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CLINICAL TRIAL

Randomized window of opportunity trial evaluating high-dose vitamin D in breast cancer patients

Angel Arnaout[1,2] · Susan Robertson[3] · Gregory R. Pond[4] · Reinhold Vieth[5] · Ahwon Jeong[2] · John Hilton[2,6] · Timothy Ramsey[7] · Mark Clemons[2,6]

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Abstract

Purpose Epidemiologic and preclinical data suggest a potential role for vitamin D in breast cancer treatment and prevention. However, results of prospective randomized trials are inconsistent. The objective of this study was to assess the effects of high-dose cholecalciferol (vitamin D3) on breast tumour proliferation and apoptosis.

Methods We conducted a prospective, randomized, phase 2, double-blinded pre-surgical window of opportunity trial. Newly diagnosed breast cancer patients were randomized to receive 40,000 IU of vitamin D3 per day or placebo for 2 to 6 weeks prior to breast surgery. The primary outcome was the relative change in proliferation (Ki67) and apoptosis (cleaved caspase 3 apoptotic assay [CC3]) in primary breast cancer cells pre and post treatment.

Results Of 83 patients randomized, 80 completed the study (43 (53.8%) vitamin D and 37 (46.3%) placebo). Mean duration of drug intake was 19 days (range 9–28 days). There were no significant differences between the control arm and the vitamin D arm in percent changes of either Ki67 index (1.6% vs. 16.7%, $p=0.25$) or CC3 (−55.9% vs. −45.9%, $p=0.28$). Serum 25-hydroxyvitamin D (25-OHD) levels were 3 times higher in the vitamin D arm (62 nmol/L vs. 246 nmol/L, $p<0.001$). Adverse effects were minimal and all classified as grade 1.

Conclusions Despite significantly higher levels of serum 25-OHD in the vitamin D-treated group, this was not associated with any significant effects on tumour proliferation or apoptosis. These findings are consistent with the lack of benefit observed in prospective prevention trials.

Trial registry *Trial registration* clinicaltrials.gov NCT01948128.

Keywords Vitamin D · Window of opportunity · Clinical trial · Breast cancer

✉ Mark Clemons
mclemons@toh.ca

1 Division of Surgical Oncology, Department of Surgery, Ottawa Hospital, Ottawa, Canada

2 Cancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, Canada

3 Division of Anatomical Pathology, Ottawa Hospital, Ottawa, Canada

4 Department of Oncology, McMaster University, Hamilton, Canada

5 Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

6 Division of Medical Oncology, Department of Medicine, University of Ottawa and Ottawa Hospital Cancer Center, Ottawa, ON, Canada

7 Center for Practice Changing Research, Ottawa Hospital Research Institute, Ottawa, Canada

Background

Although some vitamin D is derived from the diet, most is produced from sunlight-mediated conversion of dehydrocholesterol in the skin to cholecalciferol (vitamin D3). Vitamin D3 appears to have little biological activity and must undergo 2 hydroxylation in order to become biologically active [1]. The first hydroxylation occurs in the liver and gives rise to 25-hydroxyvitamin D (25-OHD), the circulating metabolite in the blood by which we assess serum vitamin D levels. A further hydroxylation in the kidney converts 25-OHD to 1,25-(OH)2D3 (calcitriol) [1, 2]. It is calcitriol that is considered to be the biologically active form of vitamin D [2].

Studies have linked serum levels of vitamin D, i.e. 25-OHD to cancer incidence and survival, with most concluding that low levels are associated with increased

incidence and decreased survival [2–4]. In addition, activation of the vitamin D receptor (VDR) by the potent metabolite calcitriol in breast tumour cells has demonstrated inhibition of cell cycle, induction of cell death through apoptosis and induction of differentiation [5, 6]. Laboratory studies have also shown that increased vitamin D action suppresses breast cancer cell migration and metastasis [7, 8]. Together, these epidemiologic and preclinical data have raised the theory that increasing the levels of vitamin D could have clinically beneficial effects against breast tumours in humans [8, 9]. However, existing randomized trials have focused on the role of vitamin D supplementation in breast cancer prevention [10–12], and evidence from patients with breast cancer is limited to a retrospective study in the adjuvant setting which showed improved disease-free survival and a prospective study in patients with metastatic breast cancer which showed no significant benefit [13, 14].

Window of opportunity trials can provide insight into biological effects and potential therapeutic efficacy of novel therapeutic strategies [15, 16]. In these studies, newly diagnosed patients receive a study agent in the time between the diagnostic breast biopsy and surgery. This allows the evaluation of agents in tissue samples obtained before and after drug exposure. Therefore, window of opportunity trials provide a rational basis for development of larger clinical trials. Here, we describe the results from a randomized window of opportunity trial evaluating the intra-tumoural effects of a high-dose oral vitamin D3 on breast cancer cellular proliferation and apoptosis.

