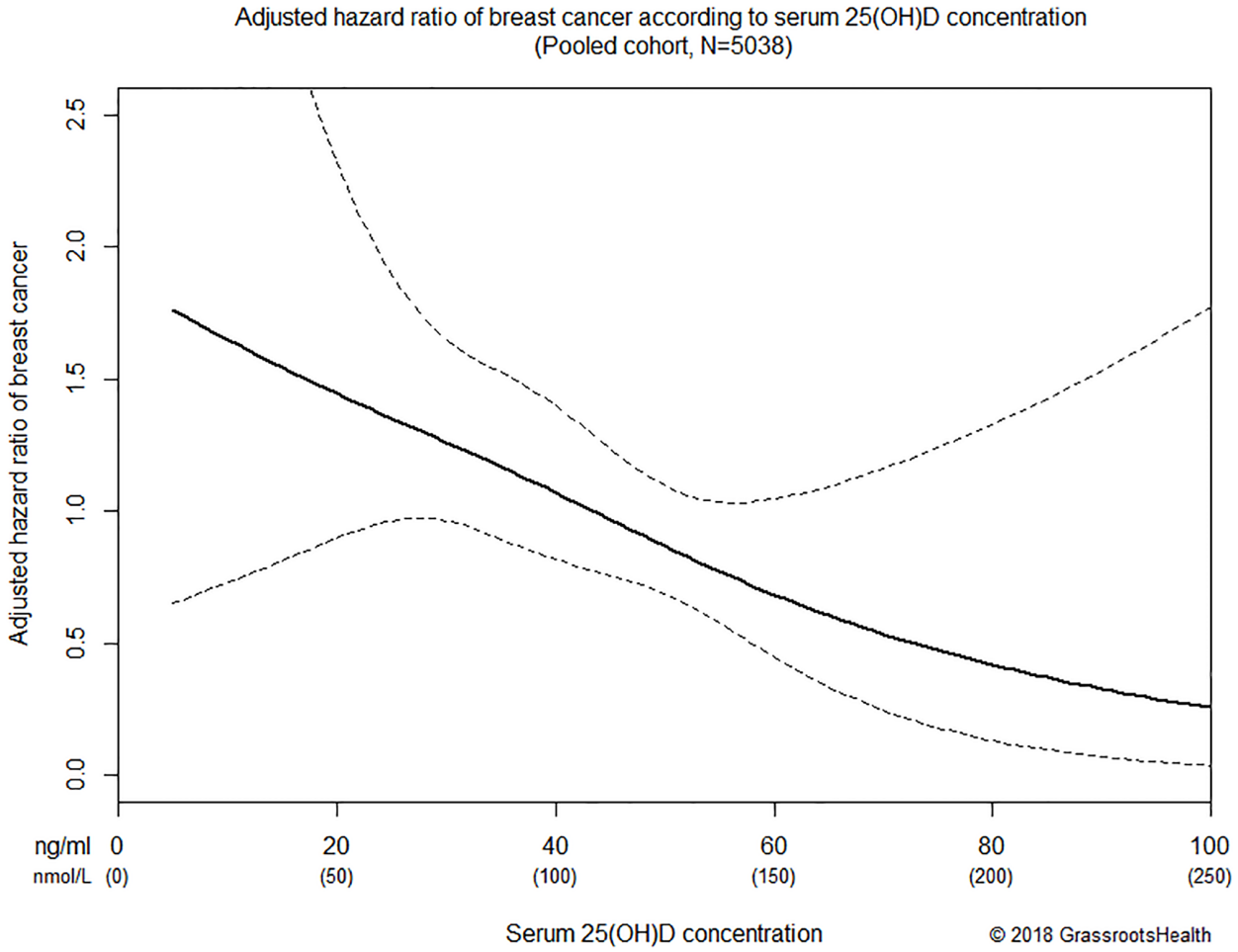

Fig 3. Association between serum 25(OH)D (as a continuous variable) and risk of breast cancer adjusted for age, BMI, smoking status, calcium supplement intake, and study of origin in the range of ≤100 ng/ml, pooled cohort (N = 5308). Solid black line represents the estimated hazard ratio for the Cox regression model with restricted cubic splines with three knots; dashed lines represent the 95% confidence interval of the estimate.

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the pre-diagnostic 25(OH)D measurement. Grant has shown that the magnitude and significance level for the relationship between 25(OH)D concentration and breast cancer risk are inversely related to the length of follow-up [39]. In this study, we performed a sensitivity analysis using only baseline 25(OH)D concentration (rather than multiple 25(OH)D values as a time varying covariate) and found a weaker association, also highlighting the diminished predictive value of 25(OH)D concentrations measured long before diagnosis.

Vitamin D may play a number of roles in the prevention of breast cancer development and progression. The biologically active form of vitamin D, $1,25(OH)_2D3$, binds to the vitamin D receptor (VDR) in normal breast epithelium and this complex regulates the cell cycle, promotes differentiation, increases cell-to-cell adhesion, protects cells from DNA damage, regulates cytokines, activates immune cells, and suppresses inflammation, all of which may act to reduce malignant transformations [6]. In breast cancer cells, this complex also activates apoptosis and other mechanisms to suppress tumor growth [6]. Additionally, other vitamin D metabolites from recently discovered alternative pathways, such as 20(OH)D3 from the CYP11A1-mediated metabolism of vitamin D, have been found to have preventive effects similar to $1,25(OH)_2D3$ [40–42]. Studies with respect to cancer treatment have demonstrated vitamin D's ability to degrade neoplasm [43] and detailed genomics have shown the profound effects vitamin D has on established neoplastic tissue [44]. These mechanisms of vitamin D action provide a possible biological explanation for a causal association between 25(OH)D and breast cancer risk and highlight the importance of assessing this association by the concentration of vitamin D metabolites in the serum and not by indirect measures such as treatment group or supplement intake amount which tend to be inadequate and prone to bias.

Whether our findings reflect prevention of the primary tumor or treatment of early stage, undiagnosed cancer by vitamin D is not clear. Of interest, the results for women who were followed and free of breast cancer at the end of the first year revealed a stronger association between 25(OH)D concentration and breast cancer risk (HR: 0.07, $P = 0.02$ for $\geq$60 vs <20 ng/ml). There was only one case of breast cancer diagnosed after one year among those with 25(OH)D concentrations $\geq$60 ng/ml. This woman's diagnosis occurred 2 months into year two. Since there is a time delay between cancer initiation and diagnosis, many undiagnosed cancers that existed at enrollment would be diagnosed during the first year. Therefore, it is possible that analyses among women free of breast cancer at one year would better assess vitamin D's specific role in prevention rather than prevention and tumor arrest combined.

While the associations between breast cancer risk and age and calcium supplement intake were in the expected directions (higher risk with increased age and lower risk with higher calcium supplement intake), the effects of these risk factors did not reach statistical significance in this analysis. Since an inclusion criterion for these cohorts was age 55 years and older, the exclusion of younger women may have diminished the effect of age in this analysis. If younger women were included in this study we would expect to see a significant increase in breast cancer risk with age and possibly the effect of age-related changes in vitamin D metabolism. Also, information on dietary calcium intake was not available for the GrassrootsHealth cohort so this analysis only assessed supplemental calcium intake. However, it is possible that dietary calcium intake or total calcium intake would have been a significant predictor of breast cancer risk. Additionally, the small proportion of current smokers may have limited the ability of this study to detect an association between smoking status and breast cancer.

Strengths of this analysis include using a wider range of 25(OH)D concentrations than most other studies and employing multiple analysis techniques with findings of a similar magnitude. Also, using serum 25(OH)D concentration is a better indicator of vitamin D status and statistically more powerful than using treatment group or intake amount because it captures the effect of multiple vitamin D input sources (supplement, sun, and food), overcomes the

inherent bias of treatment compliance, and accounts for inter-individual variability in dose response [45]. All three cohorts participated in well-designed population-based studies that included multiple measurements of serum 25(OH)D, allowing for changes in vitamin D status over the course of the observation periods. Using multiple 25(OH)D measurements also overcomes the issue of diminished predictive value of 25(OH)D measurements at enrollment over long follow-up periods. The median amount of time between the 25(OH)D measurement prior to diagnosis and the date of diagnosis was fairly short (~6 months).

Limitations of the analysis include the possible lack of generalizability to younger women and men. However, since other studies have found a significant association between higher 25(OH)D concentrations and lower breast cancer risk in younger women [10–13,18], we would expect that this inverse association is applicable to women of all ages. Also, while there were no ethnic inclusion criteria, the vast majority of participants were non-hispanic white (100% in the 2007 Lappe cohort, 99% in the 2017 Lappe cohort, and 96% in the GrassrootsHealth cohort) so these results may not be generalizable to persons of other ethnicities. While inclusion criteria were matched across cohorts and analyses were adjusted for study of origin and breast cancer risk factors, differences in demographics and methods (e.g. study design, recruitment, and data collection tools) between the cohorts may have affected pooled analyses. Median follow-up time was longer for the Lappe RCTs than the GrassrootsHealth prospective cohort; however, all rate calculations used person-time denominators and analyses accounted for varying lengths of follow-up. Additional limitations include the use of self-reported data and not being able to control for some risk factors (family history of breast cancer, diet, and estrogen use).

The current NAM recommendation of 20 ng/ml (50 nmol/L) is based solely on bone health [28], yet it is widely used as the target level for all health conditions. The findings from this study suggest that breast cancer incidence could be substantially reduced by increasing 25(OH)D concentrations well above 20 ng/ml (50 nmol/L). Fig 3 shows tight confidence bands from about 30 to 55 ng/ml, which represents a decrease in breast cancer risk of ~38%. The high end of that range, 55 ng/ml, falls within the 40 to 60 ng/ml range recommended by a consortium of scientists and physicians to prevent several diseases including breast cancer [29]. Serum 25(OH)D concentrations between 40–60 ng/ml (100–150 nmol/L) are within the physiological range, as evidenced by traditionally living Africans who have a mean 25(OH)D concentration of 46 ng/ml (range: 23–68) with 62% having concentrations between 40–60 ng/ml [46]. In the range of 60 to 100 ng/ml, the downward trend continues. The widened confidence bands stem from the decreasing number of women with 25(OH)D concentrations in this upper range. Clarifying the nature of the association in this upper range should be a high priority for future investigations.

Focusing on primary prevention and implementing evidence-based interventions is needed to substantially decrease breast cancer incidence and associated mortality and economic costs. The national cost of female breast cancer in 2010 was estimated to be $16.5 billion [47]. If women raised their 25(OH)D concentration from their current mean of approximately 30 ng/ml [48] to 55 ng/ml, the analysis from this study suggests that more than $6 billion could be saved every year in the United States. Vitamin D status is a modifiable risk factor for breast cancer, and increasing 25(OH)D concentrations via supplementation at the population level is safe and affordable.

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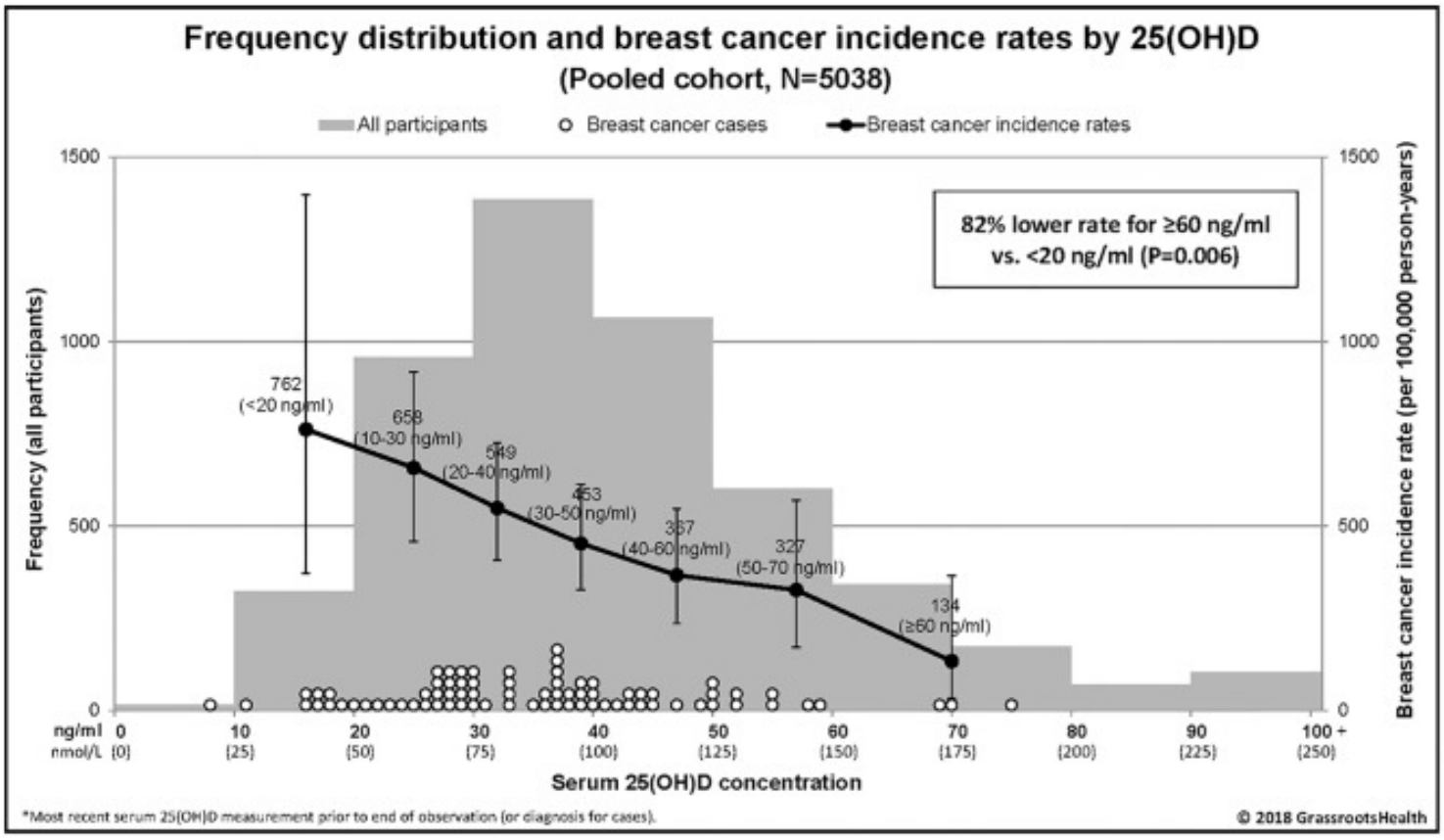
PLoS One. 2018; 13(6): e0199265. PMID: 29906273

Breast cancer risk markedly lower with serum 25-hydroxyvitamin D concentrations ≥60 vs <20 ng/ml (150 vs 50 nmol/L): Pooled analysis of two randomized trials and a prospective cohort



Plasma Vitamin D Levels, Menopause, and Risk of Breast Cancer

Dose-Response Meta-Analysis of Prospective Studies

Scott R. Bauer, ScM, Susan E. Hankinson, ScD, Elizabeth R. Bertone-Johnson, ScD, and Eric L. Ding, ScD

Abstract: Previous evidence suggests that higher circulating 25-hydroxyvitamin D (25[OH]D) levels are variably associated with lower breast cancer risk; however, prospective studies and clinical trials have been inconsistent, particularly between older and younger women of differing menopausal status. We conducted a quantitative nonlinear dose-response meta-analysis of prospective studies evaluating the association between circulating 25(OH)D and breast cancer risk, stratified by menopause. A systematic search of MEDLINE and EMBASE included studies published through May 2011. We reviewed references from retrieved articles and contacted relevant investigators for additional data from prospective studies on circulating 25(OH)D levels and incident breast cancers. Prospective studies of circulating vitamin D and breast cancer risk were reviewed, and no language restrictions were imposed. Information on study population, menopausal status, 25(OH)D levels, and relative risk (RR) estimates were extracted using a standardized protocol.

A total of 9 prospective studies were included, comprising 5206 cases and 6450 controls. Data were pooled using dose-response random-effects meta-regression models. Identifying nonlinear effects, spline models were optimized for thresholds. The relationship between circulating 25(OH)D and breast cancer risk differed by menopausal status ($p = 0.05$ for effect modification). While no association was found in premenopausal women, dose-response modeling revealed a nonlinear inverse association among postmenopausal women. Notably, a flat association was observed in the lowest range of 25(OH)D levels <27 ng/mL (RR = 1.01 per 5 ng/mL; 95% confidence interval [CI], 0.98–1.04). In contrast, postmenopausal breast cancer risk decreased with 25(OH)D levels 27-<35 ng/mL ($p = 0.02$ for nonlinear risk change), where a 5 ng/mL increase in 25(OH)D was associated with a 12% lower risk of breast cancer (RR = 0.88 per 5 ng/mL; 95% CI, 0.79–0.97), with suggestive flattening at higher doses >35 ng/mL. The significant inverse association did not appear to vary across strata of invasive/in-situ cases, body mass index adjustment, region, postmenopausal hormone use, or assay method.

In summary, this dose-response meta-analysis of prospective studies of plasma 25(OH)D suggested a breast cancer risk differential by menopause, whereby a step-wise inverse association was observed beyond a threshold of 27 ng/mL, but with flattening of effects above 35 ng/mL, in postmenopausal women. These findings help resolve prior inconsistent findings and may carry important clinical and public health implications.

