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## Changes in Circulating Levels of 25-hydroxyvitamin D<sub>3</sub> in Breast Cancer Patients Receiving Chemotherapy

Dieuwertje E. Kok<sup>a</sup> , Maaikje M. G. A. van den Berg<sup>a</sup>, Liesbeth Posthuma<sup>a</sup>, Iris van 't Erve<sup>a</sup>, Fränzel J. B. van Duijnhoven<sup>a</sup>, Wilfred K. de Roos<sup>b</sup>, Sissi Grosfeld<sup>c</sup>, Maartje Los<sup>d</sup>, Dirkje W. Sommeijer<sup>e,f</sup>, Hanneke W. M. van Laarhoven<sup>f</sup> , Renate M. Winkels<sup>a,g</sup> , and Ellen Kampman<sup>a</sup> 

<sup>a</sup>Division of Human Nutrition and Health, Wageningen University & Research, Wageningen, the Netherlands; <sup>b</sup>Department of Surgery, Hospital Gelderse Vallei, the Netherlands; <sup>c</sup>Alexander Monro Clinics, Bilthoven, the Netherlands; <sup>d</sup>Department of Medical Oncology, St. Antonius Hospital, Nieuwegein, the Netherlands; <sup>e</sup>Department of Medical Oncology, Flevohospital, Almere, the Netherlands; <sup>f</sup>Department of Medical Oncology, Academic Medical Center, Amsterdam, the Netherlands; <sup>g</sup>Department of Public Health Sciences, Penn State College of Medicine, Hershey, Pennsylvania, USA

### ABSTRACT

Cancer treatments, toxicities and their effects on lifestyle, may impact levels of vitamin D. The aim of this study was to determine serum 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) levels before, directly after and 6 months after chemotherapy in breast cancer patients ( $n=95$ ), and a comparison group of women ( $n=52$ ) not diagnosed with cancer. Changes in 25(OH)D<sub>3</sub> levels over time were compared using linear mixed models adjusted for age and season of blood sampling. Before start of chemotherapy, 25(OH)D<sub>3</sub> levels were lower in patients (estimated marginal mean 55.8 nmol/L, 95% confidence interval (95%CI) 51.2–60.4) compared to the comparison group (67.2 nmol/L, 95%CI 61.1–73.3,  $P=0.003$ ). Directly after chemotherapy, 25(OH)D<sub>3</sub> levels were slightly decreased (–5.1 nmol/L, 95%CI –10.7–0.5,  $P=0.082$ ), but ended up higher 6 months after chemotherapy (10.9 nmol/L, 95%CI 5.5–16.4,  $P<0.001$ ) compared to pre-chemotherapy values. In women without cancer, 25(OH)D<sub>3</sub> levels remained stable throughout the study. Use of dietary supplements did not explain recovery of 25(OH)D<sub>3</sub> levels after chemotherapy. We reported lower 25(OH)D<sub>3</sub> levels in breast cancer patients, which decreased during chemotherapy, but recovered to levels observed in women without cancer within 6 months after chemotherapy. Suboptimal 25(OH)D<sub>3</sub> levels in the majority of the participants highlight the relevance of monitoring in this vulnerable population.

### ARTICLE HISTORY



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### Introduction

Vitamin D, together with calcium, plays a critical role in the regulation of bone health (1,2). Hence, suboptimal levels of vitamin D are associated with low bone mineral density, osteoporosis and bone fractures, which are commonly observed in breast cancer patients (3–6). Moreover, emerging evidence suggests that low levels of circulating vitamin D at the time of breast cancer diagnosis are associated with poor clinical outcomes, such as an increased risk of cancer recurrence and mortality (7–11). These findings highlight the relevance of a sufficient vitamin D status in women with breast cancer.

Circulating levels of 25-hydroxyvitamin D (25OHD) are considered a reliable biomarker for

vitamin D status (12). Although consensus about an optimal vitamin D status has not been reached yet, vitamin D deficiencies and insufficiencies are commonly defined as plasma or serum levels  $<50$  nmol/L (or  $<20$  ng/mL) and 50–75 nmol/L (20–30 ng/mL), respectively, whereas levels of  $\geq 75$  nmol/L (or  $\geq 30$  ng/mL) are considered sufficient (13). Epidemiological and clinical studies demonstrated that up to 96% of newly diagnosed breast cancer patients had a vitamin D deficiency (10,14–21). Most studies focusing on clinical outcomes in breast cancer patients used a single measurement of 25OHD at or around cancer diagnosis. It has, however, been hypothesized that common systemic cancer treatments may interfere with 25OHD levels, possibly through altered activities

**CONTACT** Dieuwertje E. Kok  [dieuwertje.kok@wur.nl](mailto:dieuwertje.kok@wur.nl)  Division of Human Nutrition and Health, Wageningen University & Research, PO Box 17, 6700 AA Wageningen, the Netherlands.

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of drug-metabolizing enzymes, therapy-induced toxicities, avoidance of sunlight and other personal or clinical factors (22). Circulating levels of 25OHD have been shown to decline after chemotherapy in breast cancer patients in some (17,22–24), but not all studies (25). To what extent these effects are persistent over time is still unclear. Kim et al. demonstrated that 25OHD levels first declined, but recovered after the end of chemotherapy in women with breast cancer (24), whereas another study showed that 25OHD levels were still lower 8 months after chemotherapy as compared to pretreatment values (23). How potential therapy-related changes in 25OHD levels in breast cancer patients relate to fluctuations of 25OHD levels in women without cancer remains unclear.