Methods

Study design and participants

A randomized, double-blind, placebo-controlled window of opportunity trial was performed. Patients with operable breast cancer scheduled to undergo surgery were recruited from the Women's Breast Health Centre, Ottawa Hospital, Ottawa, Canada. Study eligibility included (a) invasive cancer clinically and/or radiologically ≥ 2 cm in size; (b) surgery date planned for 2–6 weeks after initial consultation; and (c) normal baseline serum and urine calcium and serum PTH. Patients were excluded if they had regular intake of vitamin D3 supplement ≥ 2000 IU/day, recurrent or metastatic breast cancer, a history of neoadjuvant hormonal therapy or chemotherapy, baseline urine hypercalciuria or a history of urolithiasis or hyperparathyroidism.

Randomization and intervention

Eligible patients were randomly allocated 1:1 to either 40,000 IU of vitamin D3 (Riva Pharmaceuticals, Canada) per day or lactose placebo with identical appearance and taste. The high dose of vitamin D3 has previously been used safely in a window of opportunity trial [17] and the literature has shown that up to 40,000 IU per day of vitamin D is unlikely to result in toxicity [18]. We therefore chose to administer 40,000 IU per day, a high dose, to allow for maximal possible effect. Both study preparations were taken once a day, for 2 to 6 weeks until the day before breast surgery. If patients were already taking standard dose vitamin D and calcium supplements, this was stopped prior to study entry. Capsules were prepared by the Ottawa Hospital Research Pharmacy according to the randomization master list, and investigators and patients were blinded to study allocations. The study protocol was approved by the Ottawa Hospital Research Ethics Boards and registered (clinicaltrials.gov: NCT01948128).

Outcomes

The primary outcome was the relative change in measures of proliferative and apoptotic response based on the Ki67 [19] and caspase 3 apoptotic assay [20] (using activated caspase 3) in primary breast cancer cells pre and post treatment with vitamin D3 or placebo. Secondary outcomes included serum 25-OHD and PTH and toxicity.

All pre-treatment diagnostic core biopsies (14 g needles) were immediately fixed in 10% neutral buffered formalin. If a post-treatment core was taken, the tissue processing was the same. If the surgical specimen was used, excisional breast specimens were sliced and exposed to formalin within 1 h. After standard tissue processing and embedding in paraffin wax, sections were cut and stained with Hematoxylin and Eosin or left unstained for immunohistochemistry.

Ki67 and caspase 3 apoptotic assay (CC3) Immunohistochemistry

Immunohistochemistry was performed with Ki67 antibody diluted 1:7 (clone MIB-1; Dako, Denmark) and Caspase-3 antibody diluted 1:5 (clone JHM62; Leica, Denmark) with 20-min retrieval with BOND Epitope Retrieval Solution 1 (citrate based pH 6.0) on BOND-Max platform, and visualized with BOND Polymer Refine Detection Kit. All samples were processed together in order to minimize variability. The ''Ki67 index'' (percentage of nuclei showing nuclear immunoreactivity of any intensity) was determined by computer image assisted count by a single pathologist (SR) blinded to the treatment arm. In each case, after a low-power scan of the entire tissue section, hot spot regions of highest activity were selected and from these 1000 tumour nuclei were counted at $\times 400$–600 magnification. For Caspase-3 immunohistochemical analysis, five hundred cells from each specimen under $\times 400$ magnification in the best-stained tumour

area of each section were counted by a single pathologist (SR) for each specimen. CC3 immunoreactivity score was defined as the percentage of stained cells.

Toxicity assessments

Participants underwent biochemical evaluation (serum creatinine, BUN, calcium, 25-OHD, PTH, urine calcium/creatinine ratios) at baseline, biweekly during follow-up, and on the day of surgery. The key safety indicator was urinary calcium excretion (ratio of millimolar concentrations of urine calcium and urine creatinine). During the study, patients were assessed biweekly for toxicity assessments using the Common Terminology Criteria for Adverse Events (CTCAE) [21]. Compliance was monitored by measuring the remaining number of capsules returned on the day of the surgery.

Statistical analyses

The randomization sequence was generated using computer software to produce randomly permutated blocks of 6. Each arm was stratified based on baseline Ki67 index of the initial diagnostic core biopsy into three levels: Ki67 low group (0–14%), Ki67 intermediate group (14.1–30%) and Ki67 high group (> 30%). Analysis was intention-to-treat and involved all patients randomized to the two study arms. All data were analysed with SPSS software (version 20) or SAS (version 9.2).

