

## ORIGINAL RESEARCH

# Pretreatment vitamin D level and response to neoadjuvant chemotherapy in women with breast cancer on the I-SPY trial (CALGB 150007/150015/ACRIN6657)

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## Introduction

Approximately 230,000 new cases of breast cancer were diagnosed in 2013; 95% of these cases are theoretically curable [1]. Those at the highest risk of distant relapse have large primary tumors, lymph node involvement, or

## Abstract

Laboratory studies suggest that vitamin D (vitD) enhances chemotherapy-induced cell death. The objective of this study was to determine whether pretreatment vitD levels were associated with response to neoadjuvant chemotherapy (NACT) in women with breast cancer. Study patients ( $n = 82$ ) were enrolled on the I-SPY TRIAL, had HER2-negative tumors, and available pretreatment serum. VitD levels were measured via DiaSorin radioimmunoassay. The primary outcome was pathologic residual cancer burden (RCB; dichotomized 0/1 vs. 2/3). Secondary outcomes included biomarkers of proliferation, differentiation, and apoptosis (Ki67, grade, Bcl2, respectively) and 3-year relapse-free survival (RFS). Mean and median vitD values were 22.7 ng/mL (SD 11.9) and 23.1 ng/mL, respectively; 72% of patients had levels deemed “insufficient” ( $<30$  ng/mL) by the Institute of Medicine (IOM). VitD level was not associated with attaining RCB 0/1 after NACT (univariate odds ratio [OR], 1.01; 95% CI, 0.96–1.05) even after adjustment for hormone receptor status (HR), grade, Ki67, or body mass index (BMI). Lower vitD levels were associated with higher tumor Ki67 adjusting for race (OR, 0.95; 95% CI, 0.90–0.99). VitD level was not associated with 3-year RFS, either alone (hazard ratio [HzR], 0.98; 95% CI, 0.95–1.02) or after adjustment for HR, grade, Ki-67, BMI, or response. VitD insufficiency was common at the time of breast cancer diagnosis among women who were candidates for NACT and was associated with a more proliferative phenotype. However, vitD levels had no impact on tumor response to NACT or short-term prognosis.

estrogen, progesterone, and HER2-negative (TN) disease [2]. Achievement of pathologic complete response following neoadjuvant chemotherapy (NACT) predicts for improved survival [3–6] in this high-risk population, particularly in those with TN or HER2+ breast cancer as demonstrated in the I-SPY1 and other trials [7–10].

Despite its potential high curability, 39,620 women died from breast cancer in 2013 [11]. Therefore, modifiable factors that improve therapeutic efficacy and decrease relapse continue to be sought.

Vitamin D (vitD) deficiency (defined by the Institute of Medicine [IOM] as  $<20$  ng/mL [12]) is common, occurring in  $\sim 40\%$  of the general adult U.S. population [13]. VitD maintains calcium homeostasis and bone metabolism, and may play a role in malignancy. Ecological studies demonstrate higher breast cancer deaths in areas with less sunlight [14]. Moreover, scientific evidence suggests that calcitriol, the steroid active metabolite of vitD, is important for regulation of the cell cycle. Through binding to vitD receptors (VDRs), ubiquitously expressed in epithelial cells, including those in the normal and malignant breast cells [15], calcitriol directly and indirectly influences transcription, resulting in inhibition of breast cancer cellular proliferation while inducing differentiation and apoptosis [15]. In vitro and in vivo data further demonstrate that calcitriol augments chemotherapy-induced cell death [16–22]. This has not been explored in breast cancer patients to our knowledge.

Because of the intracellular effects on breast cancer cells and the in vitro and in vivo experiments demonstrating enhancement of chemotherapeutic cytotoxicity with vitD pretreatment, we hypothesized that low vitD levels would be associated with impaired response to chemotherapy and more aggressive breast tumor biology resulting in higher relapse rates among women with vitD deficiency. To address these hypotheses, we measured pretreatment vitD levels in a cohort of women with newly diagnosed breast cancer enrolled on the I-SPY1 Trial (CALGB 150007/150015/ACRIN6657). Our primary aim was to determine the relationship between vitD levels and response to NACT. Our secondary aims examined the relationship between vitD levels and biomarkers of proliferation, cell death, and differentiation as well as breast cancer relapse-free survival (RFS).

## Methods

We performed a retrospective cohort study examining pretreatment vitD levels in frozen serum obtained at enrollment in patients on the I-SPY1 Trial. As previously described [7], I-SPY1 was a multicenter, prospective cohort study that examined biomarkers and radiographic predictors of response to NACT. Serial breast biopsies and breast magnetic resonance imaging (MRI) scans were obtained before and during chemotherapy treatment. All subjects enrolled on I-SPY1 had pretreatment serum samples drawn, processed, and stored at the CALGB pathology core facility.