(*Medicine* 2013;92: 123–131)

Abbreviations: 25(OH)D = 25-hydroxyvitamin D, BMI = body mass index, CI = confidence interval, IOM = Institute of Medicine, MOOSE = Meta-analysis Of Observational Studies in Epidemiology, PLCO = Prostate, Lung, Colorectal and Ovarian Cancer Screening trial, RR = relative risk, VITAL = VITamin D and OmegA-3 TriaL, WHI = Women's Health Initiative.

INTRODUCTION

Breast cancer is a leading cause of mortality in women.[3] Although a number of breast cancer risk factors are well established (for example, family history, breast density, parity, alcohol use), very few are readily modifiable. Low circulating vitamin D levels below 30 ng/mL were found in 77% of the United States population from 2000 to 2004, paralleling the increased trend of vitamin D deficiency in the last 2 decades.[30] Factors associated with lower circulating 25-hydroxyvitamin D (25[OH]D) levels include obesity, low physical activity, higher geographic latitude (marker of ultraviolet-B exposure), age, race, skin type, and smoking.[12,13,41,46,52] More importantly, circulating 25(OH)D, the best marker of vitamin D status,[38,69] is easily modifiable with 1000 IU of daily vitamin D intake increasing circulating 25(OH)D by 10 ng/mL.[37]

Preclinical experimental evidence and previous retrospective studies have suggested that vitamin D intake and higher circulating vitamin D levels may be protective against cancer,[7,8,31,66] potentially via regulation of cell division, apoptosis, and contact inhibition.[39] Vitamin D may also partially mediate the observed association between physical activity and breast cancer risk through sunlight exposure.[21,29,52] However, prospective studies in humans have been inconsistent. For example, in 2 recent studies, 1 study found no association for 25(OH)D and breast cancer risk,[2] while another found a strong inverse association.[58] Although an inverse association was also found in the Nurses' Health Study,[9] the largest prospective study to date from the Prostate, Lung, Colorectal and Ovarian Cancer Screening trial (PLCO) again found no association.[24] Furthermore, results from 3 previous meta-analyses were also inconsistent, with 2 of the studies reporting no evidence for a dose-response relationship,[14,26,68] and none of the previous studies accounted for menopause status.

Differences in study population, particularly menopausal status and the range of circulating 25(OH)D levels, may potentially account for some of these inconsistencies in observational studies. Moreover, most previous investigations only considered linear trends and compared extreme quantiles, without evaluating possible nonlinear dose-response relations or heterogeneity of baseline vitamin D levels across diverse populations.

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Therefore, to assess the dose-response relationship between circulating 25(OH)D and breast cancer risk comprehensively, we conducted a systematic review and meta-analysis of the prospective literature, particularly focusing on differences between pre- and postmenopausal women as well as potential nonlinear associations for risk of breast cancer. (See also the accompanying commentary on this study by Stearns and Visvanathan[62a] in this same issue.)

METHODS

Study Selection

We conducted a comprehensive literature search of MEDLINE (National Library of Medicine, Bethesda, MD) and EMBASE (Elsevier, Amsterdam, The Netherlands) from 1966 through May 2011. We followed the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines for searching and reporting. Search terms included MESH, EmTree, title/abstract, and synonyms of *breast cancer* combined with *vitamin D, 25-hydroxyvitamin D,* or *calcifediol.* Additional studies were searched for via references of retrieved articles, direct author contact for unpublished data, and referral by experts in the field. Studies were excluded if they did not fulfill the following criteria: a) human studies, b) prospective cohort and nested case-control studies, c) measured circulating (serum/plasma) 25(OH)D at baseline, d) reported a relative risk (RR) or odds ratio and confidence interval (CI) per vitamin D category, e) reported outcome of breast cancer risk. No language restrictions were imposed. Incident breast cancer was analyzed as the outcome of interest due to varying screening and treatments by country. In the first round of screening abstracts (n = 974), 938 articles were excluded by search criteria (Figure 1). In a second round of screening full text articles (n = 36), 27 articles were excluded: not prospective (11 articles), circulating 25(OH)D not measured (6 articles), survival among cancer cohort (5 articles), duplicate studies (3 articles), and case report (2 articles). Our search criteria yielded 9 total prospective case control studies, comprising 5206 incident cases and 6450 controls (Table 1).

Data Extraction

Data from these studies were tabulated using a standardized extraction form. Discrepancies were resolved via group discussion and review. Information extracted included lead author; publication year; population; country of origin; menopausal status; study design; average length and/or range of follow-up; number of cases and controls by quantile; adjustment for body mass index (BMI) or physical activity; mean age; 25(OHD) assay; mean/median/range of circulating 25(OH)D levels by quantile; RRs and standard error of breast cancer risk by quantile. When RR estimates were reported for more than 1 set of adjustments, we selected the most adjusted estimate.

We requested additional data via personal communications from authors of all studies in order to conduct thorough dose-response analysis and stratified analyses by menopausal status, current postmenopausal hormone use, and tumor characteristics (3 provided data by quantile, 1 provided stratified estimates and data by batch, 6 provided stratified data by menopausal status, and 5 provided stratified data by use of hormone replacement therapy). Only 1 author (Chlebowski) of the 9 contacted study authors did not provide additional de novo data. We further obtained detailed batch and subcohort data from the Nurses' Health Study I cohort (Appendix 1). Follow-up in the originally published Rejnmark study averaged only 3 months, hence subclinical influences on vitamin D levels could not be ruled out in the original report. Thus, Rejnmark (personal communication) provided an updated analysis restricted to cases diagnosed >1 year after blood draw.

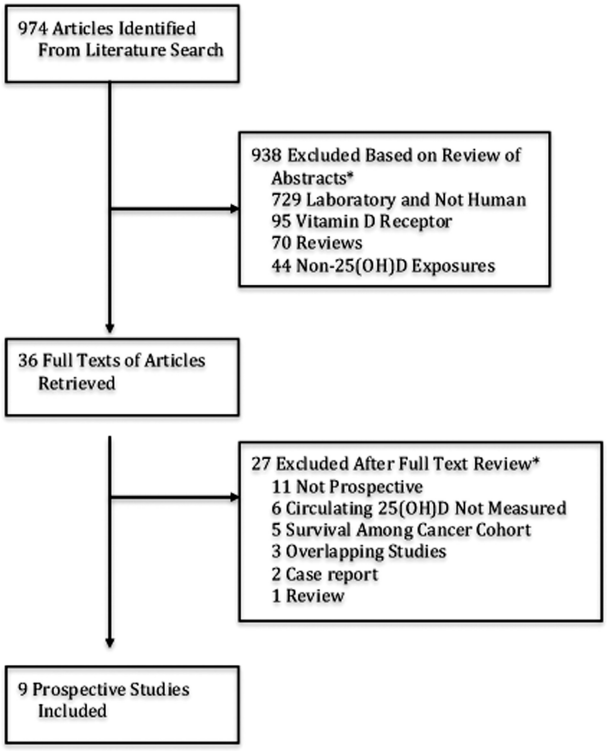

FIGURE 1. Summary of article selection process. *Studies belonging to multiple classifications were counted only once.

Vitamin D Measurements

Both immunoassay and liquid chromatography methods were used to assess circulating 25(OH)D levels. For stratified analyses, assay categories included radioimmunoassay (RIA) or chemiluminescent immunoassay (CIA) and high pressure liquid chromatography (HPLC) or isotope dilution liquid chromatography-tandem mass spectrometry. Plasma[9,23,58] and serum[1,2,15,24,50] are comparable mediums to measure circulating 25(OH)D, thus, we use circulating 25(OH)D to refer to both mediums.

Statistical Analysis

We calculated the RR as a pooled measure of the association between circulating 25(OH)D levels and breast cancer risk using both highest versus lowest category and a dose-response meta-regression analysis. A random-effects meta-regression trend estimation of summarized dose-response data, described by Greenland and Longnecker,[34,54] was used to derive the incremental dose-response RRs between circulating 25(OH)D levels and breast cancer risk. The continuous linear scale increment for the trend-estimated RR was 5 ng/mL in circulating 25(OH)D. Apparent nonlinear associations were statistically analyzed using dose-response GLST (Generalized Least-Square Trend) meta-regression and spline analysis for change in slope at specified knot-points; splined variables were created using MKSPLINE in STATA (StataCorp, College Station, TX). Goodness of fit tests and comparative chi-square statistics were subsequently used to optimize the knot-points in spline regressions and to test robustness of spline knots. Based on prior literature, test of effect

TABLE 1. Characteristics of Studies Included in the Meta-Analysis of Circulating Vitamin D and Breast Cancer Risk, Ordered by Year of Publication and Alphabetically

Source (First Author, ref.)	Year	Study Population	Country	25(OH)D Assay	25(OH)D in Controls Mean (SD) ng/mL†	Breast Cancer Outcome	No. Cases	Postmenopausal (%)	Mean Follow-Up (or Range)	Mean Age (yr)	Adjusted for BMI	Adjusted for PA
Bertone-Johnson[9]	2005	Nurses' Health Study	USA	IA*	33.1	Total, in situ, invasive	701	68	(1 mo-6.8 yr)	57	Yes	Non-influential
Chlebowski[15]	2008	Women's Health Initiative	USA	IA**	20.8 (8.4)	Invasive	895	100	7.0 yr	63	Yes	Yes
Freedman[24]	2008	PLCO	USA	IA*	26.2	Total, in situ, invasive	1005	100	3.9 yr	62	Yes (age 18–20)	No
McCullough[50]	2009	Cancer Prevention Study-II	USA	IA**	22.5 (8.9)	Total, in situ, invasive	516	100	(1 mo–6.9 yr)	70	Yes	No
Rejnmark[58]	2009	Danish women	Denmark	LC**	30.4 (11.2)	Total	9	66	(>1 yr)	58	Non-influential	Non-influential
Agborsangaya[1]	2010	Pregnant Finnish women	Finland	IA*	17.0	Total	100	0	7.4 yr	31	No	No
Almquist[2]	2010	Malmo Diet and Cancer Study	Sweden	LC*	35.5	Total, invasive	752	74	7.0 yr	57	Yes	No
Engel[23a]	2010	French E3N cohort	France	IA**	25.1 (11.0)	Invasive	615	77	(<1 yr–10 yr)	57	Yes	Yes
Eliassen[23]	2011	Nurses' Health Study II	USA	IA*	25.0 (9.6)	Total, invasive	613	0	4.8 yr	45	Yes	Non-influential

Abbreviations: IA = immunoassay (includes radioimmunoassay* and chemiluminescent immunoassay**), LC = liquid chromatography (includes high pressure liquid chromatography-tandem mass spectrometry* and isotope dilution liquid chromatography-tandem mass spectrometry**), PA = physical activity, SD = standard deviation.

†1 ng/mL = 2.5 nmol/L.

modification by menopausal status was determined a priori. Additionally, stratified meta-regressions were conducted to determine whether differences in tumor invasiveness, mean age, assay, country, mean 25(OH)D levels, or adjustment for BMI and physical activity influenced associations and explained heterogeneity across studies.[64] Linear meta-regressions were conducted in sensitivity analyses using aggregate models, where effect estimates were combined from all studies before estimating the pooled linear dose-response. To assess the presence of publication bias, we assessed the symmetry of individual study linear dose-response slopes around the pooled estimate using Begg funnel plots.[19] All analyses were conducted using STATA 10 (StataCorp, College Station, TX); $p \leq 0.05$ was considered statistically significant.

Visual Assessments of Dose-Response Relations: *Ding Spaghetti Plot*

A novel meta-analytic visual representation method was developed by Eric L. Ding to aid in detecting nonlinear relationships between circulating 25(OH)D levels and breast cancer risk among postmenopausal women (Figure 2). The *Ding Spaghetti Plot* consists of connected study-series line plots of individual study RRs, where each "spaghetti noodle" represents a RR series from the same study; and data points are represented by circles, in which the relative size of each circle reflects the analytic weight of each RR estimate (although weighting does not affect the shape of the connected line plots). Thus, RRs with smaller standard errors (that is, relatively larger sample sizes) are represented by larger data points. The aggregate graphical visual representation, via the Ding Spaghetti Plot of all studies' dose-response "noodle" plots together, allows investigators to visually identify potential nonlinear associations and different dose-response curves from multiple data series across various studies. The centrally averaged pooled dose-response curve, highlighted as the main "noodle" in the Spaghetti Plot, represents the aggregate slope between knot-points. It is accompanied by upper and lower 95% CI bands that represent the uncertainty of the central pooled dose response curve.

RESULTS

A total of 9 prospective studies with 11 study sets were included, comprising 5206 incident cases of breast cancer and 6450 controls (see Table 1). Mean 25(OH)D concentrations ranged from 17.0 to 33.1 ng/mL. BMI was evaluated as a potential confounder in 8 of 9 studies, although adjustment for physical activity was considered less often (4 of 9 studies).

Evaluating the presence of a linear dose-response relationship, we observed a borderline statistically significant inverse association between circulating 25(OH)D and breast cancer risk (RR per 5 ng/mL = 0.99; 95% CI, 0.97–1.00; Table 2). However, menopausal status was a statistically significant effect modifier of this relationship ($p_{interaction} = 0.05$), where the inverse association between circulating 25(OH)D and breast cancer risk was limited to postmenopausal women (RR per 5 ng/mL = 0.97; 95% CI, 0.93–1.00). No dose-response relationship was observed among premenopausal women (RR per 5 ng/mL = 1.01; 95% CI, 0.98–1.04). This significant menopausal effect modification was confirmed via several analytic approaches: 2-stage pooling method ($p = 0.05$ for menopause effect), linear aggregate method ($p = 0.05$ for menopause effect), and nonlinear spline models ($p = 0.05$ for menopause effect).

In our primary analysis, analyzing 25(OH)D levels to carefully assess a dose-response, results indicated a significant inverse, nonlinear association between circulating 25(OH)D and breast cancer risk among postmenopausal women, with apparent

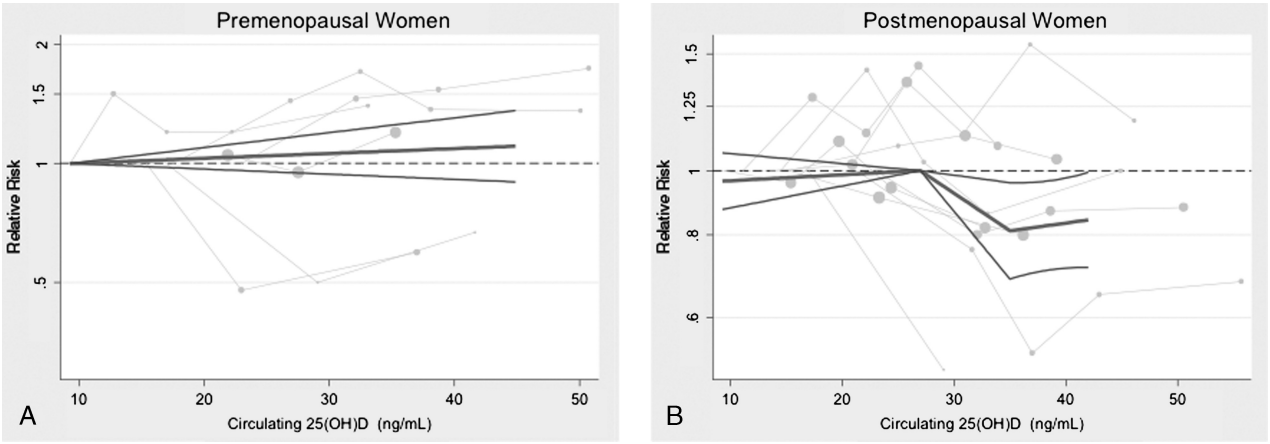

FIGURE 2. Ding Spaghetti Plot and pooled dose-response relationship between circulating 25(OH)D Levels and breast cancer risk, stratified by menopausal status (A, premenopausal, and B, postmenopausal women). The solid dark gray line represents the central pooled dose-response estimate, and the surrounding black lines represent 95% confidence interval bands. Each light gray ''spaghetti noodle'' represents a relative risk series from the same study; data points are represented by circles, with the relative size of each circle reflecting the analytic weight of each RR estimate.

Note: Quantitative RR for Figure 2:

Postmenopausal p value for nonlinear dose effect modification:
- at 27 ng/mL: p for nonlinear slope change = 0.02
- at 35 ng/mL: p for nonlinear slope change = 0.05

Point-specific RRs compared to 27 ng/mL (reference) among postmenopausal women:
- 35 ng/mL: RR = 0.81 (95% CI, 0.69–0.96), p = 0.01
- 40 ng/mL: RR = 0.83 (95% CI, 0.71–0.97), p = 0.02

Dose-response nonlinear slope RRs per 5 ng/mL increase in circulating 25(OH)D in postmenopausal women:
- <27 ng/mL range: RR per 5 ng/mL increase = 1.01 (95% CI, 0.98–1.04)
- 27–34 ng/mL range: RR per 5 ng/mL increase = 0.88 (95% CI, 0.79–0.97)
- 35–40 ng/mL range: RR per 5 ng/mL increase = 1.03 (95% CI, 0.94–1.12)

thresholds of 27 ng/mL (67 nmol/L) and 35 ng/mL (see Figure 2 and 3). Notably, while no dose-response relationship was observed among the lowest range of 25(OH)D levels <27 ng/mL (RR slope = 1.01 per 5 ng/mL; 95% CI, 0.98–1.04), higher 25(OH)D levels were associated with a reduced risk of breast cancer between 27 ng/mL and 35 ng/mL (RR slope = 0.88 per 5 ng/mL; 95% CI, 0.79–0.97), with a p for nonlinear risk change of 0.02 at 27 ng/mL. Furthermore, the reduction in risk somewhat flattened (p = 0.05 for nonlinear risk change) at highest levels ≥35 ng/mL (RR slope = 1.03 per 5 ng/mL; 95% CI, 0.94–1.12), yet remained at lower risk compared to 27 ng/mL. The nonlinear results were robust and relatively insensitive to changes in knot location. The point-specific RRs among postmenopausal women compared to a reference risk level of 27 ng/mL were RR = 0.81 (95% CI, 0.69–0.96) at 35 ng/mL, and RR = 0.83 (95% CI, 0.71–0.97) at 40 ng/mL. Moreover, effect modification by menopause was also confirmed in these spline models (p = 0.05), with no association in premenopausal women.