Descriptive statistics were used to summarize patient, tumour, treatment and outcome characteristics. The percentage change in laboratory measures was calculated as [post-surgery measure – baseline measure]/[baseline measure × 100%] and the absolute change was calculated as [post-surgery measure – baseline measure]. Primary analysis was based on a generalized linear regression model with adjustment for stratum. Secondary comparisons of measures between patients on treatment arm with patients on control arm were performed using Fisher's exact test (categorical variables), Wilcoxon rank-sum tests (continuous variables) or Cochran–Armitage test for trend (ordinal outcomes). Some dichotomization of factors occurred for statistical purposes. Given some potential violations of normality, supportive analyses were performed by performing statistical tests on linearly transformed data, specifically by applying a logarithmic transformation to the observed percent change plus 100%. Linear regression models were performed to investigate for prognostic factors of the change in Ki67 and change in caspase. All tests were two-sided and a p value of 0.05 or less was considered statistically significant. Based on the results of Dowsett et al. [22], the treatment effect (difference in means/SD of the change in Ki67) of anastrozole versus tamoxifen on the geometric mean of Ki67 was estimated to be at least 1.3-2. It was hypothesized that an effect size of at least half of what was observed for anastrozole was the minimum required to consider vitamin D for further study, hence the minimally clinically important difference was set to 0.65. Using a two-sided, alpha = 0.05, independent t-test would require a minimum of 36 patients per arm. To account for a potential loss to follow-up rate of 5%, a sample size of 38 patients per arm was targeted.

Results

Study population and characteristics

Between September 2014 and December 2015, 518 newly diagnosed invasive breast cancer patients were assessed. Of these, 98 (19%) patients were potentially eligible and approached (Fig. 1). There were 11 screen failures: abnormal urine calcium and/or PTH ($n = 7$); inadequate tissue from the diagnostic core biopsy (for baseline Ki67 and CC3 measures) and declined additional pre-treatment biopsies ($n = 3$); and current vitamin D intake of > 2000 IU/day ($n = 1$). Additionally, one patient went for surgery at another hospital, one had surgery expedited to less than 2 weeks, and two declined to participate. Overall, 83 patients were randomized: 45 (54%) to vitamin D and 38 (46%) to placebo. Further two patients did not complete their allocated intervention due to cancelled surgery and neoadjuvant therapy and one patient refused a repeat biopsy. Table 1 summarizes baseline patient, pathologic and treatment characteristics. The baseline characteristics of the tumours (Ki67 and CC3) and patient serum vitamin D levels (median 73 nmol/mL, range: 13-168) of the two groups were well matched.

Duration of treatment

The mean duration of drug intake was 19 days for both groups (range 9–28 days), while the mean wait time from consent to actual surgery date was 22 days (range 15–40 days).

Primary outcome measures

Changes in tumour Ki67 and CC3

A total of 80 patients who completed the study were included in the analysis, 37 in the control arm and 43 in the treatment arm. Baseline and post-treatment Ki67 indices and CC3 scores are listed in Table 2. The median absolute change in Ki67 index, calculated as the pre-treatment subtracted from the post-treatment value, was 0.9 (interquartile range −3.9 to +5.9) in the control arm and 4.0 (−2.2 to +12.3)