To be included in our study, subjects had to have provided written, informed consent to the parent trial and have frozen serum available for vitD testing. The eligibility criteria for I-SPY1 are described in detail elsewhere [8]. Briefly, patients had histologically confirmed breast cancer that was 3.0 cm or greater without evidence of distant metastatic disease. All patients received NACT with an anthracycline, 90% also received a taxane, and 98% ultimately underwent definitive surgery. Women with HER2-overexpressing tumors were excluded from our study because serum samples were not available.

Serum samples collected at enrollment were stored in aliquots at  $-80^{\circ}\text{C}$ . 25(OH)D levels were measured using the DiaSorin radioimmunoassay as previously described (DiaSorin, Stillwater, MN) in the Clinical Research Center of the Perelman School of Medicine at the University of Pennsylvania [23, 24]. Data were analyzed using vitD as a continuous and dichotomous variable. Cut points for the dichotomous variables were based on the IOM definitions of vitD deficiency and insufficiency ( $<20$  vs.  $\geq 20$  ng/mL;  $<30$  vs.  $\geq 30$  ng/mL, respectively).

## Outcome measurements

Our primary outcome was response to NACT and was dichotomized into response and no response. We defined response as a residual cancer burden (RCB) of 0 or 1 (complete or near-complete resolution of invasive breast cancer in the breast and lymph nodes as defined by Symmans et al. [25]). No response was defined as RCB of 2 or 3 (stable or progression of disease).

Secondary outcomes were tumor expression of Ki67, Bcl2, and tumor grade and RFS. Ki67, Bcl2, and tumor grade were measured from the original tumor biopsy specimens by one pathologist at the University of North Carolina, as previously described [26]. Ki67 and Bcl2 were dichotomized  $<10\%$  and  $\geq 10\%$  [27, 28]. Low grade was compared with moderate and high grade. Three-year RFS was collected by I-SPY1. RFS was defined according to Standardization of Events and End Points criteria [29] and began on the first day of chemotherapy. All covariable data were obtained from the I-SPY1 database; missing weight data were obtained if available from clinical sites directly.

## Statistical analysis

Patient characteristics from this study were compared to the remainder of the HER2 negative I-SPY1 study population using  $\chi^2$  analysis. We assessed vitD level distribution by generating histograms and examined the mean and median vitD levels. We compared median vitD levels in different population subgroups defined by covariables

using the Wilcoxon rank-sum test or the Wilcoxon rank-sum test for trend when a covariable had more than two groups. We used logistic regression to examine the relationships between the covariables comparing the RCB 0/1 group with the RCB 2/3 group. We also used logistic regression to examine the relationship between vitD and response. For secondary aims, logistic regression was used to examine the relationship between vitD and measures of tumor biology. Cox proportional regression analysis was performed to assess the association between vitD and RFS. Kaplan–Meier curves were created to display the experience of RFS for patients with vitD bivariable levels. We built models to adjust individually for potential confounding covariates and performed analyses to explore potential vitD and covariate interaction. Multivariate modeling was restricted to one covariate per model (trivariate models) due to the limitation in our sample size. Subjects with missing variables were excluded from analyses involving the specific variable. All statistical analyses were performed using STATA version 12.0 (College Station, TX).

For power calculations, we estimated that the mean vitD level would be 20 ng/mL with a standard deviation of 9 based on a recent NHANES study [13]. From a fixed sample size of 82 subjects with 17 responders (RCB 0/1), our study had 80% power to detect a change in odds of response of 9.5% for every 1 ng/mL change in vitD level with a two-sided test of significance level = 0.05.

## Results

Of the 221 subjects enrolled on I-SPY1, 64 were excluded from the current analysis due to HER2 positivity and 75 were excluded due to lack of remaining frozen serum. In total, 83 serum samples from 82 subjects were analyzed. Compared to those without samples, patients in the vitD cohort were more likely to be from the northern United States ( $P = 0.005$ , Table 1). Otherwise, excluded subjects did not differ from study subjects (Table 1). Pathologic response in the vitD cohort was significantly associated with HR negative (OR, 0.26; 95% CI, 0.15–0.47), moderate or high tumor grade (OR, 9.64; 95% CI, 1.26–73.93), low levels of Bcl2 (OR, 0.41; 95% CI, 0.22–0.79), and Ki67 >10 (OR, 2.46; 95% CI, 1.13–5.31).