Parsimoniously modeling linear dose-response in subgroup analyses, the association did not appear to be modified by tumor classification, study mean circulating 25(OH)D, geographic region of the study cohort, assay type, or current postmenopausal hormone use (see Table 2), although these factors were assessed among all women (since data further stratified by menopausal status were not available). Restricting the analysis to studies that adjusted for BMI did not alter the results. Physical activity was a suggestive effect modifier of the linear dose-response relationship among all women, where specifically, studies that adjusted for physical activity observed a somewhat stronger inverse association (RR per 5 ng/mL = 0.96; 95% CI, 0.91–1.01), compared to studies that did not adjust for physical activity (RR per 5 ng/mL = 1.01; 95% CI, 0.98–1.03), with $p_{interaction} = 0.10$.

Finally, we conducted a sensitivity analysis of 25(OH)D cutpoints for the 3 laboratory batches of the Nurses' Health Study, with no evidence for an effect of specific cutpoints on the results (see Appendix 1). As for assessing publication bias, the Begg test (premenopausal: p = 0.71, postmenopausal: p = 0.92), the Egger test (premenopausal: p = 0.83, postmenopausal: p = 0.88), and a funnel plot of linear dose-response slopes provided no evidence of publication bias (Appendix 2).

DISCUSSION

In the current dose-response meta-regression of prospective studies examining the association between circulating vitamin D and breast cancer risk, we observed an apparent nonlinear inverse association where higher 25(OH)D levels at or above a 27 ng/mL threshold were associated with a 12% lower risk of postmenopausal breast cancer per 5 ng/mL increase in 25(OH)D. However, no further reductions in risk of breast cancer were observed above 35 ng/mL 25(OH)D. Increases of 5 ng/mL circulating 25(OH)D will typically occur when vitamin D intake is increased 500 IU/d.[37] In contrast, no association was observed among premenopausal women. These results were consistent across multiple disease definitions and population characteristics. Data indicated that apparent inconsistencies from previous individual studies may have been due to inadequate assessment of effect modification by menopausal status and lack of spline dose-response analysis to account for a nonlinear relationship between circulating vitamin D and postmenopausal breast cancer risk. Previous conflicting reviews did not account for these dose-response and menopausal issues.[14,26,68]

Our nonlinear results are supported by other congruent findings and indications of a threshold effect, most notably in

TABLE 2. Stratified, Pooled Linear Dose-Response Relative Risks per 5 ng/mL Circulating 25(OH)D

	Number of Study Sets*	RR (95% CI) per 5 ng/mL	P for Effect Modification
Total breast cancer	11	0.99 (0.97–1.00)	
Adjusted for BMI	10	0.99 (0.97–1.00)	
Menopausal status			
Premenopausal	6	1.01 (0.98–1.04)	0.05**
Postmenopausal	9	0.97 (0.93–1.00)	
Tumor classification			
In situ tumor	3	0.93 (0.84–1.03)	0.28
Invasive tumor	9	0.99 (0.97–1.00)	
Postmenopausal hormones			
Current	5	0.99 (0.97–1.00)	0.71
Never/past	5	0.98 (0.96–1.00)	
Mean circulating 25(OH)D			
<27 ng/mL	6	0.99 (0.98–1.01)	0.85
≥27 ng/mL	5	0.99 (0.92–1.06)	
Adjusted for PA			
Yes	7	0.96 (0.91–1.01)	0.10
No	4	1.01 (0.98–1.03)	
Country			
USA	7	0.97 (0.93–1.01)	0.74
Not USA	4	0.98 (0.96–1.00)	
Assay†			
Liquid chromatography	2	1.01 (0.92–1.10)	0.70
Immunoassay	9	0.99 (0.97–1.00)	

*Bertone-Johnson contributed 3 study sets as determined by batch (except in situ was pooled for the 3 batches due to few cases).

**This significant menopausal effect modification was confirmed via several approaches: 2-stage method (p = 0.05), linear method (p = 0.05), and nonlinear spline models (p = 0.05).

†Immunoassay includes radioimmunoassay and chemiluminescent immunoassay; liquid chromatography includes high pressure liquid chromatography-tandem mass spectrometry and isotope dilution liquid chromatography-tandem mass spectrometry.

studies of dietary vitamin D and breast cancer risk. As discussed by Garland et al,[28] many earlier studies of vitamin D and breast cancer risk may have offered null results given that the mean 25(OH)D levels in the majority of those studies were below the spline threshold that we observed. A recent meta-analysis of dietary vitamin D intake and breast cancer risk supports the potential threshold effect. Although no association was found in the crude linear analysis, an inverse trend was observed comparing highest versus lowest intake when limited to vitamin D intakes greater than 400 IU/d (RR = 0.92; 95% CI, 0.87–0.97).[32] Evidence of a nonlinear relationship for the protective effect of circulating 25(OH)D has also been shown in other cancers. Notably, a prospective analysis of circulating 25(OH)D and colon cancer risk found a 3-fold decrease in risk of colon cancer above a threshold of 20 ng/mL.[27] Similarly, a possible threshold effect was observed in the Cohort Consortium Vitamin D Pooling Project of Rarer Cancers, where circulating 25(OH)D levels in women were associated with a significantly decreased risk of kidney cancer above 30 ng/mL (RR = 0.31; 95% CI, 0.12–0.85); however, as this was an unexpected finding in their subgroup analyses,[25] it warrants further replication.

These results suggest that higher-dose vitamin D interventions may yield a benefit for postmenopausal, but not premenopausal, breast cancer. One previous 4-year randomized trial of vitamin D supplementation and cancer does appear to suggest that daily supplementation with 1000 IU vitamin D plus calcium reduced total cancer mortality (RR = 0.40; 95% CI, 0.20–0.82), albeit there were few breast cancer cases.[47] Although the Women's Health

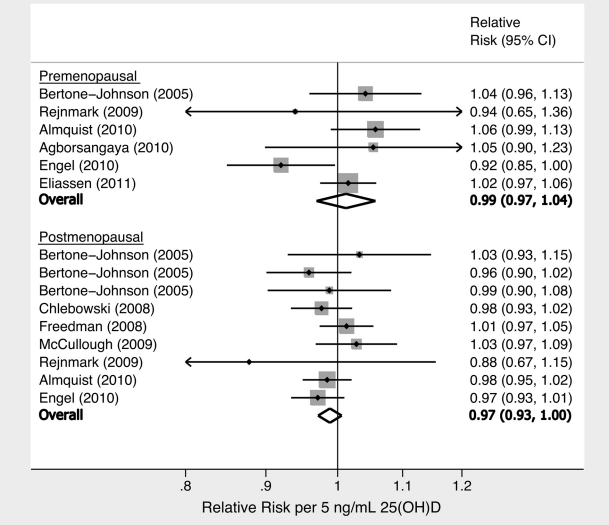

FIGURE 3. Forest plot of linear dose-response of circulating 25(OH)D and breast cancer risk, stratified by menopausal status, listed by first author and date of study. (P for menopause effect modification = 0.05.) Note: Bertone-Johnson et al contributed 3 study sets as determined by batch (except in situ was pooled for the 3 batches due to few cases).

Initiative (WHI) vitamin D plus calcium trial was, to our knowledge, the first randomized trial to specifically study vitamin D supplementation and risk of invasive breast cancer among postmenopausal women, the trial population had low baseline 25(OH)D levels and used a supplemental dose of only 400 IU/d. In concordance with the high-dose nonlinear hypotheses, the WHI trial found no reduction in breast cancer risk (RR = 0.96; 95% CI, 0.85–1.09).[15] Furthermore, in a recent reanalysis, vitamin D and calcium supplementation was associated with a significant reduction in risk of breast cancer among women who were not taking personal calcium and vitamin D supplements at randomization (RR = 0.82; 95% CI, 0.70–0.97).[11]

However, many studies and reports have conflicting evidence regarding dietary vitamin D. The recently released Institute of Medicine (IOM) guidelines for dietary intake of vitamin D and calcium stated that circulating 25(OH)D concentrations of 20 ng/mL are sufficient for 97% of the population, primarily based on bone health.[4,60] The IOM committee cited, at the time of their report, a lack of sufficient evidence supporting higher circulating 25(OH)D concentrations for protection against nonskeletal outcomes, even though this has been a controversial topic among national experts.[10]

Although the effect of menopausal status on the association between circulating 25(OH)D and breast cancer risk has not been previously studied in detail, menopause is an important effect modifier of the relationship between obesity and breast cancer.[63] In postmenopausal women, both obesity and adult weight gain are associated with an increased risk of breast cancer, primarily through increasing concentrations of circulating estrogens.[20,44] Conversely, obesity is inversely associated with risk of premenopausal breast cancer.[51] Higher estrogen concentrations are associated with an increased risk of breast cancer in postmenopausal and possibly premenopausal women.[22,36,42,43] However, higher concentrations of circulating estrogens in postmenopausal women are primarily driven by secretion of estrogen from adipose tissue, whereas ovarian production is the primary driver of estrogen concentrations in premenopausal women. Vitamin D may also inhibit growth of breast cancer cells through down-regulation of estrogen receptor expression and attenuation of estrogen signaling and synthesis.[45] Vitamin D supplementation may have interacted with concurrent estrogen treatments in the WHI, as suggested in a reanalysis of vitamin D and estrogen with colorectal cancer risk, but not breast cancer risk, in the WHI.[18] Variation in the association between 25(OH)D and breast cancer risk by menopausal status, similar to the relationship between obesity and breast cancer, may potentially be due to competitive binding of vitamin D and estrogen at lower levels of circulating 25(OH)D.

The exact mechanism behind a specific threshold is unclear; however, there are several molecular mechanisms that may account for an inverse association between circulating 25(OH)D and postmenopausal breast cancer risk. There are 3 primary pathways through which vitamin D, via the converted and tightly regulated form of 1,25(OH)D (calcitriol), may prevent breast cancer risk, including cell division, apoptosis, and contact inhibition.[39] 1,25(OH)D and a functional vitamin D receptor control cell growth and division through regulation of cyclins, cyclin-dependent kinases, and cell cycle checkpoints.[16,35,67] In addition to regulating cell division, calcitriol is needed for cells to undergo apoptosis.[6,17,40,48,49,62] Failure to undergo apoptosis following DNA damage can lead to continued proliferation and eventual malignancy. Lastly, calcitriol regulates E-cadherin, a cell adhesion molecule that is partially responsible for cellular contact inhibition.[53,57,59,61] Loss of contact inhibition is common in neoplastic cells and often predicts a poor prognosis.[55] Higher levels of prognostic circulating 25(OH)D may also be associated with increased survival among breast cancer patients.[33,56,65] These mechanisms support the biological plausibility of an inverse association between circulating 25(OH)D and breast cancer risk, although more work is needed to establish potential mechanisms of a nonlinear threshold effect.

The current study has several potential clinical implications. Most importantly, since low vitamin D levels are safely and inexpensively reversed by supplementation, low vitamin D may be one of the few modifiable risk factors for postmenopausal breast cancer. Indeed, low vitamin D status is remarkably common, particularly in older and non-white populations, which are known to have an increased risk of breast cancer.[46,52] From the national average circulating 25(OH)D level of 24 ng/mL,[30] daily supplementation of 1000 IU/d vitamin D would be needed to reach the approximate threshold of 35 ng/mL.[37,38,69] Our results highlight and reinforce the importance of ongoing higher-dose vitamin D intervention studies, such as the VITamin D and OmegA-3 TriaL (VITAL) (2000 IU/d).[5] This level of supplementation corresponds to an increase in circulating 25(OH)D levels of approximately 20 ng/mL among treatment arm participants.[37,38,69] Furthermore, our results may support ongoing efforts to increase vitamin D levels in selected populations, specifically postmenopausal women, and help refine the indications for clinical measurement of circulating vitamin D.

Although to our knowledge this is the most comprehensive meta-analysis to date of the association between circulating 25(OH)D and breast cancer risk, there are limitations. First, it is not possible to know to what degree the differences in 25(OH)D levels between study populations are due to true differences in exposure versus varying assay methods and batch-to-batch variation in laboratory results. Further, due to the nature of the published data on circulating 25(OH)D and breast cancer risk, RRs were reported by category of 25(OH)D levels rather than as a continuous variable. Thus, inconsistent assays of circulating 25(OH)D may potentially lead to some misclassification, thus reducing precision in the exact value of the optimal 25(OH)D spline knot thresholds. However, assay misclassification would be non-differentially random with respect to breast cancer, and seems unlikely to explain the significant nonlinear spline association. A future pooled analysis of individual patient-level data and circulating 25(OH)D as a continuous variable, with an embedded recalibration study to determine true differences in levels between studies, would be helpful in confirming the nonlinear inverse association as well as refine the spline thresholds.

The current meta-analysis was limited to published data, and further adjustment for individual BMI and physical activity was not possible, thus residual confounding remains a possibility. However, almost all the studies included adjusted or considered adjusting for BMI, and the results were not altered when excluding studies that did not adjust for BMI. Furthermore, stratified analyses of adjustment for physical activity suggested that studies that adjusted for physical activity observed a stronger inverse association between circulating 25(OH)D and breast cancer. Thus, residual confounding by physical activity is likely to attenuate the results, and is unlikely to explain observed associations. Not all studies reported breast cancer endpoints by tumor classification (in situ or invasive); however, authors of studies that assessed different endpoints were contacted, and stratified results were retrieved for all studies queried, which reported similar associations. Lastly, the systematic review was limited to published results or additional data provided by study investigators, and although the possibility cannot be excluded, we observed no publication bias.

In conclusion, findings from the current systematic review comprising 5206 incident cases of breast cancer and 6450 control cases suggest that the association of circulating 25(OH)D with

breast cancer risk differed a) by menopausal status, and b) nonlinearly by dose. Notably, a modest inverse association between 25(OH)D and breast cancer risk was observed among postmenopausal women, whereas no association was observed among premenopausal women. Furthermore, there is suggestive evidence of a nonlinear inverse association between circulating 25(OH)D and postmenopausal breast cancer risk, specifically at or above a threshold of 27 ng/mL. These findings highlight the potential importance of attaining a target threshold of circulating 25(OH)D levels for vitamin D among postmenopausal women to exert possible protective effects on breast cancer risk. Additional detailed dose-response assessments in large prospective studies are needed to confirm these findings. Ultimately, the benefit of vitamin D supplementation for postmenopausal women will need to be validated in large clinical trials, such as the on-going VITAL trial,[5] with adequate doses that sufficiently modify circulating 25(OH)D levels.

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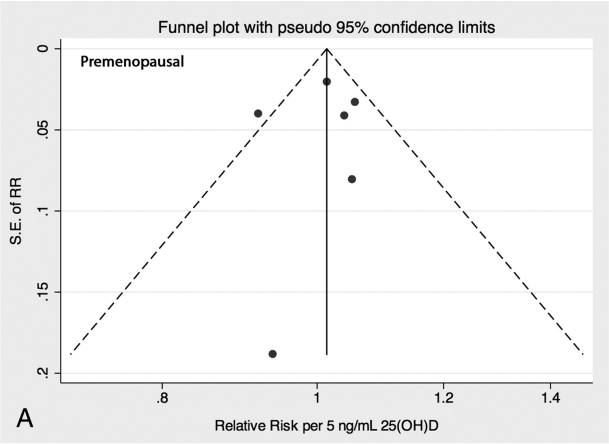
APPENDIX 1.
Data From the Nurses' Health Study and 25(OH)D Batch Cutpoints

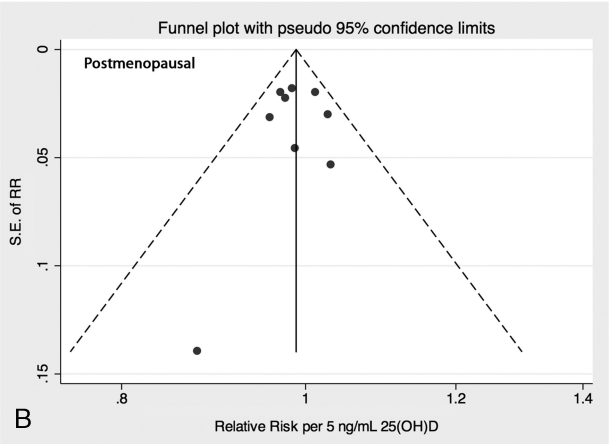
Due to variation in mean and standard deviation of circulating 25(OH)D levels between 3 different batches of distinct cases and controls in the Nurses' Health Study, the study data were extracted and analyzed with 3 sets of RRs and 25(OH)D levels by quantile for dose-response analyses.[20] Additional information from the original author (Bertone-Johnson, personal communication) facilitated the extraction of accurate information for each batch independently; thus data from the Nurses' Health Study were analyzed as 3 study sets instead of 1. No cases or controls belonged to more than 1 of these independent batches, and batches represented distinctly different person-time, which makes the 3-batches analysis identical to pooling HRs from Cox proportional hazard models stratifying on time. A sensitivity analysis was conducted using different 25(OH)D cutpoints since the variation in levels was likely due largely to laboratory batch-to-batch variation.