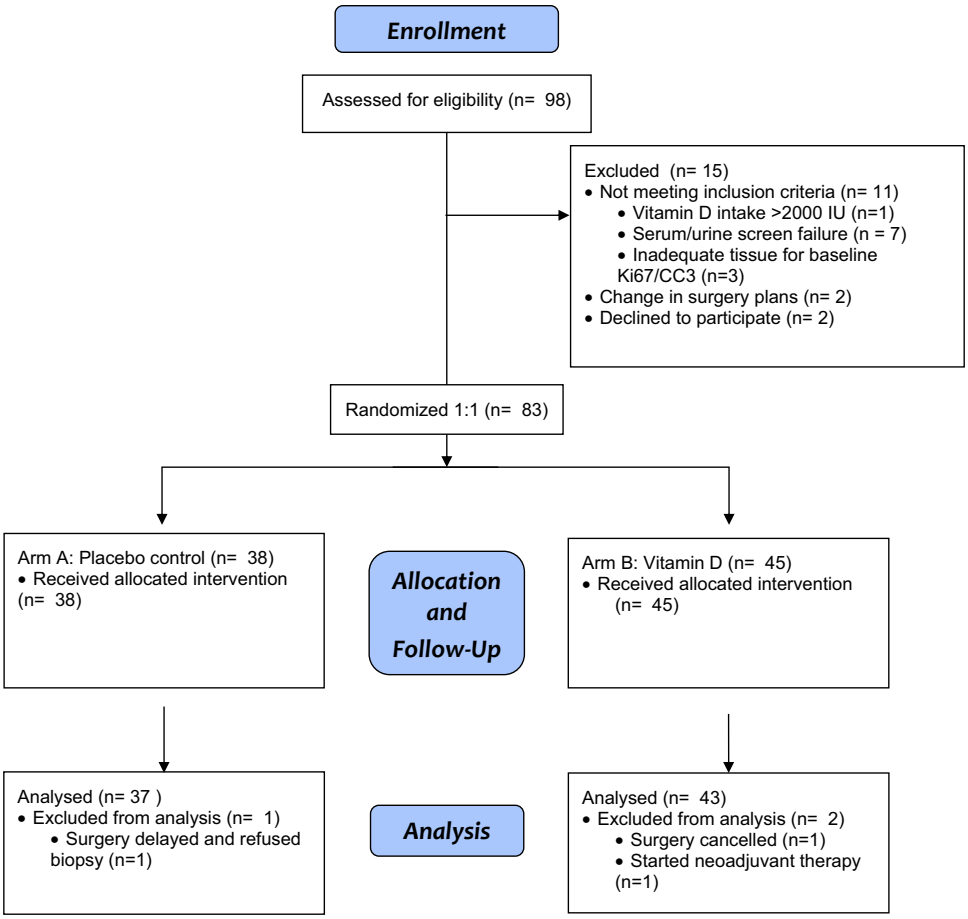

Fig. 1 Consort diagram

in the vitamin D treatment arm. The median percent change in Ki67 index was 1.6% (−15.9 to +55.0%) in the control arm and 16.7% (−5.3 to +83.0%) in the vitamin D treatment arm. The percent change in Ki67 index was not statistically significantly different between the two arms (Wilcoxon rank-sum $p=0.25$ and regression $p=0.14$ adjusted for stratum). Figure 2 demonstrates the Ki67 results before and after drug treatment. Supportive analyses based on linear regression of linearly transformed data, both with and without adjustment for hormone status (ER, PR, Her2), vitamin D levels, and other baseline characteristics, showed similar results and are not shown for simplicity.

In order to account for variability in duration of drug intake, the percent change in Ki67 index was compared between those with < 4 weeks of treatment and those with > 4 weeks of treatment. The median percent change in Ki67 index was +17.5% (IQR −2.6 to +96.7%) in those with a shorter treatment duration ($n=35$) and −38.3% (IQR −43.7% to −15.5%) in those with > 4 weeks of treatment ($n=5$). There was a statistically significant difference between these two groups ($p=0.03$).

There was a significant difference in the percent change in Ki67 following stratification based on baseline Ki67 (0–14, 14–30, 30+). For patients with a baseline Ki67 < 14 ($n=11$), the median percent change was +118.4% (IQR +17.5 to +236.1%), while those with a baseline Ki67 between 14 and 30 ($n=11$) had a median percent change of +19.4% (IQR −33.8 to +114.3%), and in those with baseline Ki-67 > 30 ($n=18$), the median percent change was +2.6% (IQR −5.7 to +17.3%) ($p=0.014$).

The CC3 score was calculated by subtracting the pre-treatment from the post-treatment value, with positive values reflecting increased apoptosis and negative values reflecting reduced apoptosis. The median absolute change in CC3 score was −13.2 (IQR −35.0 to −1.9) in the control arm and −7.7 (−39.1 to 9.8) in the vitamin D treatment arm ($p=0.33$). The median percent change in CC3 score was −55.9% (−87.9 to 23.3%) in the control arm and −45.9% (−86.3 to 67.6%) in the vitamin D treatment arm ($p=0.28$) (Fig. 2).

Secondary outcomes measures

Biochemical

Compared to the placebo control, supplementation with high-dose vitamin D was associated with alterations in