All 83 serum samples were successfully assayed for 25 (OH)D. The intra-assay coefficient of variation was 8.81%. The vitD distribution was right-skewed. The mean and median vitD levels were 22.1 ng/mL (SD 11.9) and 23.4 ng/mL, respectively (Table 2). As per the IOM definitions, 41% had deficient and 72% had insufficient vitD levels. VitD level was associated with race (median level 26.2 ng/mL in Caucasians vs. 11.5 ng/mL in non-Caucasians;  $P = 0.0001$ ), higher body mass index (BMI)

**Table 1.** Patient characteristics.

|                                      | I-SPY<br>( <i>n</i> = 221) | Vitamin D<br>( <i>n</i> = 82) | Non-vitamin<br>D <sup>1</sup> ( <i>n</i> = 75) | <i>P</i> -value <sup>1</sup> |
|--------------------------------------|----------------------------|-------------------------------|------------------------------------------------|------------------------------|
| Mean age, years (SD)                 | 48.3 (8.9)                 | 48.1 (9.0)                    | 48.0 (9.5)                                     | 0.94                         |
| Race, <i>n</i> (%)                   |                            |                               |                                                |                              |
| Caucasian                            | 165 (75%)                  | 61 (75%)                      | 59 (79%)                                       |                              |
| Non-Caucasian                        | 54 (25%)                   | 20 (25%)                      | 15 (20%)                                       | 0.51                         |
| BMI, <i>n</i> (%)                    |                            |                               |                                                |                              |
| <25 kg/m <sup>2</sup>                | 66 (30%)                   | 29 (35%)                      | 20 (27%)                                       |                              |
| 25–30 kg/m <sup>2</sup>              | 45 (20%)                   | 18 (22%)                      | 15 (20%)                                       |                              |
| >30 kg/m <sup>2</sup>                | 57 (26%)                   | 31 (38%)                      | 13 (17%)                                       | 0.32                         |
| Location, <i>n</i> (%)               |                            |                               |                                                |                              |
| North                                | 131 (59%)                  | 40 (49%)                      | 53 (71%)                                       |                              |
| South                                | 90 (41%)                   | 42 (51%)                      | 22 (29%)                                       | 0.005                        |
| Hormone receptor, <i>n</i> (%)       |                            |                               |                                                |                              |
| Positive                             | 131 (59%)                  | 50 (61%)                      | 50 (67%)                                       |                              |
| Negative                             | 90 (41%)                   | 32 (39%)                      | 25 (33%)                                       | 0.46                         |
| Grade, <i>n</i> (%)                  |                            |                               |                                                |                              |
| Low                                  | 18 (8%)                    | 8 (10%)                       | 9 (9%)                                         |                              |
| Intermediate                         | 96 (44%)                   | 30 (37%)                      | 38 (51%)                                       |                              |
| High                                 | 103 (47%)                  | 42 (51%)                      | 27 (36%)                                       | 0.13                         |
| Chemotherapy <i>n</i> (%)            |                            |                               |                                                |                              |
| AC, T or equivalent                  | 208 (94%)                  | 75 (91%)                      | 71 (95%)                                       |                              |
| Other                                | 13 (6%)                    | 7 (9%)                        | 4 (5%)                                         | 0.43                         |
| Response <i>n</i> (%)                |                            |                               |                                                |                              |
| pCR                                  | 58 (26%)                   | 12 (15%)                      | 17 (23%)                                       |                              |
| No pCR                               | 157 (71%)                  | 70 (85%)                      | 58 (77%)                                       | 0.20                         |
| Residual cancer burden, <i>n</i> (%) |                            |                               |                                                |                              |
| RCB 0                                | 58 (26%)                   | 12 (15%)                      | 17 (23%)                                       |                              |
| RCB 1                                | 18 (8%)                    | 5 (6%)                        | 4 (5%)                                         |                              |
| RCB 2                                | 86 (39%)                   | 45 (55%)                      | 24 (32%)                                       |                              |
| RCB 3                                | 41 (19%)                   | 15 (16%)                      | 15 (20%)                                       | 0.08                         |
| Outcomes, <i>n</i> (%)               |                            |                               |                                                |                              |
| Alive                                | 176 (80%)                  | 64 (78%)                      | 59 (78%)                                       | 0.56                         |
| Relapse-free                         | 160 (72%)                  | 59 (72%)                      | 57 (76%)                                       | 0.28                         |

*P* value calculated based on  $\chi^2$  analysis comparing the vitD to non-vitD HER2 negative I-SPY1 subgroups. BMI, body mass index; AC, T, doxorubicin, cyclophosphamide, paclitaxel; pCR, pathologic complete response; RCB, residual cancer burden.

<sup>1</sup>Non-vitamin D group represents the HER2 negative I-SPY1 patients without remaining serum for analysis.