To assess the effect of batch-to-batch variation in 25(OH)D cutpoints of the 3 batches used from the Nurses' Health Study, we conducted a sensitivity analysis using the lowest and highest 25(OH)D level for all 3 batches. Since the variation between batches is likely due to lab differences rather than true differences among the participants, we wanted to ensure that the different batch 25(OH)D cutpoints were not influencing the results. Accordingly, the batch cutpoint did not influence the results when the lowest or highest 25(OH)D level was used for all 3 batches ($p = 0.02$, $p = 0.02$, and $p = 0.04$ for dose-interaction).

APPENDIX 2.
Figures A and B

Funnel plot of linear dose-response slopes, by menopausal status (A, premenopausal, and B, postmenopausal women). Note there was no evidence of publication bias.





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REVIEW

Circulating vitamin D level and mortality in prostate cancer patients: a dose–response meta-analysis

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Abstract

Previous studies investigating the association of circulating 25-hydroxyvitamin D level with prognosis of prostate cancer yielded controversial results. We conducted a dose–response meta-analysis to elucidate the relationship. PubMed and EMBASE were searched for eligible studies up to July 15, 2018. We performed a dose–response meta-analysis using random-effect model to calculate the summary hazard ratio (HR) and 95% CI of mortality in patients with prostate cancer. Seven eligible cohort studies with 7808 participants were included. The results indicated that higher vitamin D level could reduce the risk of death among prostate cancer patients. The summary HR of prostate cancer-specific mortality correlated with an increment of every 20 nmol/L in circulating vitamin D level was 0.91, with 95% CI 0.87–0.97, $P = 0.002$. The HR for all-cause mortality with the increase of 20 nmol/L vitamin D was 0.91 (95% CI: 0.84–0.98, $P = 0.01$). Sensitivity analysis suggested the pooled HRs were stable and not obviously changed by any single study. No evidence of publications bias was observed. This meta-analysis suggested that higher 25-hydroxyvitamin D level was associated with a reduction of mortality in prostate cancer patients and vitamin D is an important protective factor in the progression and prognosis of prostate cancer.

Key Words
- vitamin D
- mortality
- prostate cancer
- meta-analysis

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Introduction

Prostate cancer (PCa) is one of the most common malignant tumors in male. In 2017, American Cancer Society reported 161,360 cases of newly diagnosed PCa, accounting for 20% of male tumors. Furthermore, its incidence and mortality ranked the first place and third respectively (1). The mortality of PCa was proposed to be associated with obesity, physical activity, smoking, antioxidants, etc. (2). At present, the treatment of PCa have caused serious economic burden (3). More useful treatment measures are urgently needed by people to improve the survival rate of prostate cancer patients.

The major circulating form of vitamin D in human body is 25-hydroxyvitamin D (25(OH)D), which comes from vitamin D via 25-hydroxylation process in the liver. 25(OH)D can be converted into $1,25(OH)_2D$ by 1α-hydroxylase, which is the most active hormonal metabolite of vitamin D. As a hormone, $1,25(OH)_2D$ binds to vitamin D receptor located in nucleus and functions. It is reported to play an important role in cellular proliferation (4), differentiation, apoptosis (5), angiogenesis (6) and metastasis (7). All these processes may regulate the development and progression of cancer.

A number of researches have been done to clarify the association between vitamin D and PCa. Some experimental studies indicated that vitamin D might play a crucial role in the occurrence and progression of

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PCa. One study demonstrated mutations of vitamin D receptor gene were associated with Gleason score (8). Furthermore, study showed that genetic variants in the vitamin D pathway had effects on the risk of progression, prostate cancer-specific mortality and recurrence of PCa (9). Recent studies have reported controversial results about the association of vitamin D with the survival rate of prostate cancer. For example, in newly diagnosed stage IV prostate cancer patients, no significant association of 25-hydroxyvitamin D with the prognosis of them was found (10). In contrast, other studies reported that higher 25-hydroxyvitamin D was related to improved prostate cancer prognosis (11, 12).

Therefore, the relationship between 25-hydroxyvitamin D level and mortality of PCa is still unclear. Hence, we conducted this analysis to explore whether circulating 25-hydroxyvitamin D level was correlated with the survival of PCa through a dose–response meta-analysis.

Materials and methods

Search strategy

We searched PubMed and EMBASE databases from inception to July 15, 2018, for eligible studies on the relationship between vitamin D and mortality in prostate cancer patients. The terms used to retrieve literatures were the following: (vitamin D OR 25-hydroxyvitamin D OR 25(OH)D) and (prostate cancer OR prostate carcinoma). We also referred to the reference lists from reviews or relevant papers to get more eligible researches. There was no language restriction.

Selection criteria

Reports were included in this dose–response meta-analysis if they met the criteria as follows: (1) the association between vitamin D and mortality in prostate cancer patients was reported; (2) the study type was cohort; (3) the risk estimates of mortality in prostate cancer patients, like HR and 95% CI were reported. If the same data were used in several studies, we selected the publication with the largest number of cases or more details.

Data extraction

Data were extracted from eligible studies by two researchers independently. The information collected from each study contained of the first author's last name, publication year, country, follow-up time, number of cases and person-year, risk estimates with corresponding 95% CIs and confounding factors adjusted in multivariable analysis. We extracted the risk estimates from the most completed adjusted model to decrease the risk of possible confounding. Disagreements were resolved by consensus among authors.

Quality assessment

We evaluated the quality of studies by use of the Newcastle Ottawa Scale (NOS) (13). According to its criteria, studies were assessed on the basis of three perspectives: selection, comparability and outcomes. If studies got seven or more stars, they were regarded as high quality. Differences were resolved by discussion.

Statistical analysis

We performed data analyses separately for two outcomes, namely all-cause mortality and prostate cancer-specific mortality. Pooled HRs were calculated to assess the impact of vitamin D level on the prognosis of patients. The method proposed by Greenland and Longnecker (14) and Orsini *et al.* (15) was used to estimate the HR per 20 nmol/L

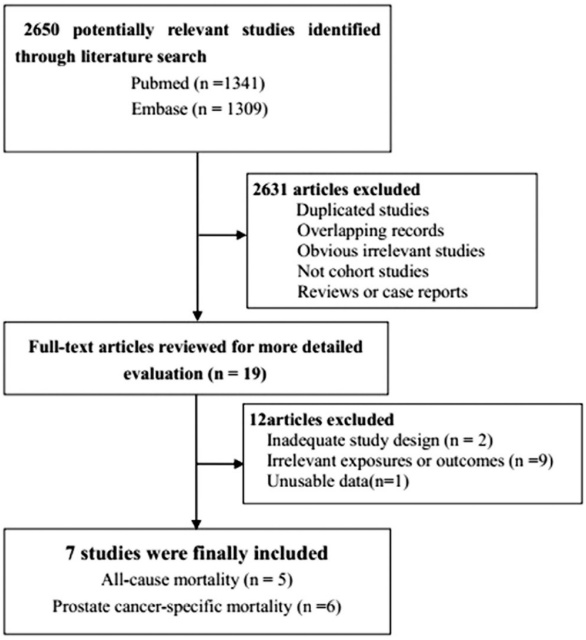

Figure 1
Flowchart of study selection in the meta-analysis.

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Table 1 The main characteristics of the included studies in the meta-analysis.

Study	Country	Study design	Time of vitamin D assessment	Participants	Follow-up	Outcomes	Age at diagnosis (years)	Adjustments	Quality
Tretli *et al*. 2009 (23)	Norway	Cohort	Postdiagnosis	160	44 months	ACM; PCSM	64.5	Patient group and age, tumor differentiation grade and the patient functional status at the time of blood collection	7
Fang *et al*. 2011 (11)	USA	Prospective cohort	Prediagnosis	1822	10 years	ACM; PCSM	68.9	Age at diagnosis, body mass index, physical activity, and smoking, Gleason score, and TNM stage	9
Holt *et al*. 2013 (35)	USA	Prospective cohort	Postdiagnosis	1476	10.8 years	PCSM	60	Season of blood draw, age and race, BMI, smoking status, and weekly exercise stage, Gleason score and primary treatment	9
Gupta *et al*. 2015 (10)	USA	Prospective cohort	Postdiagnosis	125	31 months	ACM; PCSM	60	Age, ECOG performance status, BMI, prostate specific antigen (PSA), season of blood draw, CTCA hospital, serum albumin, corrected serum calcium, bone metastasis and nutritional status	7
Mondul *et al*. 2016 (28)	Finland	Prospective cohort	Prediagnosis	1000	23 years	PCSM	69.2	Age, physical activity, cigarettes per day, and family history of prostate cancer	9
Meyer *et al*. 2016 (32)	Norway	Prospective cohort	Prediagnosis	2282	21.2 years	ACM	NA	Age, month of blood sampling and examination physical activity, BMI, smoking and education	9
Brandstedt *et al*. 2016 (34)	Sweden	Prospective cohort	Prediagnosis	943	16.6 years	ACM; PCSM	69.3	Season and year of inclusion, age at baseline, age at diagnosis, body mass index (BMI), and tumor characteristics (TNM and Gleason score)	9

ACM, all-cause mortality; BMI, body mass index; CTCA, Cancer Treatment Centers of America; ECOG, Eastern Cooperative Oncology Group; NA, not available; PCSM, prostate cancer-specific mortality; PSA, prostate specific antigen.

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increase of vitamin D level. Statistical heterogeneity among studies was evaluated with the use of Q and I^2 statistic (16, 17). For the Q statistic, we regarded P value <0.10 as statistically significant heterogeneity among studies. As to the I^2 statistic, I^2 more than 50% also suggested obvious heterogeneity. We utilized the random-effects model to combine HRs from single studies if obvious heterogeneity was observed (18). In the sensitivity analysis, studies were omitted one by one and the others were analyzed to evaluate the effect of single study on the summary risk estimates. Publication bias was assessed with the use of funnel plot and the Egger's test (19). We utilized Stata (Version 12.0) to perform this dose–response analysis. P value <0.05 was reckoned as statistically significant difference.

Results

Study selection and characteristics

The selection process was showed in Fig. 1. We retrieved 2650 articles from PubMed and EMBASE databases (Fig. 1). A majority of them were excluded from our analysis because they did not belong to cohort studies or because outcomes were not associated with our analysis, leaving 19 articles for detailed evaluation by reading full-texts (10, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37). Twelve studies were then removed after reading their full-texts. Two studies were excluded because of inadequate study design (22, 24). Nine studies were excluded because they did not contain prognosis data among prostate cancer patients (20, 21, 26, 27, 29, 32, 35, 37). One study was not qualified as a result of unusable data (36). Finally, a total of seven studies were included into our meta-analysis. The seven studies were published between 2009 and 2016 and the total number of prostate cancer participants was 7808. All of them were performed in developed countries, written in English (Table 1). Among them, three studies were conducted in USA (10, 30, 34), two in Norway (23, 31), one in Finland (28), one in Sweden (33). All studies were prospective cohort type, except one from Tretli S. It is also a cohort study but hard to define it belongs to prospective or retrospective type. Meanwhile, the vitamin D assessments were performed after diagnosis in three studies, while the others were before diagnosis of prostate cancer. All studies reported adjusted HRs. Every research was adjusted for many confounding factors, such as age, BMI, drinking history and so forth. Participants were followed up from 4 to 21 years. Five studies contained HRs of all-cause mortality among prostate cancer patients, and six reported HRs of prostate cancer-specific mortality. The quality assessment of those studies according to NOS criteria was also presented in the Table 1.

25-hydroxyvitamin D and all-cause mortality

We observed significant heterogeneity among five studies on all-cause mortality (I^2=68.9%). Figure 2A displayed the results of the dose–response analyses on all-cause mortality (Fig. 2A). A nonlinear relationship existed between 25-hydroxyvitamin D and risk of all-cause mortality in prostate cancer patients, suggesting higher 25-hydroxyvitamin D level was associated with decreased risk of death from all causes among prostate cancer patients (P=0.038). The summary HR of all-cause mortality correlated with an increment of every 20 nmol/L in circulating vitamin D level was 0.91 (95% CI:

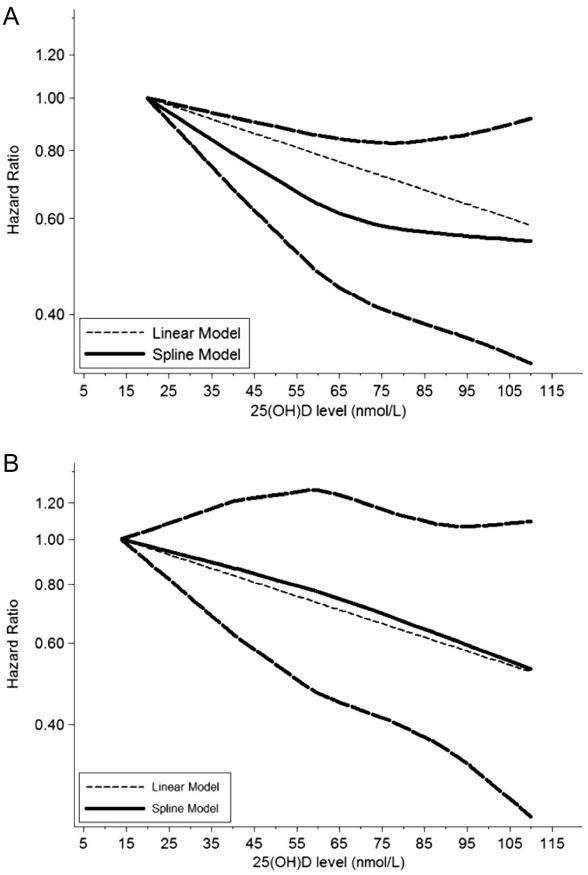

Figure 2
Dose–response relationships between 25(OH)D and risk estimates of all-cause mortality and prostate cancer-specific mortality. (A) Risk estimates with 95% CI for the association between 25(OH)D and all-cause mortality. (B) Risk estimates with 95% CI for the association between 25(OH)D and prostate cancer-specific mortality.

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0.84–0.98, $P=0.01$) (Fig. 3A). Sensitivity analysis suggested the pooled HRs were stable and not obviously changed by any individual study (Fig. 4A).

25-hydroxyvitamin D and prostate cancer-specific mortality

There was obvious heterogeneity observed among those six studies on prostate cancer-specific mortality ($I^2=53.4\%$). A nonlinear relationship between 25-hydroxyvitamin D and risk of prostate cancer-specific mortality was also presented in Fig. 2B, indicating higher vitamin D level could decrease the mortality from prostate cancer (Fig. 2B). The summary HR of prostate cancer-specific mortality correlated with an increment of every 20 nmol/L in circulating vitamin D level were 0.91 (95% CI: 0.87–0.97, $P=0.002$) (Fig. 3B). The sensitivity analysis showed the summary HRs were not markedly changed by any individual study (Fig. 4B), indicating no significant influence of single study on the results.

Publication bias

No risk of publication bias was observed in the funnel plots (Fig. 5). The outcomes from Egger's test also suggested that there were no publication bias for the analysis of all-cause mortality ($P=0.143$) and prostate cancer-specific mortality ($P=0.301$).

Subgroup analysis and meta-regression

We conducted the subgroup analysis and meta-regression to detect the source of heterogeneity, which was presented in Table 2. Stratifying by the time of vitamin D assessment,

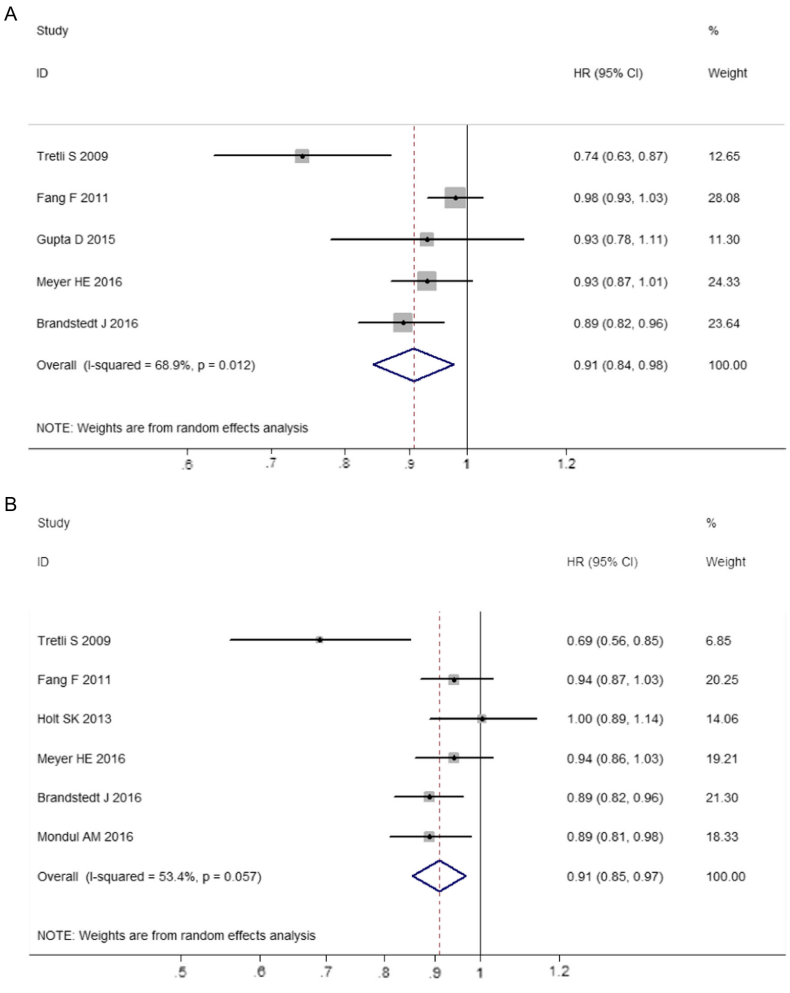

Figure 3
Summary risk estimates of mortality in prostate cancer patients associated with 20 nmol/L increment in 25(OH)D level. (A) Funnel plot of risk estimates of all-cause mortality of prostate cancer with the increment of 20 nmol/L in 25(OH)D level. (B) Funnel plot of risk estimates of prostate cancer-specific mortality with the increment of 20 nmol/L in 25(OH)D level.

