Abstract. Background: Preclinical studies suggest that 1,25-dihydroxyvitamin D [1,25(OH)2D] and celecoxib inhibit prostaglandins (PGs) associated with cancer through different mechanisms. We determined if there was synergy in their use.

Patients and Methods: A total of 36 healthy women received daily for one month/menstrual cycle: placebo, 400 international units (IU) vitamin D-3, 2,000 IU vitamin D-3, or 2,000 IU vitamin D-3 plus 400 mg celecoxib. Serum and nipple aspirate fluid (NAF) were analyzed for PGE2 and transforming growth factor (TGF)β1 and -2; serum for 25(OH)D (total, -D-2, -D-3), plasma for celecoxib; and mammary duct RNA for cyclooxygenase (COX)2. Results: 25(OH)D-3 increased (p<0.01) only in the groups receiving 2,000 IU vitamin D-3. PGE2 decreased in the breast (p=0.01) only after receiving 2,000 IU vitamin D-3; 2,000 IU vitamin D-3 alone was more effective in decreasing PGE2 than 2,000 IU vitamin D-3 plus celecoxib (p=0.018). COX2 expression decreased only in the breasts of women taking 2,000 IU vitamin D-3. Change in circulating 25(OH)D-3 correlated (p<0.01) only in the groups receiving 2,000 IU vitamin D-3. PGE2 decreased in the breast (p=0.01) only after receiving 2,000 IU vitamin D-3; 2,000 IU vitamin D-3 alone was more effective in decreasing PGE2 than 2,000 IU vitamin D-3 plus celecoxib (p=0.018). COX2 expression decreased only in the breasts of women taking 2,000 IU vitamin D-3. Change in circulating 25(OH)D-3 correlated with change in TGFβ2 in the breast. Conclusion: Vitamin D-3 reduces the PG cascade and increases TGFβ2 in a dose-dependent fashion. Adding celecoxib did not provide synergy.

There is growing interest among women in bioactive food components as part of a healthy lifestyle. Dietary vitamin D reduces breast cancer (BCa) risk by controlling cell growth, and doses up to 2,000 IU/day can be taken long-term without toxicity. The odds of developing BCa among women with vitamin D levels above 42 ng/ml, requiring a dose of approximately 2,000 IU/day, was 50% less than in women with vitamin D levels of 0-11 ng/ml (1). Preclinical (2) and epidemiological (3) studies suggest that the anti-inflammatory agent celecoxib also reduces BCa risk.

In vitro studies suggest that the active form of vitamin D-3, 1,25-dihydroxyvitamin D-3 [1,25(OH)2D-3], regulates prostaglandin metabolism, leading to a decrease in cancer cell growth (4). 1,25(OH)2D-3 reduces mRNA and protein levels of cyclooxygenase (COX)2, the key PGE2 synthesis enzyme, and up-regulates 15-hydroxyprostaglandin dehydrogenase (15-PGDH), the enzyme initiating PG metabolism, resulting in lower PGE2 levels and lower cell proliferation (4). Celecoxib inhibits COX2 in a different way, through direct binding to the molecule, preventing the conversion of arachadonic acid to PGE2 and other PGs. 1,25(OH)2D-3 has been reported to act in synergy with melatonin to inhibit BCa cell growth in vitro (5). Thus, there is potential for a synergistic effect of vitamin D with celecoxib in the breast, with vitamin D acting to reduce production of COX2, celecoxib to prevent the conversion of arachadonic acid to PGE2, and vitamin D also increasing the metabolism of PGE2 which is produced. 1,25(OH)2D-3 is also reported to act in synergy with melatonin to inhibit BCa cell growth in vitro (5).

The purpose of this study was to determine if there was a synergistic effect of vitamin D supplementation with celecoxib in the breast. The presumption was that increased intake of vitamin D-3 would raise blood levels of 25(OH)D, which in turn would be converted to 1,25(OH)2D-3 in the breast and reduce COX2 activity. Since celecoxib prevents the conversion of arachadonic acid to PGE2, and 1,25(OH)2D-3 also increases the metabolism of PGE2 which is produced, the combination of these two compounds could have a synergistic effect.