Table 1 Baseline patient, pathologic and treatment characteristics

	Control $n=38$	Treatment $n=45$	All patients $n=83$
Age, mean (SD), years	53.7 (12.1)	54.6 (12.9)	54.2 (12.4)
BMI, median (range), kg/m^2	26.6 (19.8, 41.3)	25.3 (18.4, 61.3)	26.6 (18.4, 61.3)
Tumour characteristics			
Primary pathologic tumour type: ductal, No. (%)	30 (79.0)	36 (80.0)	66 (79.5)
Pathologic grade, No. (%)			
1	2 (5.3)	2 (4.4)	4 (4.8)
2	13 (34.2)	15 (33.3)	28 (33.7)
3	21 (55.3)	26 (57.8)	47 (56.6)
Unknown	2 (5.3)	2 (4.4)	4 (4.8)
ER			
Positive	25 (65.8)	33 (73.3)	58 (69.9)
Negative	9 (23.7)	9 (20.0)	18 (21.7)
Unknown	4 (10.5)	3 (6.7)	7 (8.4)
PR			
Positive	21 (55.3)	29 (64.4)	50 (60.2)
Negative	13 (34.2)	13 (28.9)	26 (31.3)
Unknown	4 (10.5)	3 (6.7)	7 (8.4)
HER2			
Positive	7 (18.4)	8 (17.8)	15 (18.1)
Negative	28 (73.7)	34 (75.6)	62 (74.7)
Unknown	3 (7.9)	3 (6.7)	6 (7.2)
Pathologic tumour size, median (range), cm	3.4 (1.7, 9)	3.2 (1.6, 10)	3.3 (1.6, 10)
Pathologic T stage			
T1c	6 (15.8)	5 (11.1)	11 (13.3)
T2	19 (50.0)	27 (60.0)	46 (55.4)
T3	9 (23.7)	27 (60.0)	16 (19.3)
Unknown	4 (10.5)	6 (13.3)	10 (12.1)
Pathologic N stage			
N0	12 (31.6)	16 (35.6)	28 (33.7)
N1	14 (36.8)	12 (26.7)	26 (31.3)
N2	8 (21.1)	7 (15.6)	15 (18.1)
N3	0 (0.0)	4 (8.9)	4 (4.8)
NA	4 (10.5)	6 (13.3)	10 (12.1)
Baseline Ki67, median (range), %	25.1 (1.1, 96)	28.7 (3.6, 89.7)	25.9 (1.1, 96)
Low: 0–14%, No. (%)	10 (26.3)	11 (24.4)	21 (25.3)
Intermediate: 14.1–30%, No. (%)	11 (29.0)	13 (28.9)	24 (28.9)
High: > 30%, No. (%)	17 (44.7)	21 (46.7)	38 (45.8)
Baseline CC3, median (range), %	29.6 (2.5, 83.7)	24.6 (0.5, 84.0)	28.2 (0.5, 84.0)
Post-treatment adjuvant therapy			
Adjuvant chemotherapy, No. (%)			
Yes	27 (71.1)	32 (71.1)	59 (71.1)
No	7 (18.4)	8 (17.8)	15 (18.1)
Unknown	0 (0.0)	1 (2.2)	1 (1.2)
Adjuvant radiation, No. (%)			
Yes	34 (89.5)	42 (93.3)	76 (91.6)
No	2 (5.3)	1 (2.2)	3 (3.6)
Unknown	2 (5.3)	2 (4.4)	4 (4.8)

CC3 cleaved caspase 3

Table 2 Ki67 labelling index (%) and cleaved caspase 3 (CC3)

	Control $n=37$	Treatment $n=43$	p value
Ki67 labelling index, median (IQR), %			
Baseline	25.1 (13.0, 60.8)	28.7 (14.2, 52.0)	0.72
Post surgery	28.0 (17.6, 48.4)	34 (17.5, 53.8)	0.74
% change in Ki67	1.6 (−15.9, 55.0)	16.7 (−5.3, 83.0)	0.25
Absolute change in Ki-67	0.9 (−3.9, 5.9)	4.0 (−2.2, 12.3)	0.11
Cleaved caspase 3 (CC3), median (IQR), %			
Baseline	29.6 (15.8, 45.9)	24.6 (8.5, 46.7)	0.61
Post surgery	9.0 (4.2, 18.2)	10.0 (5.8, 24.6)	0.39
% change in CC3	−55.9 (−87.9, 23.3)	−45.9 (−86.3, 67.6)	0.28
Absolute change in CC3	−13.2 (−35.0, −1.9)	−7.7 (−39.1, 9.8)	0.33