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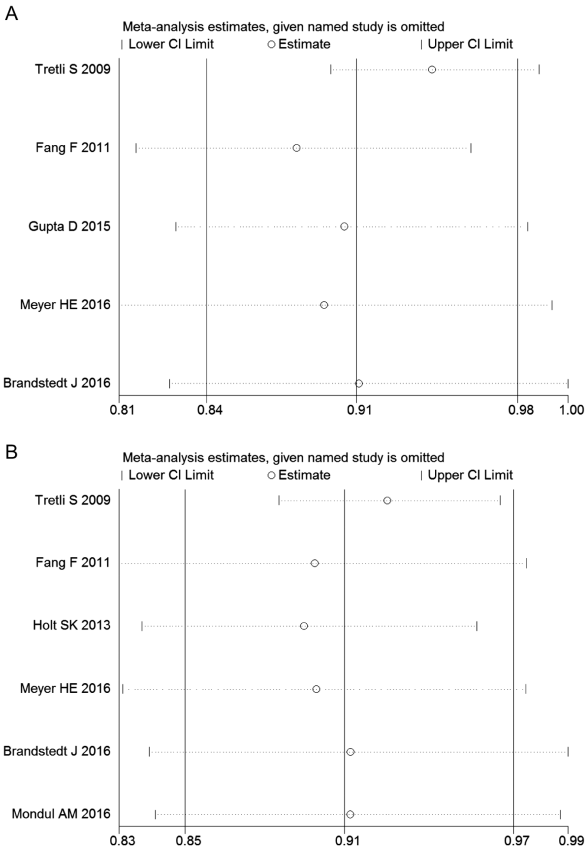

Figure 4
Sensitivity analysis by excluding studies by turns suggested that the pooled HRs were not significantly changed by any individual study. (A) Sensitivity analysis of the association between 25(OH)D and all-cause mortality of prostate cancer. (B) Sensitivity analysis of the association between 25(OH)D and prostate cancer-specific mortality.

the HR of prostate cancer-specific mortality was 0.91 (95% CI: 0.88–0.95) for prediagnosis studies and 0.84 (95% CI: 0.58–1.21) for postdiagnosis ones. The HR of all-cause mortality was 0.94 (95% CI: 0.88–0.98) in prediagnosis subgroup. Restricting the analysis among more than 10-year follow-up yielded a HR of 0.92 (95% CI: 0.89–0.96) and 0.94 (95% CI: 0.89–0.98) for prostate cancer-specific mortality and all-cause mortality respectively, which was slightly higher than the overall results. Moreover, there was no evidence of significant heterogeneity between subgroups with the use of meta-regression analyses.

Discussion

The role of circulating 25-hydroxyvitamin D and survival outcomes among prostate cancer patients remains unclear and controversial. This meta-analysis is the first one to focus on the relationship between 25-hydroxyvitamin D and mortality in prostate cancer, involving 7808 participants with survival outcomes. The results calculated from seven eligible studies indicated higher vitamin D level was significantly associated with decreased all-cause mortality and prostate cancer-specific mortality. Further dose–response analysis showed that every 20 nmol/L increment in 25-hydroxyvitamin D level was associated with a 9% lower risk of all-cause mortality and prostate cancer-specific mortality. By conducting the subgroup analysis, we found the results were consistent in prediagnosis and more than 10-year follow-up subgroups. The assessment of vitamin D before diagnosis was more likely to get rid of the influence of prostate cancer on the level of vitamin D and long follow-up time enabled researchers to calculate the outcome events more precisely. Based on the above findings, we conclude that higher circulating vitamin D

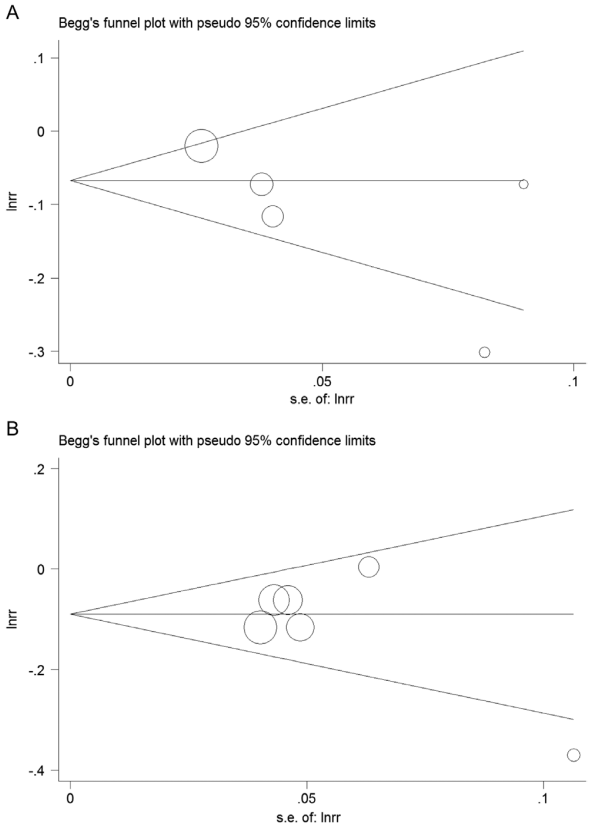

Figure 5
Publication bias. (A) Publication bias of the association between 25(OH)D and all-cause mortality of prostate cancer. (B) Publication bias of the association between 25(OH)D and prostate cancer-specific mortality.

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Table 2 Summary risk estimates of the associations between vitamin D level and prostate cancer mortality.

Study characteristics	No. of studies	HR	95% CI	I^2 (%)	*P* value 1	*P* value 2
Studies of PCM	6	0.91	0.87–0.97	53.4	0.057	
Country						0.294
Europe	4	0.88	0.81–0.95	57.9	0.068	
USA	2	0.96	0.90–1.03	0	0.389	
Time of vitamin D assessment						0.36
Postdiagnosis	2	0.84	0.58–1.21	89.1	0.002	
Prediagnosis	4	0.91	0.88–0.95	0	0.675	
Follow-up						0.055
Less than 10 years	1					
More than 10 years	5	0.92	0.89–0.96	0	0.479	
Studies of ACM	5	0.91	0.84–0.98	68.9	0.012	
Country						0.295
Europe	3	0.87	0.79	68.5	0.042	
USA	2	0.98	0.93–1.03	0	0.576	
Time of vitamin D assessment						0.246
Postdiagnosis	2	0.83	0.66–1.04	71.5	0.061	
Prediagnosis	3	0.94	0.89–0.98	53.9	0.114	
Follow-up						0.246
Less than 10 years	2	0.83	0.66–1.04	71.5	0.061	
More than 10 years	3	0.94	0.89–0.98	53.9	0.114	

P value 1 for heterogeneity within each subgroup. *P* value 2 for heterogeneity between subgroups with meta-regression analysis.
ACM, all-cause mortality; CI, confidence interval; HR, summary hazard ratio; PCSM, prostate cancer-specific mortality.

level is associated with a lower risk of death from prostate cancer.

Numerous experimental studies have been done to elucidate the mechanism by which vitamin D affect the prostate cancer survival. According to previous studies, $1,25(OH)_2D$ could cause cell cycle arrest and induce apoptosis, inhibiting cell proliferation in several prostate cancer cell lines (38, 39, 40). $1,25(OH)_2D$ played a protective role in preventing normal human prostate epithelial cell lines from oxidative stress in since it increased both the expression and activity of antioxidants, such as glucose-6-phosphate dehydrogenase and glutathione (41). Ben-Shoshan and colleagues demonstrated that $1,25(OH)_2D$ inhibited angiogenesis by reducing HIF-1α expression in various human prostate cancer cell lines (42). In terms of animal model evidence, Ray and colleagues indicated that a diet deficient in vitamin D rather than vitamin D-sufficient diet accelerated growth of human prostate cancers insensitive to androgen therapy in athymic mice (43). Another study reported that a higher vitamin D3-supplemented diet led to significant tumor shrinkage in mice bearing PC-3 prostate cancer xenografts (44). Moreover, vitamin D could prevent the metastasis of prostate cancer according to several animal and cell experiments (45, 46). Therefore, there is some evidence supporting the protective effect of vitamin D in prostate cancer. However, the underlying molecular mechanisms are still not fully clarified, and more studies are needed to explore them.

Some studies reported that 25-hydroxyvitamin D concentration was correlated with prostate cancer pathology. Researchers found lower 25-hydroxyvitamin D concentrations were positively correlated with higher Gleason grade and tumor stage (47, 48). The findings above provide some explanations for the prognostic role of 25-hydroxyvitamin D in prostate cancer.

Previous studies reported conflicting results about the vitamin D and prostate cancer incidence. One meta-analysis showed positive association between high level of vitamin D and increased incidence of prostate cancer (49). Some studies also suggested that high incidence of aggressive prostate cancer in African Americans might be partly due to deficient concentrations of serum vitamin D (50, 51). In the contrast, one Mendelian randomization study showed null relationship between vitamin D and risk of prostate cancer (52). Other studies also failed to find a positive relationship between vitamin D and prostate cancer risk (47, 53). The conflicting findings in the relationship between vitamin D and prostate cancer risk may result from the some factors, such as different populations, various study design and different confounding factors. The findings in our study suggest that vitamin D is more likely to be a suppressive and protective factor during the development of prostate

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cancer. Therefore, there is still controversy on the role of vitamin D in prostate cancer, which need to be elucidated in future researches.

There is also some evidence from clinical trials on the roles of vitamin D in prostate cancer. In a clinical trial, low-grade prostate cancer patients took 4000 IU of vitamin D3 every day for a whole year and had a biopsy after the supplementation (54). Results of biopsy revealed a decreased number of positive cores and no increase in Gleason score (54). Several randomized clinical trials showed that oral vitamin D3 modestly decreased the level of PSA (55) and reduced the PSA rise rate (56, 57). However, a vitamin D supplementation trial showed no influence on free or total PSA level in African American population (58). At present, the evidence from clinical trials on the roles of vitamin D in prostate cancer is still limited, and more clinical trials are needed.

There are potential limitations existing in our study which should be considered. For one thing, although all studies adjusted for confounding factors, some potential confounding factors related to vitamin D remained residual. For another, some studies included in our meta-analysis tested the circulating vitamin D level postdiagnosis or post treatment, thus it is difficult to get rid of the possibility of reverse causality. What is more, the limited number of included studies restricted us to find the source of heterogeneity.

Based on the results mentioned earlier, we can draw the conclusion that higher vitamin D level is significantly associated with a risk reduction of all-cause mortality and prostate cancer-specific mortality, indicating vitamin D may exert a protective effect in the progression and prognosis of prostate cancer. More cohort studies and randomized clinical trial are needed to further illustrate the role of vitamin D in the pathogenesis and prognosis of prostate cancer.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement
Zhen-yu Song designed the study. Qiuming Yao, Zhi-yuan Zhuo and Zhe Ma extracted the data. Zhen-yu Song and Qiuming Yao performed the analyses. Zhen-yu Song wrote the draft. Gang Chen revised it critically.

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Association between serum 25(OH)D and death from prostate cancer

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Abstract

Based on observations that for certain cancers, mortality varies according to sun exposure, vitamin D has been proposed to influence on disease progression. This study aims to investigate whether serum levels of 25(OH)D are associated with prognosis in patients with prostate cancer. In total, 160 patients with a serum sample in the JANUS serum bank were included. For 123 patients a pre-treatment serum sample was taken, whereas 37 of the patients had received hormone therapy prior to the blood collection. The serum level of 25(OH)D was classified as low (< 50 nmol l^{-1}), medium (50–80 nmol l^{-1}) or high (>80 nmol l^{-1}). A Cox proportional hazard regression model was used to assess the association between serum 25(OH)D and cancer mortality. During follow-up, 61 deaths occurred, of whom 52 died of prostate cancer. The median time of follow-up was 44.0 months (range, 1.2–154.6). Serum 25(OH)D at medium or high levels were significantly related to better prognosis (RR 0.33; 95% CI 0.14–0.77, RR 0.16; 95% CI 0.05–0.43) compared with the low level. Analysis restricted to patients receiving hormone therapy gave a stronger association. The serum level of 25(OH)D may be involved in disease progression and is a potential marker of prognosis in patients with prostate cancer.

Keywords: **25(OH)D, serum, prostate cancer, prognosis, mortality**

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REVIEW Open Access

Pancreatic cancer associated with obesity and diabetes: an alternative approach for its targeting

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Abstract

Background: Pancreatic cancer (PC) is among foremost causes of cancer related deaths worldwide due to generic symptoms, lack of effective screening strategies and resistance to chemo- and radiotherapies. The risk factors associated with PC include several metabolic disorders such as obesity, insulin resistance and type 2 diabetes mellitus (T2DM). Studies have shown that obesity and T2DM are associated with PC pathogenesis; however, their role in PC initiation and development remains obscure.

Main body: Several biochemical and physiological factors associated with obesity and/or T2DM including adipokines, inflammatory mediators, and altered microbiome are involved in PC progression and metastasis albeit by different molecular mechanisms. Deep understanding of these factors and causal relationship between factors and altered signaling pathways will facilitate deconvolution of disease complexity as well as lead to development of novel therapies. In the present review, we focuses on the interplay between adipocytokines, gut microbiota, adrenomedullin, hyaluronan, vanin and matrix metalloproteinase affected by metabolic alteration and pancreatic tumor progression.

Conclusions: Metabolic diseases, such as obesity and T2DM, contribute PC development through altered metabolic pathways. Delineating key players in oncogenic development in pancreas due to metabolic disorder could be a beneficial strategy to combat cancers associated with metabolic diseases in particular, PC.

Keywords: Pancreatic cancer, Obesity, Insulin resistance, Diabetes, Adiponectin, Leptin, Gut microbiota, Inflammation

Background

The pancreas contains exocrine and endocrine cells. The endocrine cells secrete insulin, glucagon, and somatostatin, whereas exocrine cells are involved in the secretion of digestive enzymes. Pancreatic cancer (PC) is lethal malignancy and approximately, 95% of PC has an exocrine cell origin. It is very difficult to diagnose at an early stage due to the lack of symptoms and deep retroperitoneal of pancreas. This PC type is commonly known as pancreatic ductal adenocarcinoma (PDAC), with a 5-year survival rate of ~7.2% in the United States (US) [1]. PC has become the third leading cause of cancer-related deaths with an estimated new cases of 55,440 and deaths of 44,330 in 2018 [2]. The lifetime risk of developing PC in any one person is 1.6% and it is expected to surpass colon cancer in mortality by year 2030 [3]. PC is frequently diagnosed at an advanced stage, when the cancer has metastasized to distant organs like the liver, lung, lymph node and peritoneal cavity [4]. Unfortunately by the clinical presentation, 85% of the tumors are unresectable [5, 6] which translates to poor prognosis and high mortality in the absence of effective chemo- and radiotherapies. Risk factors for PDAC include age (high percentage in elderly), sex (high incidence in men), gene mutations, cigarette smoking (nearly one quarter of all PC cases), obesity, chronic pancreatitis, and diabetes [7, 8].

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In PC, pancreatic stellate cells form a dense stromal tissue, which is referred to as a desmoplastic reaction. Stellate cells are responsible for limiting vascularization, which leads to hypoxia, tumor progression, invasion, and metastasis [9–13]. In PC, a compendium of mutations occur in various oncogenes like Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and tumor suppressor genes (*INK4A/p16*, *Tp53* and *SMAD4*) [14]. Mutations in the *KRAS* oncogene, observed in more than 90% of PC tumors, leads to constitutively active Ras protein that results in uncontrolled cell proliferation. Further, inactivating mutations in *INK4A/p16* and *Tp53* results in the loss of cell cycle and apoptotic regulation [4]. Differential expression of epidermal growth factor receptor (*EGFR*), mucins (*MUC1*, *MUC6* and *MUC5AC*) and matrix metalloproteinases (MMPs) occurs during precursor development [15]. Mutations in *INK4A/p16* (90%) appear in PanIN-2, whereas *Tp53* (85%) and *SMAD4* (55%) mutations are found in PanIN-3. Since PanINs represent precancerous ductal lesions, these mutations are considered early molecular biomarkers for PC [15]. A combination of biomarkers (EGFR, ERK, SIAH, Ki67 and HIF-α) can predict survival rates for patients with resectable PC. In fact, a combination of these biomarkers is more strongly associated with pathological features including tumor size, tumor grade, margin and lymph node status compared to a single marker [7, 16, 17]. In a multicenter study, to differentiate PC from chronic pancreatitis and their benign controls, mucin (MUC5AC) alone or in combination with CA19-9 could be a potential diagnostic/prognostic biomarker [18].