Patients and Methods

Participants. Candidates were enrolled in an institutional review board-approved protocol (ClinicalTrials.gov Identifier NCT01769625). Prior to enrollment and at the end of the study, participants had serum chemistries and complete blood count drawn to insure normalcy and no change related to treatment. Participants had to lack a history which
would make them at high risk (≥1.67% at 5 years) for breast cancer, had to not have taken nonsteroidal anti-inflammatory medications within two weeks of enrollment, nor while on study (except celecoxib if randomized to receive this). They could not be pregnant. A urine pregnancy test was to be obtained within 48 h of enrollment unless the candidate had not had a menstrual cycle in the last 12 months, she was >55 years old, or had undergone hysterectomy. Of 36 individuals who enrolled, eight withdrew before completion: five elected not to continue, two developed cold-like symptoms, one a rash. Participants were randomized in double blind fashion to one of four treatment groups for each month: placebo, 400 IU or 2,000 IU vitamin D-3 daily, or 2,000 IU vitamin D-3 plus 400 mg celecoxib daily. Vitamin D-3 doses were obtained from Tishcon (Westbury, NY, USA); celecoxib was obtained from Pfizer (New York, NY, USA). Due to low enrollment, with potential participants indicating they did not want to enroll if they might receive placebo, the protocol was revised such that additional recruits were randomized to one of three groups which included vitamin D-3.

Sample collection. We asked permission to collect three sample types from each participant: blood, breast nipple aspirate fluid (NAF), and mammary ductoscopy (MD) samples. NAF collection was attempted from at least one breast, and if agreed, MD samples were collected from the same breast both before and after treatment. Scientists performing the biomarker analyses were blinded as to the participant’s treatment assignment. Participants were asked to take their last dose of medication the morning of collection. All samples were collected within six hours of their last medication dose.

Blood was collected and serum or plasma separated using standard clinical laboratory techniques. NAF was aspirated by a trained physician or nurse clinician using a modified breast pump (7). After nipple aspiration was completed and if the participant agreed, a topical anesthetic was applied to the nipple. This was followed by a nipple block with injected lidocaine. Whenever possible, the same duct from which NAF was collected was entered with a mammary ductoscope (Solos Endoscopy, Boston, MA, USA).

Sample analysis. **PGE2:** Each NAF and serum specimen and standard was be analyzed in duplicate. Because of variability in the concentration of biomolecules, NAF PGE2 biomarker levels were calculated per mg of total protein. Total protein in NAF was analyzed using a Pierce BCA Kit (Rockford, IL, USA). PGE2 was measured using the high sensitivity PGE2 immunoassay kit from Oxford Biomedical Research (Oxford, MI, USA) per manufacturer’s instructions.

**25(OH)D:** Serum levels of 25(OH)D-2, 25(OH)D-3, and total 25(OH)D were determined as previously described (8). Briefly, liquid chromatography-mass spectrometry (LC-MS/MS) was performed to determine the contribution of 25(OH)D-2 and 25(OH)D-3 to serum 25(OH)D using a TSQ Quantum Ultra triple mass spectrometer (Thermo Finnigan Corp., San Jose, CA, USA).

**Celecoxib:** High performance liquid chromatography (HPLC) MS analysis was performed using an Agilent 1100 Series with an G1969 high resolution time of flight (TOF) MS system with an electrospray (ESI) source (Agilent, Santa Clara, CA, USA). The chromatographic and mass spectral data were acquired using MassHunter software (Agilent). Samples were purified using solid-phase extraction (SPE) with Bond Elute C18 cartridges.

**TGFβ1 and -2:** The concentrations of TGFβ1 and β2 in NAF and serum samples were measured by an immunoassay kit from R&D Systems (Minneapolis, MN, USA), following the manufacturer’s instructions. The samples were analyzed in duplicate.

### Results

**Participants.** All participants were white, non-Hispanic females. A total of 32% of the women were clinically obese (BMI >30). Overall, 61% of the women were college educated and the remaining women had either a high school diploma or post-graduate education (25% and 14%, respectively). In total, 75% of the women were premenopausal, 18% were taking hormone replacement therapy at the time of study and 33% were taking birth control pills. Table 1 summarizes the demographic composition of the women in our data set. There were no significant differences in any of the variables between the treatment groups.

**25(OH)D and celecoxib levels increase after treatment.** Fifty-four serum samples from 27 participants (half before and half after study completion) were analyzed for 25(OH)D-2, 25(OH)D-3 and total 25(OH)D. 25(OH)D-3 represented more than 95% total 25(OH)D for all participants both before and after treatment. There was not a significant difference between the groups at baseline. Controlling for confounders, there was a significant difference (p<0.01) in the change in serum 25(OH)D-3 and total 25(OH)D based on treatment, with both groups...
receiving 2,000 IU vitamin D increasing with treatment, whereas values for those receiving placebo or 400 IU actually declined slightly (Table II, Figure 1A).

Celecoxib was only detected in the plasma of participants randomized to the 2,000 IU vitamin D plus celecoxib arm. As expected, levels of the agent significantly increased in this group after treatment ($p<0.01$).

**PGE$_2$ decreases in the breast after vitamin D-3 treatment.** There was a significant difference ($p=0.01$) in treatment effect in NAF PGE$_2$ (Table II, Figure 1B). Moreover, treatment with 2,000 IU vitamin D-3 significantly lowered PGE$_2$ levels in the breast as measured in NAF compared to 2,000 IU plus celecoxib ($p=0.018$). There was no significant treatment effect on PGE$_2$ levels in serum (Table II).