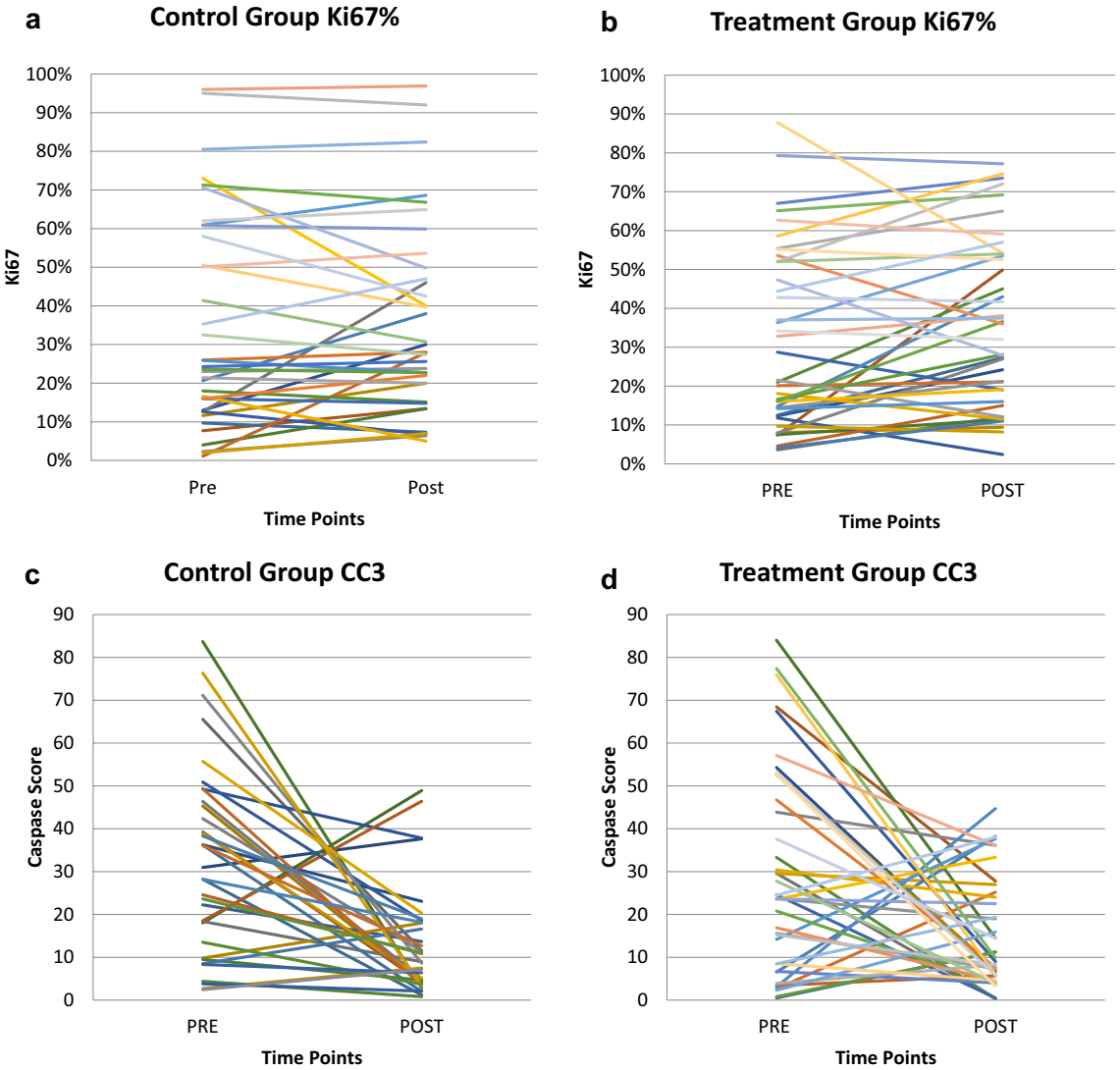

Fig. 2 Change in Ki67 index (**a**, **b**) and cleaved caspase 3 (CC3; **c**, **d**) pre and post vitamin D supplementation

serum BUN (0.5 mmol/L vs. −0.2 mmol/L, $p=0.017$), PTH (−1.1 pmol/L vs. 0 pmol/L, $p=0.023$), urine calcium/creatinine ratio (0.1 vs. −0.2, $p<0.001$), and serum 25-OHD levels (164.0 nmol/mL vs. −4.0 nmol/mL, $p<0.001$) (Table 3).

Toxicity

Adverse effects are summarized in Table 3. All adverse effects were classified as grade 1 and no patients developed hypercalcemia. Fatigue was the only adverse event with a significant difference between the two groups ($p=0.01$).

Discussion

There can be few supplements that have received as much attention as vitamin D. Combined data from epidemiological, animal models and cell line studies would appear to provide a rationale for investigating vitamin D as a potential anti-cancer treatment [1, 3, 23, 24]. Indeed, a retrospective study of vitamin D supplementation during adjuvant chemotherapy in HER2-positive breast cancer patients demonstrated improved disease-free survival [13]. However, recent large prevention trials showed that vitamin D supplementation does not lower cancer incidence [11, 12], and vitamin D supplementation in breast cancer patients with bone metastasis showed no palliative benefit or change in bone resorption [14]. In this context of conflicting evidence, window of opportunity trials provide a unique opportunity to evaluate the potential biological effects of agents in vivo [25, 26]. The current study evaluated the effects of daily high-dose vitamin D3 supplementation (40,000 IU) on tumour proliferation and apoptosis in breast cancer patients. The proliferate maker Ki67 and the apoptosis marker cleaved caspase 3 (CC3) were chosen as they have both been widely used in window trials and are associated with response to a range of active therapies [19, 20]. Despite a minimum of 2 weeks of study medication, we were unable to demonstrate any significant effect on either cancer cell proliferation or apoptosis. This is despite the expected physiological effects of vitamin D3 shown by decreases in PTH and the significant threefold elevation in blood 25-OHD levels in the vitamin D-treated arm.

So are the findings of this study simply confirming that high-dose vitamin D3 has no demonstrable anti-cancer effect in this pre-operative setting or are there challenges with the window of opportunity trial design that may explain these results? To our knowledge there are only two window trials evaluating vitamin D in the window setting. In one, a non-randomized trial of 33 post-menopausal breast cancer patients treated with calcitriol (0.50 µg/day) for 30 days, showed no significant difference in Ki67 compared with a historical control group [27]. A study in men with prostate cancer administered with 400, 10,000, or 40,000 IU/day of vitamin D (mean duration of treatment 33.6 ± 0.5 days) showed that higher doses lowered serum PTH and PSA compared to lower dose ($p<0.0001$ and $p=0.017$, respectively), but Ki67 expression did not differ significantly [17].