Due to generic symptoms (weight loss, fatigue, jaundice, abdominal pain and nausea) common across multiple other pathologies , early identification of PC is difficult [19, 20]. Recent studies suggest that PC develops from a precursor lesion of <5 mm in diameter and may take an average of 20 years to metastasize [20]. Therefore, it provides a window of opportunity to diagnose and treat PC if it is detected at an early stage [21]. To date, efforts are being made in multiple directions to develop early diagnostic test for PC including histopathological tests on fine needle aspirates, serological tests, imaging (computed tomography/magnetic resonance imaging), and analysis of genetic mutation markers [21–23]. Regarding PC treatment, gemcitabine (a nucleotide analogue) is the preferred first-line option but survival is often less than ~5 months. Combination therapy with gemcitabine and erlotinib (an inhibitor of EGFR) increased the 1-year survival rate to 23% as compared to 17% in the gemcitabine plus placebo group in a randomized phase III clinical trial [24]. Other drugs such as folfirinox/nab-paclitaxel with gemcitabine also increase survival [25–27]. In a clinical trial, metastatic PC patients were treated by administration of folfirinox (5-fluorouracil with leucovorin, irinotecan, and oxaliplatin) had shown greater efficacy for metastatic cancer; however, few limitations were observed due to its cytotoxicity [28]. However, in a systematic study, over 30 years (from 1986 to 2016) weighted median overall survival was improved with folfirinox alone [3]. In addition to the above chemotherapeutic agents, different treatment options for PC patients includes Capecitabine and 5-fluorouracil (5-FU) along with platinum-based or other cancer drugs (leucovorin, exatecan, and irinotecan) [27]. Therefore novel treatment strategies are needed to improve the overall survival in PC patients.

Obesity, insulin resistance and diabetes

Obesity has become a serious threat worldwide and is considered an epidemic. It occurs due to changes in lifestyle (physical inactivity, intake of high fat/caloric diet, high sugar diet) and is also associated with lifestyle including cigarette smoking and alcohol consumption. Additionally, genetic factors such as mutation in the leptin pathway leads to monogenic obesity while chromosomal abnormalities results in syndromic obesity [29]. In the body, adipose tissue (AT) plays an important role in the storage of triglycerides (TG), which come from the diet. It is classified as brown and white AT, where brown AT (BAT) is predominantly located in the cervical area and utilizes TG to generate heat (a process called as thermogenesis). Disappearance of BAT has been observed during the aging process and recently it has gained significant attention. White AT is present in the subcutaneous layer, omentum and retroperitoneal cavity, where it stores excess fat. According to the lipid burden hypothesis, AT stores sufficient lipids in the form of droplets. Excess storage of lipids leads to hypertrophy (increase in cell size) and hyperplasia (increase in cell number) [30]. Moreover, in obesity, heavy traffic of lipids inside the body leads to release of excess TG in the form of free fatty acids (FFAs) into the circulation. Further, these FFAs accumulate in non-adipose tissues such as the pancreas, muscle, liver, heart and kidney, resulting in insulin resistance and diabetes [31].

Obesity is a multifactorial disease associated with several metabolic disorders including insulin resistance, glucose intolerance, dyslipidemia, and elevated blood pressure. All these disorders are collectively called metabolic X syndrome [32]. Further, obesity is a strong risk factor for type 2 diabetes mellitus (T2DM), cardiovascular diseases and even many types of cancers such as pancreatic, hematological, prostate and breast cancers [33]. Recent studies have revealed that obesity and PC are strongly associated. For instance, a body mass index greater than 35 is one of the risk factors for PC in both men and women [33, 34]. Moreover, studies have suggested that both obese mice and patients develop PC

lesions following an increase in fat mass [35, 36] and show infiltration of fat cells in the pancreas as a consequence of PC development [37, 38]. Insulin resistance is a hallmark of T2DM, in which insulin fails to trigger adequate glucose uptake, leading to accumulation of circulatory glucose as well as increased insulin levels. These increased insulin levels in T2DM patients may be associated with PC growth by binding to its receptors located on the pancreas. For example, we still don't know if the insulin resistance that characterizes T2DM promotes PC or if the reverse is true (Fig. 1). In the present review, we have attempted to compress all the available literature on obesity-and diabetes-associated molecules involved in PC development. Several molecules have been characterized in obesity-associated PC, whereas less is known about factors unique to diabetes-associated PC. These molecules are expected to be the focus for future investigations of the molecular oncology of cancer.

Obesity associated pancreatic ductal adenocarcinoma

Obesity is associated with pancreatic and other types of cancers [39–41]. Individuals with abdominal adiposity have a 50% increased risk of PC development compared to lean individuals [42]. In the U.S., about 70% of the adult population is overweight and has a two-fold increased risk of PC incidence and mortality [39, 42]. However, link between obesity and PC is still not fully understood [43]. The current theory is that excess TG in obesity leads to an increase in size and number of adipocytes, which results in devascularization, hypoxia, and ultimately macrophage infiltration. In this condition, adipocytokines including adiponectin, leptin, tumor necrosis factor-alpha (TNF-α), interleukins, and monocyte chemoattractant proteins are secreted locally leading to inflammation. Evidence suggests that increased levels of adipocytokines, altered gut microbiota, and inflammation are involved in PC progression [39, 44]; thus, this review focuses on the possible oncogenic roles of these factors in PC.

Adipocytokines

Besides storing excess energy as TG, AT secretes several factors regulating energy metabolism in various organs. These adipokines including adiponectin, leptin, resistin, and ghrelin play an important role in glucose and lipid metabolism. Among them, adiponectin and leptin are the most important and are therefore the focus here in discussing obesity-associated PC.

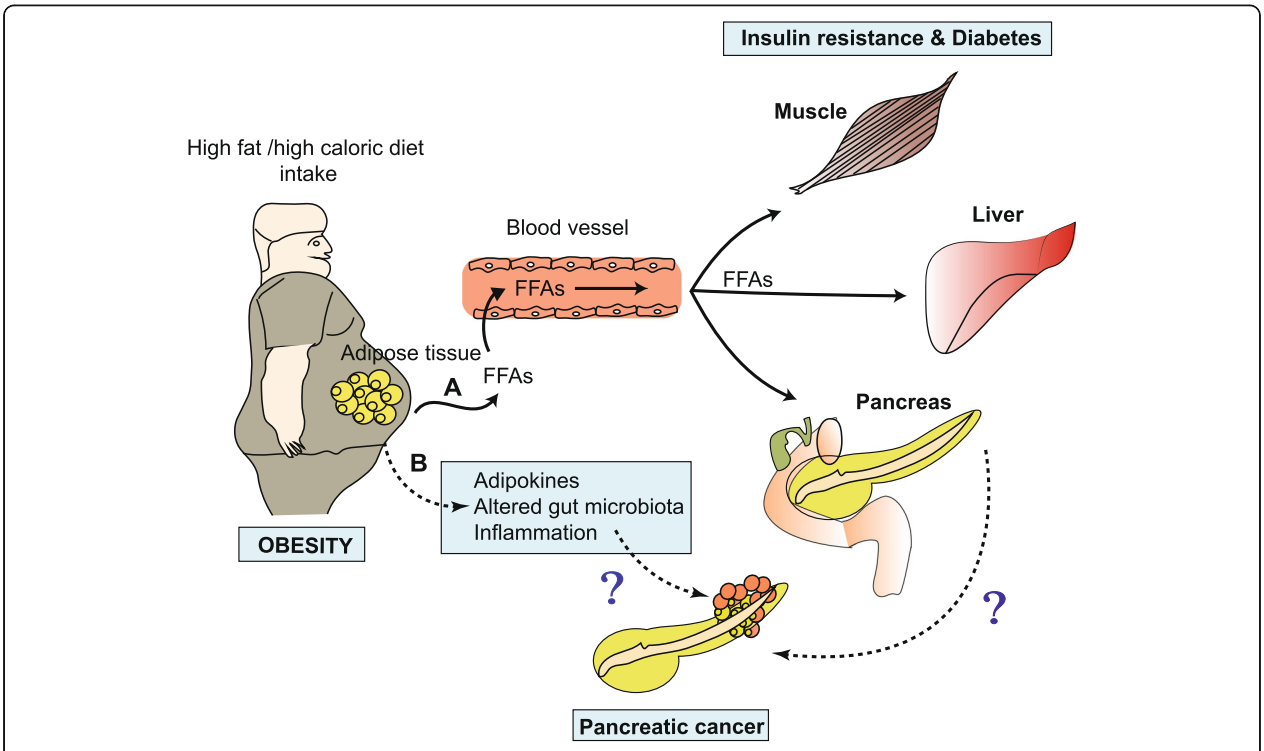

Fig. 1 A schematic representation of obesity- and diabetes-associated pancreatic cancer. High fat/caloric intake results in accumulation of excess fat, which further leads to development of obesity. **a**. In obesity, adipose tissue releases free fatty acids (FFAs), which enter circulation and accumulate in the non-adipose tissues such as muscle, liver and pancreas that leads to insulin resistance and diabetes. **b**. Along with FFAs, adipokines, altered gut microbiota and inflammatory markers contribute to pancreatic cancer development through unknown mechanisms.

Adiponectin

Adiponectin is also referred to as AdipoQ, which acts on several tissues to control energy homeostasis and insulin sensitivity [45, 46]. It regulates carbohydrate as well as lipid metabolism through the adenosine monophosphate -activated protein kinase (AMPK) pathway. The expression of circulatory AdipoQ is decreased in obesity and diabetes. However, the role of circulating AdipoQ in PC remains debatable regarding its impact on pancreatic tumor progression. Adiponectin serve as negative regulator that mediate its function by acting on its two receptors i.e. AdipoR1 and AdipoR2. Mechanistically, AdipoQ increases insulin synthesis and secretion by preventing apoptosis of pancreatic β-cells through activation of ERK and AKT pathways [47] (Fig. 2). Huang et al. demonstrated that subcutaneous implant of mouse pancreatic cell lines (H7 and Panc02) in AdipoQ knockout (APNKO) mice has reduced tumor weight and size as well as increased apoptosis by up-regulating cleaved caspase-3 as compared to wild type (WT) littermates. In addition, knockdown of AdipoR1, the major receptor of AdipoQ in these mouse cell lines (H7 and Panc02) followed by subcutaneous injection reduced tumor weight, size, and expression of Ki-67 (proliferation marker). Further, AdipoQ was observed to decreases apoptosis and increases PC cell proliferation and migration

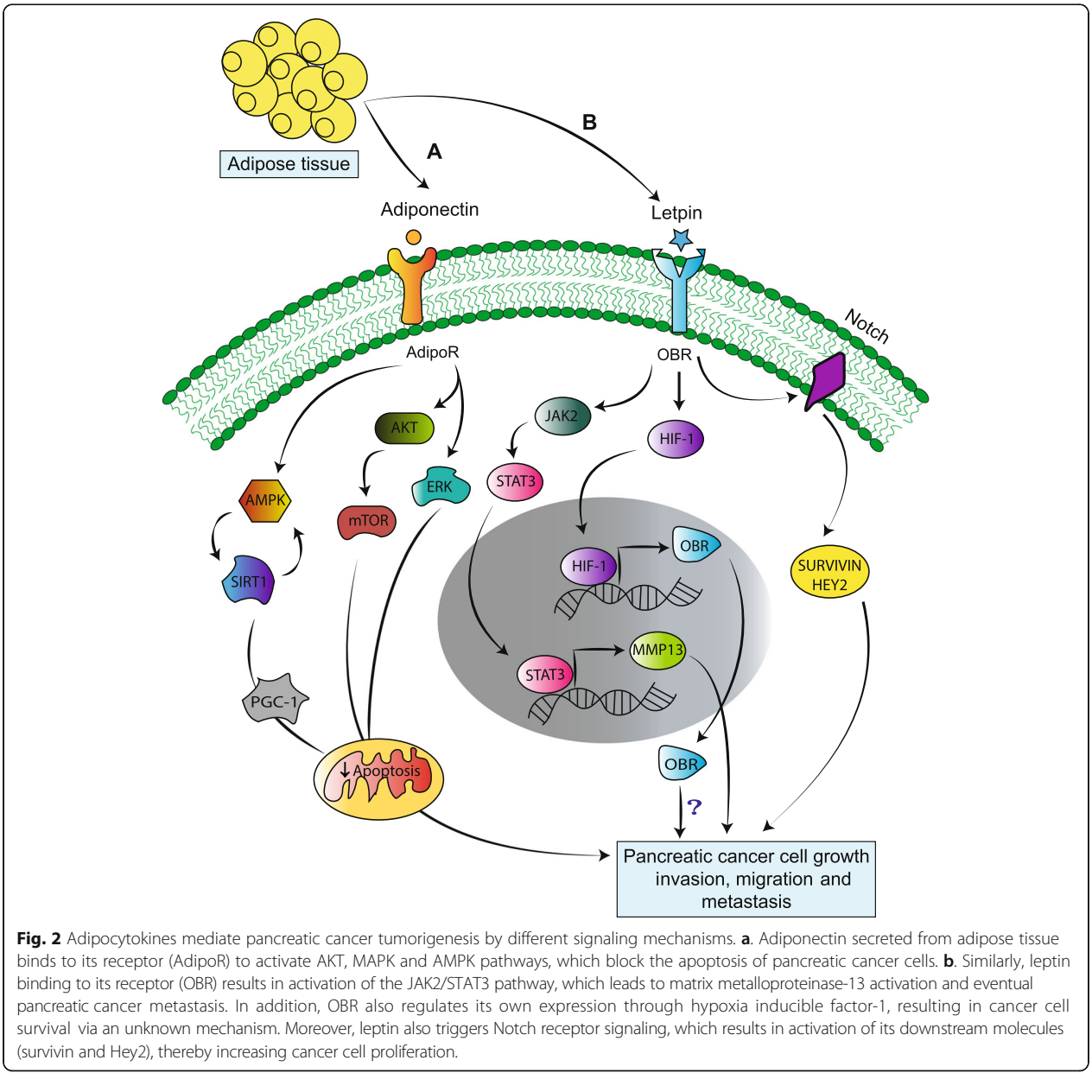

Fig. 2 Adipocytokines mediate pancreatic cancer tumorigenesis by different signaling mechanisms. **a**. Adiponectin secreted from adipose tissue binds to its receptor (AdipoR) to activate AKT, MAPK and AMPK pathways, which block the apoptosis of pancreatic cancer cells. **b**. Similarly, leptin binding to its receptor (OBR) results in activation of the JAK2/STAT3 pathway, which leads to matrix metalloproteinase-13 activation and eventual pancreatic cancer metastasis. In addition, OBR also regulates its own expression through hypoxia inducible factor-1, resulting in cancer cell survival via an unknown mechanism. Moreover, leptin also triggers Notch receptor signaling, which results in activation of its downstream molecules (survivin and Hey2), thereby increasing cancer cell proliferation.

by activating the AMPK-Sirt1-PGC1α pathway [48] (Fig. 2). Similarly, in a case-control study, Dalamaga et al. studied the blood levels of AdipoQ in PC and control cases both before and after controlling for age, gender, BMI, smoking status, alcohol consumption, history of diabetes, and family history of PC. Higher AdipoQ levels were associated with PC. At tissue level, utilizing 16 tumor tissues, the authors observed positive or strong positive expression of AdipoR1 in 87.5% of cases while positive or strong positive expression of AdipoR2 was observed in >97% cases. Based on this, the investigators suggested to investigate the role of AdipoQ as a marker for early detection of PC. Further, Kadri et al. observed no correlation between adiponectin levels and PC [49]. Similarly, Pezzilli et al. did not observe any significant correlation among adiponectin levels and PC at serum level [50]. However, retrospective and prospective studies indicate that early detection of low circulatory AdipoQ levels may or may not be associated with the development of PC, because single nucleotide polymorphisms of the AdipoQ gene are common [51–54] and the presence of these SNPs in AdipoQ, but not its receptors, are associated with altered serum adiponectin levels [55].