**TGFβ1 and -2.** Neither TGFβ1 nor -2 levels in NAF or serum were influenced by treatment (Table II). On the other hand, there was a significant inverse correlation ($p=0.027$).
Moreover, the change in level of TGFβ2 as measured in NAF directly correlated with the change ($p=0.014$, $r=0.60$) in 25(OH)D-3 levels after treatment (Figure 2).

Discussion

In women, BCa is the most common type of cancer and the most common cause of cancer-related death worldwide (9). BCa incidence is expected to increase appreciably in the coming decades (10). The prevention of cancer requires the use of therapy which is efficacious with minimal toxicity. Only two medications (tamoxifen and raloxifene) to reduce risk of developing BCa are approved by the FDA, and only in high-risk women. Both drugs have side effects that limit their use. Vitamin D is available over the counter and currently used for bone health. Celecoxib is used by thousands of women for arthritis relief without complications, especially when taken as a single daily dose as in our study.

Dietary vitamin D reduces BCa risk by controlling cell growth, and doses used in this study can be taken long term without toxicity (4). The anti-inflammatory agent celecoxib has also been shown to reduce BCa risk (11). This study was designed to provide organ-specific information using cells exfoliated from the breast ductal epithelium, the cells that give rise to the overwhelming majority of breast cancers.

Reports evaluating the benefit of vitamin D supplementation in cancer prevention have been mixed (12). While the reasons for this are speculative, a common thread in many of the negative studies has been the relatively low dose used in the intervention arm (generally <1000 IU per day). Our study found that 2,000 IU vitamin D-3/day (but not 400 IU/day) significantly increased 25(OH)D-3 and 25(OH)D levels. Concomitant with the increase in circulating 25(OH)D-3, we observed a decrease in COX2 and PGE2.

Although there was not a significant difference between 25(OH)D-3 levels at baseline, levels were, on average, highest in the group receiving 2,000 IU vitamin D-3 and lowest among those receiving 2,000 IU vitamin D-3 plus celecoxib. Both groups receiving 2,000 IU vitamin D-3 had a significant increase in circulating levels of vitaminD3 after treatment. Despite the increase in vitamin D-3, we did not observe an additive or synergistic effect of vitamin D-3 with celecoxib. PGE2 and COX2 levels decreased in the breasts of women treated with 2,000 IU vitamin D-3 alone, but not in those treated with 2,000 IU vitamin D3 plus celecoxib. Indeed, if anything, the effect was antagonistic, with an increase in COX-2 and PGE2 in the breast greater than that with both 400 IU vitamin D-3 and placebo.

Vitamin supplementation is known to reverse the effects of corticosteroids on calcium metabolism in humans (13), and 1,25(OH)2D-3 analogs have been reported to have both additive and antagonistic effects in preclinical colorectal cancer study (14).

There are limitations of the current study. The first is the limited sample size. Because of the limited sample size, our findings should be validated in a larger study. A second limitation is that only women of normal risk were evaluated. Findings in high-risk women, or those with newly-diagnosed BCa may be different.

In the circulation, we observed that levels of 25(OH)D-3 are inversely correlated with levels of TGFβ1, which is consistent with an earlier report (6). On the other hand,

Figure 1. Changes in biomarkers with treatment. A: Serum 25-hydroxyvitamin D-3 levels increased in women receiving 2,000 IU vitamin D-3 (with and without celecoxib), but not in women receiving a lesser dose. B: Nipple aspirate fluid prostaglandin E2 decreased in women receiving 2,000 IU vitamin D-3 daily, but not in the other groups. C: Cyclooxygenase 2 decreased in dose dependent fashion after treatment with vitamin D-3.
vitamin D-3 was reported to enhance the secretion of TGFβ1 and -2 (15). We observed a direct correlation between change in circulating 25(OH)D-3 and TGFβ2 levels in the breast. TGFβ2 is thought to have a breast cancer preventive function in healthy organisms. Transgenic mice that overexpress TGFβ exhibit increased apoptosis in the mammary epithelium throughout mammary development (16), and TGFβ mediates proapoptotic effects during involution (17). We previously observed that TGFβ2 significantly increased in the breast milk during weaning, as the breast initiated involution back to the pre-pregnant state (18).

In conclusion, 25(OH)D-3 and total 25(OH)D levels rose in the circulation in women receiving 2,000 IU vitamin D-3 daily (with and without celecoxib), but not with a lesser dose. A dose of 2,000 IU vitamin D-3 daily lowered levels of cancer promoting COX2 and PGE2 in the breast. Celecoxib did not enhance this effect. Vitamin D-3 supplementation increased levels of TGFβ2 in the breast, which is thought to have a chemopreventive effect.

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References


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