( $P$ -trend=0.01 comparing those <25 kg/m<sup>2</sup> to those 25–30 kg/m<sup>2</sup> to those >30 kg/m<sup>2</sup>), season of blood draw (median 17.5 ng/mL if drawn during winter or spring compared to 27.3 ng/mL summer or fall,  $P = 0.009$ ) and geographic location of blood draw (median level from subjects from northern cities was 25.8 and 18.3 ng/mL in those from southern cities,  $P = 0.011$ ). Notably, the vitD/geographic association was the reverse of expected because 80% of subjects from southern cities were of non-Caucasian race ( $P = 0.002$ , data not shown); thus, the high proportion of non-Caucasian subjects in south-

**Table 2.** Vitamin D levels among different study subgroups.

|                          | N  | Mean (SD),<br>ng/mL | Median,<br>ng/mL | Interquartile<br>range, ng/mL | P-value <sup>1</sup> |
|--------------------------|----|---------------------|------------------|-------------------------------|----------------------|
| Total                    | 82 | 22.7 (11.9)         | 23.1             | 13.1, 30.5                    | N/A                  |
| Age (years)              |    |                     |                  |                               |                      |
| <45                      | 25 | 26.7 (15.1)         | 26.8             | 15.3, 32.9                    | 0.14<br>(trend)      |
| 45–55                    | 39 | 22.1 (9.7)          | 22.6             | 12.9, 30.3                    |                      |
| >55                      | 18 | 20.3 (10.4)         | 19.3             | 12.0, 27.4                    |                      |
| Race                     |    |                     |                  |                               |                      |
| Caucasian                | 61 | 25.9 (11.7)         | 26.2             | 16.0, 32.5                    | 0.0001               |
| Non-Caucasian            | 20 | 14.4 (8.1)          | 11.5             | 8.5, 19.4                     |                      |
| BMI (kg/m <sup>2</sup> ) |    |                     |                  |                               |                      |
| <25                      | 29 | 27.0 (11.5)         | 27.3             | 18.8, 32.5                    | 0.01<br>(trend)      |
| 25–30                    | 18 | 19.7 (7.7)          | 20.2             | 13.6, 25.6                    |                      |
| >30                      | 31 | 19.8 (12.1)         | 14.4             | 9.1, 31.2                     |                      |
| Location                 |    |                     |                  |                               |                      |
| North                    | 40 | 26.4 (11.6)         | 25.8             | 17.9, 32.5                    | 0.011                |
| South                    | 42 | 20.0 (11.5)         | 18.3             | 9.2, 28.5                     |                      |
| Season of blood draw     |    |                     |                  |                               |                      |
| Winter/spring            | 31 | 19.6 (9.4)          | 17.5             | 12.4, 30.5                    | 0.009                |
| Summer/fall              | 45 | 27.2 (12/4)         | 27.3             | 20.0, 32.6                    |                      |
| Hormone receptor         |    |                     |                  |                               |                      |
| Positive                 | 50 | 24.4 (12.0)         | 24.9             | 11.3, 29.5                    | 0.21                 |
| Negative                 | 31 | 21.0 (11.6)         | 20.7             | 14.2, 31.4                    |                      |
| Response                 |    |                     |                  |                               |                      |
| pCR                      | 12 | 25.3 (8.7)          | 26.8             | 16.0, 31.2                    | 0.44                 |
| no pCR                   | 69 | 22.6 (12.4)         | 22.7             | 12.9, 30.4                    |                      |
| Residual cancer burden   |    |                     |                  |                               |                      |
| RCB 0/1                  | 17 | 23.9 (9.9)          | 26.8             | 16.0, 31.2                    | 0.34<br>(trend)      |
| RCB 2                    | 45 | 23.5 (12.7)         | 22.7             | 13.1, 30.5                    |                      |
| RCB 3                    | 15 | 21.6 (12.6)         | 21.4             | 12.0, 27.1                    |                      |

BMI, body mass index; pCR, pathologic complete response; RCB, residual cancer burden.

<sup>1</sup>Comparison of median vitamin D values (across strata when testing for trend) using Wilcoxon rank-sum test or Wilcoxon rRank-sum test for trend.