Inhibitory role of AdipoQ in halting tumor progression has also been observed [49]. In this regard some clinical studies suggest that circulating AdipoQ inhibit tumor cell proliferation by decreasing AKT and beta catenin levels across multiple malignancies (breast, colon and prostate) [56, 57]. In the case of PC, the molecular mechanism by which up-regulated AdipoQ levels inhibit cancer progression is still unclear; possibilities include 1) increasing insulin sensitivity via phosphorylation of insulin receptors, which down-regulates insulin/IGF-1 signaling, 2) down-regulating the expression of inflammatory cytokines that inhibit NF-κB activation, 3) directly activating the AMPK pathway to activate the p53 tumor suppressor gene, and 4) promoting cancer cell apoptosis via peroxisome proliferator-activated receptor gamma (PPARγ) activation and inhibiting angiogenesis [58, 59]. One study fed genetically engineered PC mice ($Kras^{G12D}$/Pdx-1-Cre) with a calorie-restricted diet and observed delays in formation of pancreatic intraepithelial neoplasms (PanIN) [60, 61]. Delayed progression of PanIN to PDAC was accompanied by increased AdipoQ and Sirt1 levels as well as decreased mTOR and IGF-1 expression [61]. In another study, Kato et al. incubated recombinant AdipoQ with the Pan02 murine cell line and noted decreased cell proliferation and increased apoptosis at 5 and 10 μg/ml, respectively. Further, orthotopic implantation of Pan02 cell line showed a significant increase in tumor volume by higher vascularization (more microvessel density) and decreased apoptosis in AdipoQ knockout mice as compared to WT animal [58, 62]. Overall, the findings from this study suggested AdipoQ to be a tumor suppressive role in PC by directly inhibiting proliferation and inducing apoptosis [62]. Interestingly, a recent study by Messaggio and co-workers showed that the decreased expression of AdipoQ receptors in pancreatic tumor tissues as compared to adjacent normal tissue. To elucidate the role of AdipoQ, its agonist AdipoRon was applied to both mouse and human cell lines and was found to inhibit PC tumor growth and proliferation by down-regulating leptin-induced STAT3 signaling. These results suggest AdipoRon could be a potential therapeutic agent for PC [63].

Leptin

Leptin was the first adipokine identified in AT in 1993; it controls food intake and energy expenditure *via* a feedback mechanism in the brain [64]. After secretion from AT, leptin enters into circulation and reaches a level depending upon the AT size [65]. Under normal physiological conditions, leptin decreases appetite and increases fatty acid oxidation through its receptor (OBR or LEPR). However, in obesity and diabetes, elevated circulatory levels of leptin do not drive the same appetite feedback responses [66]. Like AdipoQ, leptin has a role in PC pathogenesis. In PC tumor cells, leptin binds to both full-length receptor (OBR1) as well as the short form (OBRs) to mediate downstream signaling [67]. Leptin receptor (OBR) and hypoxia inducible factor-1 (HIF-1) are predominantly co-expressed in PC cell lines and tissues during hypoxic conditions. HIF-1 binds to the hypoxia-responsive element (HRE) in the OBR promoter, regulating OBR transcription. Co-expression of OBR and HIF-1 in PC tissues was associated with poor prognosis, decreased overall survival and increased metastasis to distant organs in PC patients (Fig. 2). Silencing of HIF-1 inhibited leptin receptor expression in PC cells, suggesting that a positive feedback loop between HIF-1 and leptin/OBR mediates PC progression [67]. In another *in vitro* study, recombinant human leptin promoted PC cell migration and invasion but had no effect on proliferation [68]. The migration of PC cells occurred via the janus kinase 2 and signal transducer and activator of transcription 3 (JAK2/STAT3) pathway, which targets its downstream effector matrix metalloproteinase 13 (MMP13). The *in vivo* impact of leptin-over expressing PC cells was tested by orthotopic implantation into athymic nude mice, which led to greater tumor growth and lymph node metastasis. Over expression of leptin in PC cells and mouse tumors resulted in up-regulation of MMP13 levels, suggesting that leptin/MMP13 signaling is important for metastasis. In addition, MMP13 levels correlated with OBR expression in lymph node metastatic human PC tissues. The authors concluded that PC cell migration, invasion and

metastasis occur via the JAK2/STAT3/MMP13 pathway [68] (Fig. 2).

A high fat/caloric diet leads to obesity, insulin resistance and increased leptin levels, all of which contribute to pancreatic adiposity. The accumulation of lipid molecules into the pancreas leads to activation and deposition of inflammatory cytokines (e.g., interleukin-6), which potentiate PC cell growth, migration and invasion [69]. Leptin activates Notch signaling and its receptors, leading to activation of its downstream molecules (survivin and Hey2) required for PC proliferation (Fig. 2). Notch signaling also up-regulates stem cell markers (CD44, CD24 and ESA) in PC cells. Inhibition of leptin (by IONP-LPrA2) after subcutaneous implantation of PC cells delayed tumor onset and decreased tumor size as well as cancer stem cell markers [70]. In another study by the same group reported that BxPC-3 and MiaPaCa-2 PC cells were treated in the presence of 5-FU, leptin, notch inhibitor (DAPT) and leptin inhibitor (IONP-LPrA2). They observed that decreased 5-FU cytotoxicity (by decreasing pro-apoptotic markers), increased cell proliferation and anti-apoptotic factors was due to leptin treatment. Moreover, IONP-LPrA2 reduced PC tumorspheres (treated with 5-FU) *via* notch signaling and suggesting that leptin might be involved in reducing the cytotoxic effect of chemotherapeutic drug and facilitating chemoresistance [71]. The leptin-notch signaling axis targeting has been projected as potential mediator for benefitting PC patients with obesity. Overall, the effect of AdipoQ and leptin in the progression of PC is still under investigation in obese people and further studies are warranted before targeting these adipokines in PC therapy.

Gut microbiota and inflammation

The gut microbiome (hidden organ) comprises at least 10^{14} microorganisms belonging mostly to the phyla *Firmicutes* and *Bacteroidetes*, which play an important role in obesity and other metabolic disorders [72]. Recent evidences suggest that diet, environmental factors and microbial components can contribute to the development of cancer in liver and pancreas through a gut-liver/pancreas axis [73]. As shown in Fig. 3, a high-fat diet can alter the gut microbiome and trigger an inflammatory cascade. Gram-negative bacteria secrete lipopolysaccharide (LPS), which induces low-grade inflammation through its binding to toll-like receptors (TLRs) and CD14 co-receptors present on monocytes, macrophages and neutrophils [74, 75]. Furthermore, altered gut microbiota may lead to decreased intestinal tight junction proteins (ZO-1 and occludin), which allows LPS entry into circulation [76]. Binding of LPS to its up-regulated receptors (CD14 or TLRs) on immune cells induces PC cell proliferation [77, 78]. Additionally, these immune cells also play a role in cancer cell invasion, angiogenesis and metastasis [79–81] by recruiting myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) adaptor molecules. Activation of these molecules leads to inflammation by up-regulating p44/42 mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK) and NF-κB pathways (Fig. 3). Therefore, an altered gut microbiota may promote cancer by driving inflammatory responses [82]. In support of this, germ-free (absent microflora) mice are less prone to carcinogenesis probably due to a decrease in tumor-associated inflammation [83, 84]. Similar results were observed when WT mice were treated with broad-spectrum antibiotics to inhibit the microbiota [85]. As final evidence, antigenic peptide secreted from *Helicobacter pylori* (which causes gastric ulcers) has been associated with PC pathogenesis [86]. *H. pylori* components translocate into the pancreas from the gut and activate NF-κB, thereby increasing the expression of pro-inflammatory cytokines involved in PC initiation and progression [87]. A recent study by Sethi et al. demonstrated that the gut microbiome modulation may have impact on tumor growth in a mouse model. Initially, the authors orally administered a cocktail of broad-spectrum antibiotics to C57BL/6J mice for 15 days. Then at 15 days, a pancreatic cell line derived from $Kras^{G12D/+}$; $Trp53^{R172H/+}$; $Pdx1^{cre}$ (KPC) mice was injected subcutaneously or intrasplenically (to induce liver metastasis). Results of this study showed that absence of gut microbiota led to a significant decrease in subcutaneous tumors, and decreased degree of liver metastasis. Besides, an absence of gut microbiota shows a significant increase in anti-tumor mature T cells [Th1 (IFN gamma^{+}CD4^{+}CD3^{+}) and Tc1 cells (IFN gamma^{+}CD8^{+}CD3^{+})] in the tumor microenvironment with an unknown mechanism. Finally, the relative abundance of *Bacteroidetes* and *Firmicutes* phyla decreased in fecal samples upon antibiotic administration in KPC mice. The authors concluded that modulation of gut microbiota on tumor progression could be a novel immunotherapeutic strategy [88].

In general, pancreatic tumors depend on carbohydrate metabolism for their survival, growth and resistance to chemotherapy. Dietary carbohydrates are usually fully metabolized in the small intestine, with the exception of resistant starch. Gut microbiota further process the starch in the large intestine through fermentation, and as a result, short-chain fatty acids (acetate, butyrate and propionate) are released. Resistant starch by avoiding degradation in small intestine imparts several health benefits via decreasing circulatory glucose levels, body weight, and inflammation without causing any side effects [89]. Interestingly, media engineered to mimic resistant starch (low glucose concentration) decreased PC cell proliferation compared

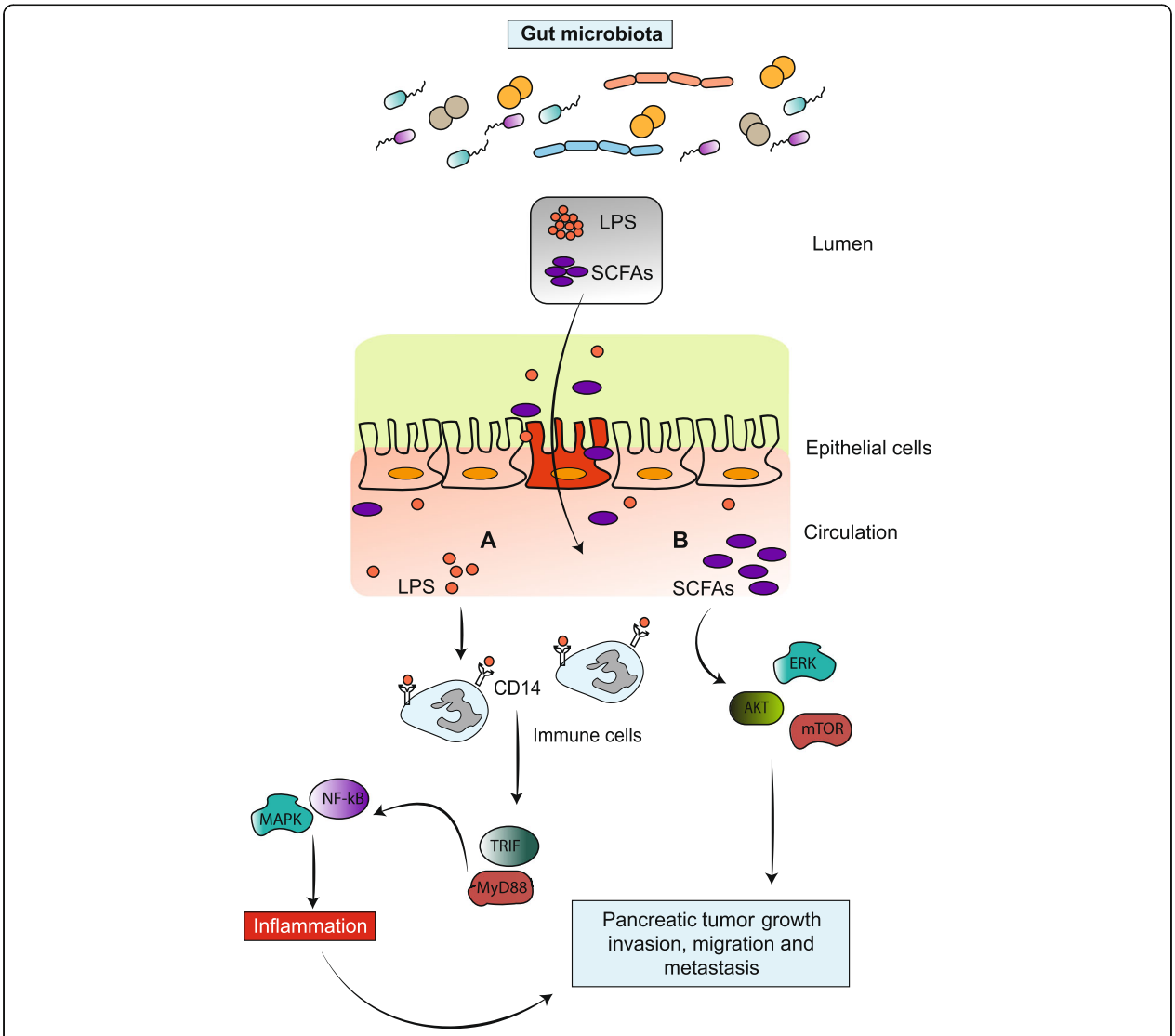

Fig. 3 Altered gut microbiota is responsible for pancreatic cancer progression. **a**. High-fat diet intake alters the gut microbiota composition. Altered gut microbiota secrete lipopolysaccharides (LPS), which enters circulation by damaging intestinal tight junction proteins. Circulatory LPS then binds to the toll like receptor on immune cells to recruit MyD88 or TRIF adaptor molecules. These molecules further activate MAPK and NF-κB pathways to activate several inflammatory cytokines, leading to cancer cell proliferation. **b**. Short-chain fatty acids (SCFAs) are released from resistant starch by the gut microbiota that enters the circulation. Afterward, SCFAs bind to G-protein-coupled receptors to activate the MAPK signaling pathway, triggering cancer cell proliferation.

with control media. The decrease in cell proliferation is due to down-regulation of ERK and mTOR signaling (Fig. 3). Similarly, mice bearing sub-cutaneous PC tumors fed a resistant starch diet showed lower tumor weight than controls on a normal diet. Additionally, resistant starch also inhibits the growth of inflammation-causing organisms including *Bacteroides acidifaciens*, *Ruminococcus gnavus*, *Clostridium cocleatum* and *Escherichia coli* in mice by modulating gut microbiota [90].

Early metastasis (primarily at lymph nodes and liver) and chemoresistance are responsible for PC aggressiveness. However, treatment with gemcitabine, first line therapy for metastatic PC, results in altered gut microbiota, which affects PC growth. Administration of gemcitabine in nude mice bearing subcutaneous PC cell line tumors leads to increased growth of *Proteobacteria* and *Akkermansia muciniphila,* which potentiate inflammation and/or mucin degradation. The imbalance of gut microbiome due to gemcitabine treatment also disrupts the intestinal integrity; this, in turn, favors the entry of microorganisms or their components into the circulation to reach distant organs. In the pancreas, the microbe-associated molecular patterns (such as LPS and endotoxins) on the microbial surfaces bind to

TLRs, activating inflammation through NF-kB signaling. In addition, gemcitabine-treated mice have greater LPS-induced inflammation and lower levels of inosine (a naturally occurring metabolite of adenosine), which has anti-inflammatory and immunosuppressive effects [91]. Furthermore, fecal microbiota obtained from KPC mice was recolonized into antibiotic treated WT mice which shows higher bacterial population access into the pancreas. Ablation of gut microbiota in *Ptfla*^{Cre}; LSL-*Kras*^{G12D} (KC) mice by oral antibiotics were recolonized with feces derived from WT or KPC mice and pancreatic tumor growth acceleration was observed in KPC derived feces only. Similarly, recolonization of feces (from KPC animal bearing pancreatic tumors) in germ free (GF)-KC mice shows increased pancreatic tumor growth as compared to GF-WT mice. This tumor acceleration might be associated with a decrease in activated T-cell infiltration in GF condition. They hypothesized that antibiotic treatment results in an increased intratumoral CD8:CD4 T-cell ratio which activates immunogenicity in PC. Future studies are warranted to identify microbial signatures that influence growth of PC tumors [92]. Taken together, a better understanding of the role of gut microbiota in PC tumor progression could open up new avenues in PC therapy development.

In obesity, pro-inflammatory cytokines are released from AT macrophages and infiltrate into AT; however the exact mechanism for these events is not known. In obese rats and humans, elevated inflammatory cytokine TNF-α activates other cytokines, in particular, IL-6, promoting angiogenesis and metastasis [93–95]. Therefore, the possible common mechanism by which obesity induces inflammation in several cancers (pancreatic, lymphoma and glioblastoma) might be through TNF-α-induced NF-κB signaling [96–98]. In addition, TNF-α secreted from cancer cells triggers cancer associated fibroblasts to stimulate macrophage infiltration [99, 100]. This infiltration occurs in several cancers through TNF-α-induced IL-6 to up-regulate STAT3 signaling [101]. Mice with PC tumors and diet-induced or genetic obesity expressed significantly higher STAT3 in the PC tumors. The up-regulation of STAT3 can drive PC progression through the activation of anti-apoptotic and proliferative proteins (Bcl-X_L, Mcl-1, Survivin, c-Myc and cyclin D1) as well as matrix metalloproteinases [102–104]. Currently, studies are focused on the role of AT-derived inflammatory cytokines in modulating signaling pathways that can indirectly influence the progression of PC.

Glucose metabolic enzymes

Despite the harsh hypoxic environment, PC survives in part due to expression of HIF1-α, which prevents apoptosis and increases the synthesis of glycolytic enzymes and transporter proteins [105]. According to the Warburg effect, the cancer cell depends on glycolysis to produce energy instead of aerobic respiration [106–108]. The most important rate-limiting glycolytic enzymes are pyruvate kinase (PKM2), which catalyzes the conversion of phosphoenol pyruvate to pyruvate, and lactate dehydrogenase (LDHA), which then catalyzes conversion of pyruvate to lactate. The glycolytic pathway releases high-energy phosphates in the form of nicotinamide adenine dinucleotide, which enters the mitochondria for energy synthesis. LDHA is overexpressed throughout carcinogenesis, while PKM2 expression increases during the transition of cystic lesions to cancer. A possible explanation is that cystic lesions require high levels of LDHA, which induces PKM2 splicing in a later stage of tumor proliferation [109]. Furthermore, activation of EGFR initiates translocation of PKM2 to the nucleus where it binds to β-catenin, resulting in up-regulation of cyclin D1, Stat3, Oct4 and HIF, which induce cell proliferation [110, 111]. Therefore, both glycolytic enzymes (PKM2 and LDHA) are possible targets for PC treatment in preclinical studies.