One potential reason for these negative results is that the doses of vitamin D3 used were not high enough. Given that the current recommendation by the Food and Nutrition Board of the Canadian Institute of Medicine is 600 IU/day of vitamin D for adults (< 70 years) for daily optimum maintenance of health purposes [28], the dose of vitamin D3 for the current study would certainly be deemed, "high". Second, was it possible that patients already had "sufficient" vitamin D3 so that supplementation was unlikely to have a physiological effect? 25-OHD, the circulating hormone precursor to the active tissue metabolite 1,25(OH)2D, is the accepted clinical indicator of vitamin D status and provides a comprehensive measure of vitamin D intake from all sources (diet, sunlight and supplementation). Although there is no ''standard'' cut-off definition of vitamin D status, a widely accepted classification using serum 25-OHD levels defines deficiency at < 50 nmol/L (20 ng/mL), insufficiency at 50–74 nmol/L (20–30 ng/mL), and an optimal range of ≥ 75 nmol/L (30 ng/mL) [29, 30]. Our results demonstrated that 19 patients (42%) in the vitamin D group and 23 patients (61%) in the placebo group were deemed "insufficient" or "deficient" at baseline, with a median 25-OHD level of 86 (range 31–168) and 67 (range 13–118), respectively. After the trial, all patients in the vitamin D group reached sufficiency with a median of 246 nmol/L (range 128–313) while 26 patients (70%) remained insufficient with a median of 72 nmol/L (range 12–115) in the placebo group ($p<0.001$). An exploratory analysis stratifying by baseline serum 25-OHD levels did not produce any significant associations with changes in Ki67 after treatment.

It is possible that the lack of effect on proliferation or apoptosis may be due in part to either a lack of statistical power or the short interval of treatment. The potential for length of treatment on Ki67 was assessed. Despite a statistically significant difference between those with < 4 weeks of treatment and > 4 weeks of treatment, a conclusion could not be made due to the small sample size. Window of opportunity trials do not allow longer duration of treatment without delaying surgery, which is a recognized challenge with such design.

Even in the absence of observed potential therapeutic benefit, there is the potential for harm that must be considered. Within the prevention studies, neither the ViDA study (oral vitamin D3 initial bolus of 200,000 IU followed by monthly doses of 100,000 IU) [11] nor the VITAL study (daily vitamin D3 2000 IU plus marine $n-3$ fatty acids at 1 g) [12] showed any significant toxicity in terms of hypercalcaemia (VIDA) nor hypercalcemia, kidney stones,

Table 3 All safety-related biochemical measures and side effects

	Control $n=37$	Treatment $n=43$	p value
Serum creatinine, median (range), μmol/L			
Baseline	58 (42, 81)	62 (43, 79)	0.25
Post surgery	58.5 (39, 74)	62 (23, 88)	0.34
Change in creatinine	−0.5 (−14, 16)	0 (−29, 12)	0.76
Serum BUN, median (range), mmol/L			
Baseline	4.6 (3.0, 8.1)	4.3 (1.8, 7.8)	0.33
Post surgery	4.2 (2.7, 7.6)	4.6 (1.0, 8.7)	0.27
Change in BUN	−0.2 (−2.7, 2.0)	0.5 (−2.4, 1.8)	0.017
Serum total calcium, median (range), mmol/L			
Baseline	2.30 (1.12, 2.44)	2.31 (2.14, 2.49)	0.43
Post surgery	2.25 (2.06, 2.41)	2.28 (1.69, 2.57)	0.047
Change in calcium	−0.06 (−0.26, 1.09)	−0.01 (−0.58, 0.14)	0.085
Serum PTH, median (range), pmol/L			
Baseline	5.8 (2.3, 9.1)	5.1 (2.0, 8.7)	0.064
Post surgery	5.7 (1.7, 18.9)	3.4 (1.8, 11.7)	<0.001
Change in PTH	0 (−4.6, 11.0)	−1.1 (−3.9, 4.6)	0.023
Urine Ca/Creatinine ratio, median (range)			
Baseline	0.5 (0.04, 1.0)	0.4 (0.1, 0.9)	0.12
Post surgery	0.3 (0.1, 1.3)	0.5 (0.1, 4.0)	0.002
Change in urine ratio	−0.2 (−0.7, 0.7)	0.1 (-0.5, 3.6)	<0.001
Vitamin D, median (range)[a], nmol/L			
Baseline	67 (13, 118)	86 (31, 168)	0.055
Post surgery	62 (12, 115)	246 (128, 313)	<0.001
Change in vitamin D	−4.0 (−31.0, 20.0)	164.0 (65.0, 244.0)	<0.001
Vitamin deficiency and insufficiency at baseline, *n* (%)			
<50 nmol/L	6 (15.8)	6 (13.3)	0.23
50–74 nmol/L	17 (44.7)	13 (28.9)	
75+nmol/L	15 (39.5)	26 (57.8)	
Vitamin deficiency and insufficiency post surgery, *n* (%)			
<50 nmol/L	6 (16.2)	0 (0.0)	<0.001
50–74 nmol/L	20 (54.1)	0 (0.0)	
75+nmol/L	11 (20.8)	42 (100.0)	
Adverse events, No. (%)			
Constipation	2 (5.4)	3 (7.0)	1.00
Dizziness	5 (13.5)	4 (9.3)	0.73
Headache	3 (8.1)	4 (9.3)	1.00
Joint pain	3 (8.1)	1 (2.3)	0.33
Heart burn	0 (0.0)	2 (4.7)	0.50
Fatigue	10 (27.0)	2 (4.7)	0.010
Dry mouth	5 (13.5)	2 (4.7)	0.24
Metallic taste	2 (5.4)	0 (0.0)	0.21
Nausea	3 (8.1)	1 (2.3)	0.33
Upset stomach	3 (8.1)	2 (4.7)	0.66
Loose bowel movement	2 (5.4)	2 (4.7)	1.00