**Table 3.** Vitamin D and response: examination in three models.

| Model (N)                   | Vitamin D (continuous)<br>OR (95% CI)<br>P-value | Vitamin D deficiency<br>OR (95% CI)<br>P-value | Vitamin D insufficiency<br>OR (95% CI)<br>P-value |
|-----------------------------|--------------------------------------------------|------------------------------------------------|---------------------------------------------------|
| Univariate analysis         |                                                  | ≥20 vs. <20 ng/mL                              | ≥30 vs. <30 ng/mL                                 |
| Vitamin D (82)              | 1.01 (0.96, 1.05) <i>P</i> = 0.391               | 0.75 (0.14, 2.19) <i>P</i> = 0.535             | 1.54 (0.49, 4.80) <i>P</i> = 0.253                |
| Multivariate analysis       |                                                  |                                                |                                                   |
| Race (81)                   | 1.01 (0.97, 1.07) <i>P</i> = 0.323               | 0.83 (0.26, 2.68) <i>P</i> = 0.408             | 1.68 (0.51, 5.53) <i>P</i> = 0.271                |
| HR (82)                     | 1.02 (0.97, 1.07) <i>P</i> = 0.571               | 0.83 (0.27, 2.54) <i>P</i> = 0.708             | 1.75 (0.53, 5.81) <i>P</i> = 0.263                |
| Grade (80)                  | 1.01 (0.96, 1.06) <i>P</i> = 0.443               | 0.82 (0.26, 2.62) <i>P</i> = 0.527             | 1.66 (0.47, 5.84) <i>P</i> = 0.312                |
| Ki67 (73)                   | 1.02 (0.97, 1.07) <i>P</i> = 0.506               | 0.98 (0.30, 3.15) <i>P</i> = 0.606             | 1.78 (0.50, 6.30) <i>P</i> = 0.452                |
| BMI (78)                    | 1.00 (0.96, 1.05) <i>P</i> = 0.133               | 0.69 (0.22, 2.13) <i>P</i> = 0.219             | 1.37 (0.41, 4.55) <i>P</i> = 0.166                |
| Stratified analysis         |                                                  |                                                |                                                   |
| HR <sup>+</sup> (50)        | 1.00 (0.93, 1.07) <i>P</i> = 0.908               | 0.26 (0.04, 1.58) <i>P</i> = 0.915             | 1.19 (0.19, 7.33) <i>P</i> = 0.574                |
| HR <sup>-</sup> (32)        | 1.03 (0.97, 1.10) <i>P</i> = 0.468               | 1.93 (0.43, 8.61) <i>P</i> = 0.646             | 2.43 (0.47, 12.54) <i>P</i> = 0.367               |
| Ki67 Low <sup>1</sup> (22)  | 1.01 (0.93, 1.10) <i>P</i> = 0.932               | 0.71 (0.05, 9.70) <i>P</i> = 0.517             | 4.33 (0.33, 57.65) <i>P</i> = 0.821               |
| Ki67 High <sup>1</sup> (51) | 1.02 (0.96, 1.08) <i>P</i> = 0.373               | 1.05 (0.29, 3.84) <i>P</i> = 0.354             | 1.29 (0.28, 5.91) <i>P</i> = 0.460                |

OR, odds ratio; CI, confidence interval; HR, hormone receptor status; BMI, body mass index.

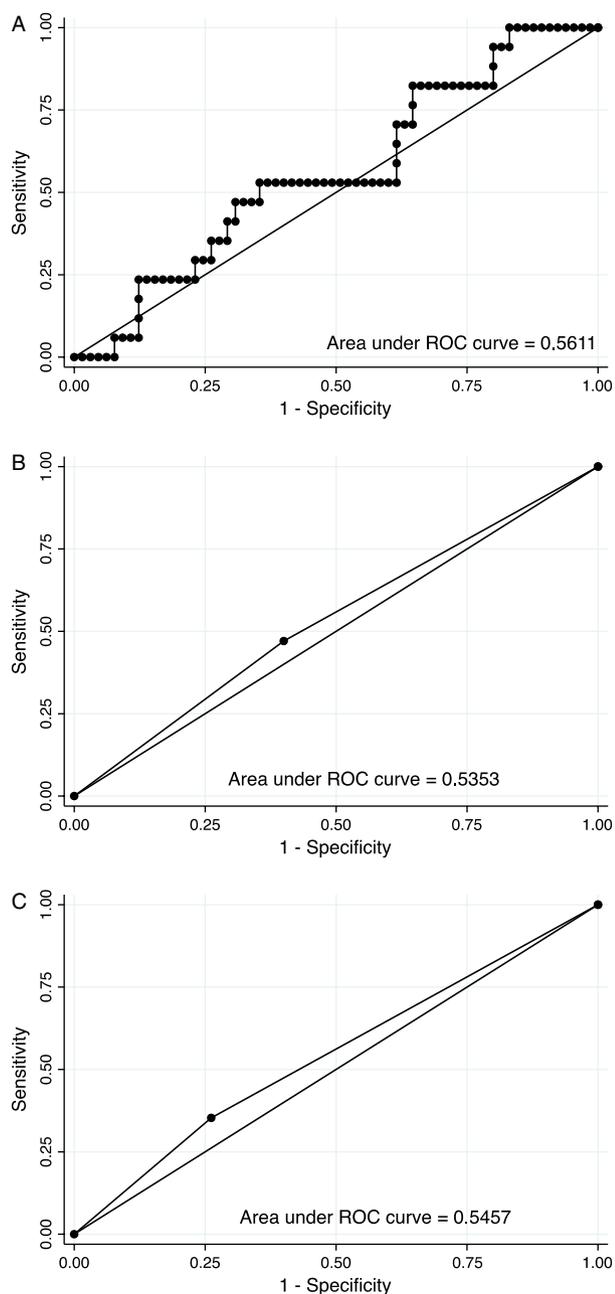
<sup>1</sup>There is no evidence of effect modification by HR or Ki67, *P* interaction = 0.43 for HR and 0.95 for Ki67 in continuous model.

ern locations accounted for this inverse association. VitD levels were not associated with HR.