Hepatocyte growth factor

In addition to adipokines, pre-adipocytes as well as mature AT secrete cytokines and growth factors that have a role in tumor growth. In pancreatic tumor progression, cross-talk between PSC and PC is mediated through several growth factors including platelet-derived growth factor, transforming growth factor, vascular endothelial growth factor and hepatocyte growth factor (HGF) [112, 113]. HGF has received much attention due to its mitogenic signal and its angiogenic effects on AT [114, 115]. In the case of obesity, HGF is released from the AT and the resulting circulatory levels contribute to pancreatic cell proliferation [116]. Exogenous supplementation of HGF induces proliferation in a murine pancreatic cell line (Pan02) through its receptor c-MET, whereas in the absence of c-MET, HGF had no direct effects in a murine pancreatic cell line and indirectly inhibited apoptotic cell death [117]. HGF inhibition by means of neutralizing antibody (AMG102) inhibited tumor growth and metastasis as compared to gemcitabine treatment [118]. Over expression of c-Met renders PC cells resistant to gemcitabine and radiation [44, 119] through an unknown mechanism. As one possibility, Cui and co-workers demonstrated that the Forkhead box M1 (FOXM1) transcription factor regulates c-MET expression via ERK, AKT and STAT3 pathways, creating a positive feedback loop that promotes tumor growth. Further, inhibition of c-MET, FOXM1, ERK, AKT and STAT3 signaling pathways with their respective inhibitors abolished the c-MET positive loop [120]. Therefore, the HGF/c-MET feedback loop regulates tumor

proliferation, invasion and migration [121] and may be a novel target for growth factor-induced tumor growth.

Hyaluronan

In obesity, TG accumulates in the pancreas along with other organs and results in inflammation, higher expression of cytokines and remodeling of extracellular matrix (ECM). Hyaluronic acid or hyaluronan (HA) is a glycosaminoglycan and ubiquitous component of ECM which increases interstitial fluid pressure (IFP) and also reduces entry of chemotherapeutic drugs in PC tumors [122]. In tumor progression, the cross-talk between cancer cells and ECM is very important. Normally, HA synthesized by hyaluronan synthase (HAS) and secreted into the ECM under controlled conditions. However, increased expression of HA was observed in insulin-resistant mice aorta [123] and in the pancreas of diabetic mice [124]. In addition, expression of HA in the ECM is associated with diet-induced insulin resistance and was reversed upon treatment with the drug pegylated recombinant human hyaluronidase (PEGPH20), which improves insulin sensitivity in muscle tissue [125].

The PC stroma cells and the ECM express abundant HA to maintain a supportive tumor microenvironment [126]. Binding of HA to its receptors [cluster of differentiation-44 (CD44) or receptor for HA-mediated motility (RHAMM)], activates Ras and PI3K signaling, leading to increased cell proliferation, migration, and metastasis. Further, the activated PI3K pathway in cancer cells also increases drug resistance via activation of a multi-drug receptor [127–129]. The HA receptor CD44/RHAMM mediates cell-cell/matrix interactions and up-regulation of HA (around 12-fold increase) is observed in PC [130–133]. PC cells increase expression of HA via epigenetic regulation (decreased DNA methylation) and concomitant up-regulation of its enzyme HAS [134]. HA exists in low and high molecular weight forms. *In vitro* treatment with low molecular weight HA (25-75 kDa) increased PC cell motility compared to treatment with high molecular weight HA (400-600 kDa) [135, 136]. In conclusion, inhibition of HA synthesis may be a possible therapeutic strategy against PC and obesity-associated PC. Recently, PEGPH20 has gained interest to target HA for improving intratumoral microenvironment in PC. The different concentrations of HA along with mouse PC cells were implanted in immunodeficient mice that showed high IFP which reduce delivery of chemotherapeutic drugs. So targeting HA, a single high dose of PEGPH20 had a significant reduction on IFP in KPC mice. Further, a combination of PEGPH20 and gemcitabine showed decrease in cell proliferation and increased apoptosis in KPC mice [137]. In a randomized phase II clinical study, metastatic PC patients (231 were selected from a total of 279 patients) were treated with nab-paclitaxel/gemcitabine (AG) or PEGPH20 + nab-paclitaxel/gemcitabine (PAG). Patients (n=84) who had HA-high tumors showed improvement in the progression-free survival, overall survival and reduction in the thromboembolic (TE) incidence by PAG alone. Furthermore, PAG treatment was accompanied by more muscle spasm, neutropenia, myalgia and TE as compared to AG. Overall, srudy finding suggested that tumor HA could be a promising therapeutic target for PC patients with high HA [138].

Diabetes mellitus associated pancreatic ductal adenocarcinoma

Obesity is associated with insulin resistance and T2DM, which in turn is a potential risk factor for PC. In a post-prandial state, insulin maintains the levels of circulating glucose and FFAs. Insulin resistance is a condition in which the adipose and muscle tissues and to a lesser extent the pancreas, brain, liver and kidney are unable to respond to insulin. Insulin resistance is a hallmark of T2DM, leading to down-regulation of insulin signaling pathways (at the post-receptor level) in these tissues [139]. Of the diabetic population, 12% are diagnosed with type 1 diabetes, 80% with T2DM, and 8% with pancreatic diabetes (acute and chronic) [140]. About 80% of the PC population have insulin resistance or frank diabetes and are diagnosed at the metastasis stage. However, recent-onset diabetic patients developing diabetes at later age (average age greater than or equal to50) accompanied with weight loss and exceesive exocrine damage (PC associated diabetes mellitus) were higher risk for PC than long term diabetic population [141]. Pharmacological therapies like metformin (lowers blood glucose and insulin levels), sulfonylurea (promotes secretion of insulin from the pancreas) and insulin analogues (glargine) are available to treat diabetes [142, 143]; however these treatments often fail after prolonged usage. However, a case-control study at M.D. Anderson Cancer Center from 2004 to 2008 recruited 973 PDAC patients among them 259 were diabetic. The diabetic patients who received metformin had a lower risk of PC compared to those who were not given metformin; whereas, insulin or insulin secretagogues administered diabetic patients had a higher risk of PC [144].

As mentioned, T2DM is also a major risk factor for several cancers including PC. Epidemiological studies indicate that T2DM patients have a 1.8-fold increased risk for PC development [145]. However, the literature suggests that insulin resistance and diabetes may be a consequence of PC (up to 50-80% of cases). Clinical studies reveal that 0.85% (8 out of 2122) to 7% (6 out of 86) of diabetic patients were first diagnosed with PC [146, 147]. In PC, the increase in circulatory FFAs secreted from AT causes lipotoxicity in β-cells, resulting in

PC-associated diabetes mellitus (PCDM) [145, 148, 149]. After tumor resection, the increased survival of PC patients was associated with greater insulin sensitivity [150]. Recently, the American Diabetic Association classified PCDM, which is induced by chronic pancreatitis and pancreatic surgery, as type3c diabetes mellitus [151]. Still, evidence describing how diabetes leads to PC or *vice versa* is lacking. Some of the key molecules secreted from AT are being considered for the treatment of PCDM, which we focus on below.

Adrenomedullin and extracellular vesicles (exosomes)

Adrenomedullin (AM) is expressed by F-cells of the pancreas and plays a role in PC along with its receptor (adrenomedullin receptor ADMR). In 1993, AM was initially isolated from an adrenal medulla tumor called a pheochromocytoma. It is also expressed in AT and acts on pancreatic β-cells to inhibit insulin secretion; however, its effects on β-cells are poorly understood [152]. The circulatory levels of AM are very low under normal conditions; however, its levels are elevated in PC to cause insulin resistance [153]. Pancreatic beta, endothelial and stellate cells express ADMR. Its autocrine function in modulating tumor growth and progression has been evaluated in certain PC cell lines, i.e. Panc-1, BxPC3, and MPanc96 as well as human PSC and endothelial cells [154]. In a study, treatment with AM antagonist reduced PC tumor growth which indicating that AM plays a role in promoting PC progression. Furthermore, silencing of ADMR inhibited tumor growth and metastasis in liver and lung tissues of xenograft mice [155]. In PCDM, plasma levels of AM were significantly higher compared to diabetic patients alone, and its expression is higher in tumor and hypoxia conditions [152, 156].

AM is transported in the pancreas by extracellular vesicles, which contain proteins, lipids, and nucleic acids and are secreted by all cell types into circulation [157]. These vesicles play an important role in the transportation of biological components to other cells and tissues [158]. Extracellular vesicles form exosomes (30-100 nm) by inward or reverse budding of vesicular bodies called microvesicles (100-1000 nm) or by outward blebbing of membrane and apoptotic bodies (500-2000 nm) [159, 160]. Exosomes derived from PC cells have the capacity to promote metastasis in a tissue such as liver by residing in a pre-metastatic niche. The niche contains macrophage migration inhibitory factor engulfed by Kupffer cells, which induces secretion of fibronectin in the liver. The secreted fibronectin inhibits infiltration of macrophages and neutrophils derived from the bone marrow and promotes tumor growth [161]. PC exosomes control the specific site of organ metastasis by producing integrins, molecules that mediate cell adhesion. For example, Kupffer, lung fibroblast and epithelial cells recognize integrins such as αvβ5, α6β1 and α6β4, respectively, and subsequently recruit PC cells to these organs [162]. PC exosomes can also transfer AM to pancreatic β-cells (via caveolin-dependent endocytosis and micropinocytosis), which causes insulin resistance through ADMR-AM interactions. Furthermore, the presence of exosomal AM results in β-cell damage by increasing the production of reactive oxygen/nitrogen species and by increasing endoplasmic reticular stress markers (Bip and Chop) [163]. PC cell exosomes containing AM enter AT by the same mechanism that occurs in pancreatic β-cells. Internalization of AM results in lipolysis by activation of hormone-sensitive lipase through p38 and MAPK/ERK pathways; the resulting effect is growth and differentiation of the cancer cells [164]. Another similar peptide to AM is AM-2, which was identified in rats in 2004. AM2 has a similar function as AM in promoting angiogenesis, tumor development, progression and metastasis through MAPK signaling [165]. However, no studies have examined the role of AM-2 in PCDM.

Vanin and matrix metalloproteinase

Another important molecule is vanin 1 (VNN1, pantetheinase) present on the surface of epithelial and myeloid cells and highly expressed in the gut and liver tissue [166, 167]. VNN1 is mainly responsible for the breakdown of pantetheine to pantothenic acid (vitamin B_5) and cysteamine [168]. It is actively involved in inflammation, migration, stress, and glucose and lipid metabolism. Alteration of glucose and lipid metabolic pathways in the liver leads to development of insulin resistance and eventual T2DM. Mice exhibiting diet-induced obesity and Zucker diabetic fatty rats (model for T2DM) have more VNN1 activity in plasma as well as higher expression in the liver compared to normal controls [169]. Based on gene expression profiling, PCDM patients express higher VNN1 and MMP9 levels in peripheral blood as compared to patients with T2DM alone [170]. VNN1 reduces inflammation in PCDM by altering the levels of cysteamine and glutathione. VNN1 along with cysteamine protect the pancreatic β-cells from the oxidative stress generated during streptozotocin-induced diabetes in animals [171]. Enhanced γ-glutamylcysteine synthetase activity observed in vanin-$1^{-/-}$ deficient mice with low levels of cysteamine resulted in an accumulation of endogenous glutathione (GSH) levels [172, 173]. By contrast, over expression of VNN1 decreased expression of GSH and PPARγ, resulting in increased oxidative stress in PCDM [174], through an unknown mechanism. These findings suggest that decreased GSH and PPARγ might contribute to islet dysfunction in PCDM and that vanin-1 and MMP9 could serve as novel pharmacological targets to treat early asymptomatic PCDM patients.

Impact of obesity and diabetes on acinar, ductal and islet cells

The pancreas islets, acinar cells, and ducts of the gland make up approximately 2-3%, 85% and 5% of the volume, respectively. Similar to other organs, pancreas size is regulated by genetic as well as environmental factors (food intake) [175]. Feeding chronic high fat diet to Zucker diabetic fatty rats (model for both obesity and T2DM) showed excessive fat accumulation in pancreatic acinar cells and later resulted in acinar cell injury and pancreatic fibrosis [176]. In another study, feeding high fat or high calorie-diets to *Pdx-1*^{Cre} and LSL-*Kras*^{G12D} mice caused increased PSC activation, stromal fibrosis and infiltration by inflammatory cells [177]. In case of T2DM, both islets and peri-islet exocrine tissue of pancreas have an activated PSC. The activated, as well as quiescent PSC express receptors for insulin and insulin-like growth factor, however in activated PSC; insulin enhances cell proliferation and production of extracellular matrix proteins as compared to quiescent PSC [178]. Moreover, obese and T2DM patients show a ten-folds and four-folds increase in pancreatic ductal cell replication (more Ki67 expression), respectively. The increased pancreatic ductal cell replication is a risk factor towards pancreatitis and pancreatic cancer in obesity and or type 2 diabetes subjects [179].

Obesity and diabetes associated PC stem cells

Studies are suggesting that tumor initiation, progression, and resistance to chemotherapy is due to the presence of a small subset of a cell population within the tumor called cancer stem cells [180–182]. The presence of stem cell markers in normal pancreas might be involved in the progression of PC and resistance to drugs [183]. In obesity, leptin treatment affected PC progression and increased pancreatic cancer stem cell markers such as CD24/CD44/ESA, ALDH, CD133, and Oct-4. Further, the expression of leptin receptor was decreased by tumor suppressor micro RNAs that specifically target pancreatic stem cell markers (Met, ABCB1, and CD44) to reduce their expression [184]. Leptin is also involved in the growth of PC tumorspheres and resistance to the chemotherapeutic drug (gemcitabine) [185] by increasing the stem cell markers (CD24, CD44, ESA, CD133, and ALDH) in MiaPaCa-2 PC cell line. Additionally, leptin up-regulates the expression of ABCB1 (an ATP binding transporter protein) in PC tumorspheres suggesting its role in stem cell stimulation and chemoresistance [70]. In case of diabetic population, hyperglycemia is a hallmark of T2DM which stimulates PC by promoting a epithelial to mesenchymal transition and expression of pluripotency stem cell markers (Sox2, Oct4, and Nanog) via activating transforming growth factor-beta 1 [186]. Further, studies are needed to understand the exact molecular mechanisms involved in metabolic diseases associated with PC stem cells.

Conclusions

Several studies suggest that obesity and T2DM increase the risk for PC development and its pathogenesis. However, mechanistic interplay responsible for development and progression of pancreatic tumor remains obscure. Recent studies on key players associated with obesity and diabetes such as adipocytokines, gut microbiota, adrenomedullin, hyaluronan, vanin and matrix metalloproteinase have deciphered unknown linkage present across PC as well as PCDM. These mediators play central role in promoting obesity-and diabetes-associated pancreatic cancer, however, to date studies involving therapeutic targeting and harnessing their biomarker potential are still in infancy. Henceforth, based on literature survey, we suggest that there is an urgent need to delineate biomarkers as well as therapeutic target(s) involved in the obesity and T2DM associated PC development making inroads to prevent this highly lethal malignancy.

Abbreviations

AdipoQ: Adiponectin; ADMR: Adrenomedullin receptor; AG: Nab-paclitaxel/gemcitabine; AM: Adrenomedullin; AMPK: Adenosine monophosphate-activated protein kinase; AT: Adipose tissue; ECM: Extracellular matrix; EGFR: Epidermal growth factor receptor; FFA: Free fatty acids; GF: Germ-free; HA: Hyaluronan; HAS: Hyaluronan synthase; HGF: Hepatocyte growth factor; HIF: Hypoxia inducible factor; HRE: Hormone response element; IFP: Interstitial fluid pressure; IL: Interleukin; KC: *Kras*$^{G12D/+}$;*Ptf1a*^{Cre}; KPC: *Kras*$^{G12D/+}$ *Trp53*$^{R172H/+}$; KRAS: Kirsten rat sarcoma viral oncogene homolog; OBR: Leptin receptor; LPS: Lipopolysaccharide; MMPs: Matrix metalloproteinases; PAG: PEGPH20 + nab-paclitaxel/gemcitabine; PanIn: Pancreatic intra-epithelial neoplasia lesions; PC: Pancreatic cancer; PCDM: PC-associated diabetes mellitus; PDAC: Pancreatic ductal adenocarcinoma; PEGPH20: Pegylated recombinant human hyaluronidase; PFS: Progression-free survival; PSC: Pancreatic stellate cells; T2DM: Type 2 diabetes mellitus; TE: Thromboembolic event rate; TG: Triglycerides; TLR: Toll like receptor; TNF: Tumor necrotic factor; VNN: Vannin; WT: Wild-type

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Authors' contributions

RP, SR and SKB planned the manuscript. SR, WMJ, SC, SV, SK and SKB revised and corrected the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Competing interests

SKB is one of the co-founders of Sanguine Diagnostics and Therapeutics, Inc. WMJ is Chief Scientific Officer of Sanguine Diagnostics and Therapeutics, Inc. Other authors declare no competing interests.

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