BUN blood urea nitrogen, *PTH* parathyroid hormone

[a]1 nmol/L = 0.4 ng/mL

parathyroid condition or kidney failure (VITAL). However, a study of importance in an era of increased adjuvant bisphosphonates use in patients with early-stage breast cancer [31] was a phase 2 trial exploring the effects of high-dose vitamin D3 in breast cancer patients with bone metastases [14]. In this study, patients with metastatic breast cancer who were on bisphosphonates took 10,000 IU of vitamin D3 and 1000 mg of calcium supplementation each day for 4 months. A small but statistically significant increase in serum calcium was seen, as well as unmasking of primary hypercalcemia in 2 (5%) patients related to primary hyperparathyroidism [32]. In the current study with vitamin D3 of 40,000 IU/day for up to 6 weeks, physiological responses in PTH were seen. Even though serum 25-OHD levels had increased significantly from baseline with vitamin D3 supplementation, the classic safety indices for vitamin D excess, namely plasma and urine calcium concentrations, were unaffected. Furthermore, kidney and liver function were not impaired by vitamin D dosing, and all reported side effects were minor and unrelated to study intervention. There were no significant differences in patient-reported outcomes in the current study apart from grade 1 fatigue scores [33].

This clinical trial has limitations such as being single centre and relatively small. The challenges with the window model are well recognized. For example, variability in Ki67 staining as a result of a number of factors, including duration of tissue ischemia, formalin quality, duration of fixation, immunohistochemical technique used and assessor differences, is described and controlled for in our study [34, 35]. The major remaining variable that is impossible to control for is sampling variability using pre-intervention core biopsies. This variable can be significant when considering tumours greater than 2 cm. In this study, "hot spot" assessments were compared, i.e. counts in most proliferative areas rather than a comparison to average Ki67. However, as the whole tumour could be expected to respond, the hot spot methodology was selected. Despite these challenges, Ki67 remains the most validated biomarker for window trials [36]. These changes should be equally present in both the treatment and control arms in a randomized controlled clinical trial and we observe this in our study.

Future trials could evaluate administering vitamin D synthetic agonists such as calcipotriol or the active tissue metabolite 1,25$(OH)_2$D, calcitriol [37, 38]. However, the relative advantages and disadvantages of these strategies must be carefully evaluated [39]. Perhaps further insights on the effects of oral vitamin D3 supplementation would be obtained by examining tissue metabolite levels, namely of the potent 1,25-(OH)2D3. Additional analysis stratifying by vitamin D receptor (VDR) levels may provide insight into the effect of vitamin D supplementation on breast cancer subtypes. In addition to proliferation and apoptosis, vitamin D is known to have other anti-cancer effects, such as inducing autophagy, promoting cell differentiation and inhibition of metastasis [1], which may be explored in future studies. Tissue specimens were banked in the current study and if readers are interested in obtaining them they should contact the study team.

In conclusion, despite significantly higher levels of serum 25-OHD in patients receiving daily 40,000 IU vitamin D3 supplementation for a minimum of 2 weeks in this window of opportunity trial, this was not associated with any significant effects on tumour proliferation or apoptosis. However, given the observed lack of a therapeutic effect in the tumour samples evaluated in this study, a lack of consistent effect in reported clinical trials, and with the potential consequences for long-term toxicity high-dose vitamin D3 should not be routinely recommended to cancer patients.

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Compliance with ethical standards

Conflict of interest Dr Vieth is an unpaid advisor to the Vitamin D Society, and receives royalties from partial ownership and a patent pertaining to vitamin D supplementation.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Ottawa Hospital Research Ethics Board) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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