The median vitD level in those achieving a response (RCB 0/1) to NACT was 26.8 ng/mL compared to 21.9 ng/mL in those without a response (RCB 2/3) (Table 2). Univariate analysis did not demonstrate a statistically significant association between vitD levels and response to NACT (OR, 1.01; 95% CI, 0.96–1.05) (Table 3). Individual adjustment by potential patient-related confounders (race and BMI) or tumor and response-related confounders (HR, Ki67, and grade), did not alter the association. When stratified by HR or Ki67, there was no evidence of effect modification (*P* interaction 0.43 for HR and 0.95 for Ki67).

We further explored the vitD/response relationship by dichotomizing vitD based on the IOM definitions of vitD deficiency and insufficiency. We found no evidence of a significant association between response to NACT and vitD deficiency (OR, 0.75; 95% CI, 0.14–2.19). VitD sufficiency was associated with increased odds of response compared to those with insufficient vitD levels, although this was not statistically significant (OR, 1.54; 95% CI, 0.49–4.80) (Table 3). Receiver operating characteristic curve (ROC) analysis showed that vitD was not a good predictor of response (Fig. 1).

We also performed exploratory analyses to assess the relationship between vitD and biomarkers of tumor biology, including Ki67, tumor grade, and Bcl2 phosphorylation. We found a significant relationship between Ki67 and vitD level (OR, 0.95; 95% CI, 0.91–0.99; *P* = 0.017; Table 4); for every one unit increase in serum vitD level, the odds of having a Ki67 >10 decreased by 5%. This relationship remained unchanged after adjustment for



**Figure 1.** ROC curves: vitamin D levels predicting response to neoadjuvant chemotherapy. (A) ROC to predict response using vitD as a continuous variable. (B) ROC to predict response in those with vitD levels  $\geq 20$  or  $< 20$  ng/mL. (C) ROC to predict response in those with vitD levels  $\geq 30$  or  $< 30$  ng/mL.

race, HR, grade, or Bcl2 expression (Table 4). Stratifying by HR or response status revealed that neither variable modified the Ki67/vitD relationship ( $P$  interaction = 0.20 for HR and 0.51 for response).

We did not find a statistically significant relationship between vitD level and breast cancer RFS, with a mini-

**Table 4.** Vitamin D and tumor characteristics.

|                                 | <i>N</i> | OR   | 95% CI     | <i>P</i> -value <sup>1</sup> |
|---------------------------------|----------|------|------------|------------------------------|
| Univariate analysis             |          |      |            |                              |
| Ki67                            | 73       |      |            |                              |
| Ki67 $\leq 10$                  | 22       | 1.00 | 0.91, 0.99 | 0.017                        |
| Ki67 $> 10$                     | 51       | 0.95 |            |                              |
| Bcl2                            | 71       |      |            |                              |
| Bcl2 low                        | 30       | 1.00 | 0.98, 1.06 | 0.348                        |
| Bcl2 high                       | 41       | 1.02 |            |                              |
| Grade                           | 80       |      |            |                              |
| Grade 1                         | 8        | 1.00 | 0.93, 1.04 | 0.612                        |
| Grade 2/3                       | 72       | 0.98 |            |                              |
| Multivariate analysis with Ki67 |          |      |            |                              |
| HR                              | 73       | 0.95 | 0.90, 1.00 | 0.04                         |
| Race                            | 73       | 0.95 | 0.90, 0.99 | 0.030                        |
| Bcl2                            | 73       | 0.95 | 0.90, 1.00 | 0.036                        |
| Grade                           | 73       | 0.95 | 0.91, 0.99 | 0.022                        |
| Stratified analysis with Ki67   |          |      |            |                              |
| Ki67, HR+                       | 45       | 0.93 | 0.87, 0.99 | 0.027                        |
| Ki67, HR-                       | 28       | 1.02 | 0.89, 1.17 | 0.755                        |

OR, odds ratio; CI, confidence interval; HR, hormone receptor status. <sup>1</sup> $P$  value was calculated based on the standard Wald statistic.

**Table 5.** Vitamin D and recurrence-free survival.

|                                                 | <i>N</i> | H <sub>z</sub> R | 95% CI    | <i>P</i> -value <sup>1</sup> |
|-------------------------------------------------|----------|------------------|-----------|------------------------------|
| Univariate analysis                             |          |                  |           |                              |
| Vitamin D, continuous                           | 82       | 0.98             | 0.95–1.02 | 0.391                        |
| Deficiency                                      | 82       | 0.77             | 0.34–1.75 | 0.535                        |
| Insufficiency                                   | 82       | 0.53             | 0.18–1.57 | 0.253                        |
| Multivariate analysis with continuous vitamin D |          |                  |           |                              |
| HR                                              | 82       | 0.99             | 0.95–1.03 | 0.571                        |
| Grade                                           | 80       | 0.99             | 0.94–1.04 | 0.778                        |
| Response                                        | 82       | 0.99             | 0.95–1.02 | 0.441                        |
| Age                                             | 82       | 0.98             | 0.95–1.02 | 0.383                        |
| Race                                            | 81       | 0.97             | 0.94–1.02 | 0.323                        |
| Chemo                                           | 82       | 0.99             | 0.95–1.02 | 0.444                        |
| Bcl2                                            | 71       | 0.99             | 0.95–1.03 | 0.578                        |
| Ki67                                            | 73       | 0.99             | 0.95–1.03 | 0.506                        |
| BMI                                             | 78       | 0.96             | 0.92–1.01 | 0.133                        |
| Stratified analysis                             |          |                  |           |                              |
| HR+                                             | 50       | 1.00             | 0.95–1.05 | 0.908                        |
| HR-                                             | 32       | 0.98             | 0.93–1.03 | 0.468                        |
| RCB 0/1                                         | 17       | 0.99             | 0.96–1.03 | 0.583 <sup>2</sup>           |
| RCB 2/3                                         | 60       | 0.64             | 0.26–1.61 | 0.349 <sup>2</sup>           |
| BMI $< 25$                                      | 29       | 0.99             | 0.94–1.05 | 0.828                        |
| BMI $\geq 25$                                   | 49       | 0.93             | 0.87–1.01 | 0.073                        |

H<sub>z</sub>R, hazard ratio; CI, confidence interval; HR, hormone receptor status; BMI, body mass index; RCB, residual cancer burden.

<sup>1</sup> $P$  value based on the standard Wald statistic.

<sup>2</sup>There is no effect modification by response status ( $P$  interaction = 0.099).

mum follow up of 3 years (Table 5). This relationship was not altered after individual adjustment by patient-related (age, race, or BMI) or tumor-related (HR, Ki67,

grade, or Bcl2) variables. It was also not altered by stratification on HR, response, or Bcl2 expression ( $P$  interaction 0.75 for HR, 0.10 for response, and 0.21 for Bcl2). Finally, we explored whether 3-year RFS was related to vitD deficiency and insufficiency. There was no statistically significant relationship in our analysis, although the Kaplan–Meier curves do show some separation between those above and below the deficiency and insufficiency thresholds (Fig. 2).

## Discussion

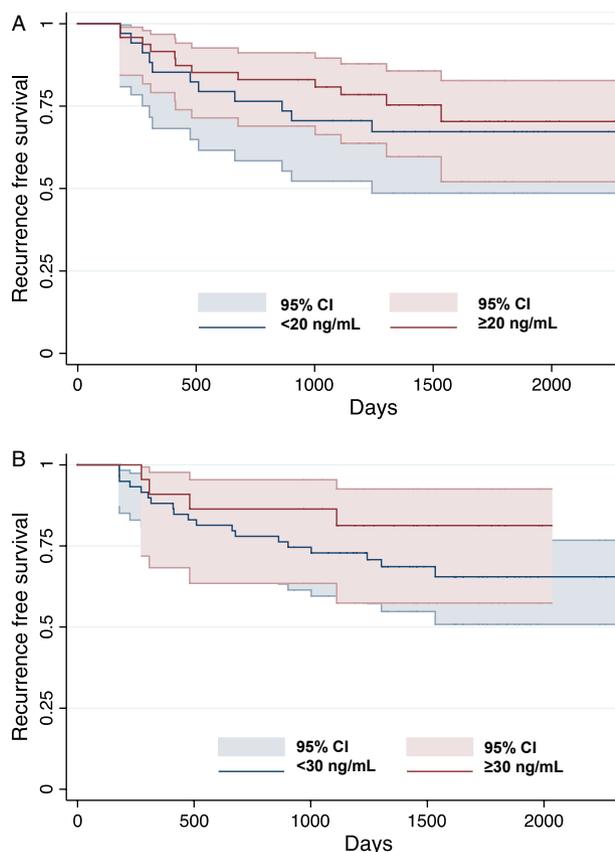
This study examined the relationship between vitD levels and response to NACT in breast cancer patients. As expected, vitD insufficiency was highly prevalent in our population: 42% were deficient (consistent with general U.S. population statistics) and 72% had insufficient levels per IOM definitions. Significantly lower vitD levels were observed in those of non-Caucasian race, with higher BMI and whose pretreatment serum was drawn in the

winter or spring months as expected from previous studies [30–36]. Despite adequate power to show a 9.5% change in odds of response for every one unit change in vitD level, we did not find an association between vitD level and response to NACT. In exploratory analyses, we did find a significant inverse association between vitD level and Ki67; for every one unit decrease in vitD level the odds of having a highly proliferative breast tumor (Ki67 >10) increased by 5%. This suggests that higher vitD levels may suppress proliferation of breast tumors, although this is speculative. There was no association between vitD level and RFS.

The rationale for our study was based on scientific evidence demonstrating that calcitriol binds to VDRS in breast cancer cells resulting in direct and indirect inhibition of proliferation through regulation of genes encoding cyclin proteins, cyclin-dependent kinases (CDK), and CDK inhibitors and indirect promotion of differentiation, apoptosis, and angiogenesis in breast cancer cells [15, 37–39]. When combined in vitro, calcitriol augmented MCF7 breast cancer cell line cytotoxicity via reduction in super oxide dismutase mRNA levels and suppression of Bcl2 protein levels rendering the cells more susceptible to doxorubicin [16] and paclitaxel [19], respectively, compared to either chemotherapeutic alone [16, 17, 19, 40]. In vivo models combined calcitriol analogs with doxorubicin, paclitaxel, or tamoxifen and demonstrated enhanced antitumor effect compared these agents alone [18, 19, 41]. Thus, calcitriol appeared to increase chemotherapy-induced cell death in cancer cell lines and animal models, but whether calcitriol influences chemotherapy efficacy in breast cancer patients had not previously been explored.

We acknowledge several limitations to this study. First, our sample size was fixed at 82 subjects with 17 responders in HER2 negative subjects. Thus, we were unable to build full multivariate models, though we did perform trivariate analyses to assess for confounding by individual covariables. When vitD was used as a continuous variable, we did not find evidence for confounding by any of the variables we assessed. In trivariate analysis, the only covariable that significantly contributed to the vitD/response relationship was HR. Although HR was significant in the model, the vitD/response relationship remained insignificant. Thus, we doubt that a full model would change these results. Despite the small sample size, our study was adequately powered to definitively detect whether a relationship exists between vitD levels and response.

Second, we note that the vitD levels in our population were skewed with only 28% having sufficient levels (>30 ng/mL). The OR for univariate and adjusted models comparing subjects with vitD insufficiency to those with sufficient levels were consistently over 1.0 suggesting an



**Figure 2.** Kaplan–Meier analysis using IOM cutoffs to dichotomize vitamin D. (A) VitD deficiency and RFS (comparing those with levels  $\geq 20$  to  $< 20$  ng/mL). (B) VitD insufficiency and RFS (comparing those with levels  $\geq 30$  to  $< 30$  ng/mL).

association between vitD sufficiency and response. We were not powered to answer this question, and further studies are needed to specifically explore this association. Furthermore, it is scientifically plausible that vitD levels needed to optimize breast health and response to chemotherapy have no relationship with the IOM cutoffs, since these were based on bone events. Supporting this idea is the unadjusted dose-response curve calculated by Mohr et al. in a recent meta analysis that estimated a 50% lower risk of breast cancer in subjects with serum vitD levels above 47 ng/mL compared to those with levels <47 ng/mL [42]. If these vitD levels were also needed to optimize response to chemotherapy, our study would not be able to identify such an association since so few subjects had levels above 30 ng/mL.

Third, there are potentially important unmeasured confounding variables, such as smoking status, physical activity, diabetes mellitus type 2 status, or vitD supplement use. None of these were collected as part of the I-SPY study. While these variables have been associated with breast cancer relapse, there has been no defined association between these variables and response to breast cancer chemotherapy which was our primary endpoint. Thus, we doubt that inclusion of any of these covariables would uncover a statistically significant relationship between vitD and response. Finally, we do not know who was taking vitD supplements and at what dose. It is plausible that some might have taken high doses, and this might bias our results toward the null.

We also did not find evidence that vitD levels were of prognostic significance for those with a new breast cancer diagnosis. Our RFS analyses were negative, although few events had occurred, limiting the power of this analysis (post hoc analysis revealed 10% power to detect a difference in relapse between those with vitD levels  $\geq$  and <30 ng/mL). Moreover, Hatse et al. have found a statistically significant difference between vitD and relapse over time [43]. We note that the Kaplan–Meier curves for those with vitD levels above and below the insufficient range do separate, favoring those with levels above 30 ng/mL.

Our study suggests that insufficient or deficient vitD levels do not impair or predict the efficacy of NACT in breast cancer patients. We do not know whether the vitD status of our patients was known at the time of NACT. Therefore, our study was not designed to examine whether vitD can enhance the effect of chemotherapy. There is one phase III prospective randomized trial that completed enrollment and compares standard versus high-dose vitD supplementation on time to breast cancer progression. Thus, it still remains to be seen whether vitD supplements can enhance the cytotoxic effects of chemotherapy in breast cancer patients.

## Conflict of Interest

None declared.

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