The aim of this study was to determine changes in vitamin D levels by measuring serum 25-hydroxyvitamin D<sub>3</sub> 25(OH)D<sub>3</sub> levels before, directly after, and 6 months after chemotherapy in patients with stage I–III breast cancer, and within a similar time frame in a comparison group of women without cancer.

## Subjects and Methods

### Study Participants

This study was conducted as part of the COBRA study, which is an observational multicenter study among breast cancer patients receiving chemotherapy and a comparison group of women not diagnosed with cancer. Design of the study and recruitment of the participants have been previously described in detail (26). Briefly, 181 eligible patients with incident stage I–IIIB breast cancer were recruited between May 2013 and November 2016 from 12 academic and peripheral hospitals in the Netherlands prior to start of neoadjuvant or adjuvant chemotherapy. The comparison group consisted of 180 women who had a similar age ( $\pm$  2 years) and who were recruited via the breast cancer patients. All participants needed to be at least 18 years old and be able to communicate in Dutch. Exclusion criteria were: a history of cancer except basal cell carcinoma, previous treatment with chemotherapy, being pregnant or the intention to get pregnant during the study period, dementia or other mental conditions that made it impossible to comply with the study procedures. This study was approved by the Medical Ethical Committee of Wageningen University & Research (Protocol no. NL40666.081.12) and all participants provided written informed consent before enrollment. For the current study, circulating 25(OH)D<sub>3</sub> levels were analyzed in all participants who completed the study by the end of March 2016 (i.e.,

recruited up to April 2015) and who had blood samples available, resulting in a total of 441 samples from 147 participants ( $n=95$  patients and  $n=52$  women from the comparison group). Recruitment of the larger cohort continued after March 2016 (for other research objectives) and therefore a proportion of the original cohort has not been considered.

### Blood Sampling and Vitamin D Measurements

Sample collection took place at three moments during the study period, namely before start of chemotherapy (T1), 1–3 weeks after the last cycle of chemotherapy (T2), and 6 months after chemotherapy (T3). For women in the comparison group, blood samples were collected at similar time points namely at baseline (T1), 6 months after baseline (T2), and 12 months after baseline (T3). Nonfasted blood samples were collected in 8.5-mL serum tubes (Becton Dickinson B.V.). Serum was collected after centrifugation and stored at  $-80^{\circ}\text{C}$  until analyses. All samples were shipped at the same time and analyzed in the same laboratory (department of Clinical Chemistry, Canisius Wilhelmina Hospital, Nijmegen, the Netherlands). Levels of 25(OH)D<sub>3</sub> were measured using isotope-dilution liquid chromatography (LC) tandem-mass spectrometry (MS/MS) (27). The interassay coefficients of variation were as follows: 5.3%, 3.1% and 2.9% at 25(OH)D<sub>3</sub> concentrations of 39.0, 92.5 and 127.0 nmol/L, respectively. The lower limit of quantification (LLOQ) was 1 nmol/L. No samples showed 25(OH)D<sub>3</sub> levels below the LLOQ. The current method was also able to quantify levels of 25(OH)D<sub>2</sub>; however, since 25(OH)D<sub>2</sub> levels were below the LLOQ (2 nmol/L) for all except one patients, these values were not considered for the current analyses. The vitamin D status was defined as deficient ( $<50$  nmol/L), insufficient (50–75 nmol/L) or sufficient ( $\geq 75$  nmol/L) (13).

### Data Collection

Demographic and general characteristics, including age, height, smoking status (current, former, never), educational level and menopausal status (pre- or postmenopausal), were collected from a general questionnaire provided at baseline (T1). Educational level was defined as low (primary school and lower vocational education), medium (vocational education), or high (higher professional education and university). The validated Short QUestionnaire to ASsess Health enhancing physical activity (SQUASH) was used to calculate adherence to the Dutch physical activity guideline (i.e., at least 30 min moderate intense

physical activity for a minimum of 5 days per week) as described previously (28,29). Body weight was determined by use of a dual-energy X-ray absorptiometry (DEXA)-scanner in the hospitals at baseline (T1). Body mass index (BMI) was calculated as body weight divided by squared height ( $\text{kg}/\text{m}^2$ ). Use of dietary supplements during the previous month was reported as part of a food frequency questionnaire provided at baseline (T1) and 6 months after chemotherapy (T3). Specific dietary supplements containing vitamin D as well as multivitamins were considered with regard to supplemental vitamin D intake. Most multivitamins available in the Netherlands contain vitamin D. The consistency of dietary supplement use was determined based on intake at baseline (T1) and 6 months after the end of chemotherapy (patient group) or 12 months after baseline (comparison group) (T3). Participants were categorized as consistent users, consistent nonusers, participants who started using supplements ('starters') and participants who stopped using supplements ('stoppers') during the study. In case of missing data on supplement use at one of the indicated time points, consistency of supplement use was classified as unknown. Information on clinical characteristics, including adjuvant or neoadjuvant chemotherapy and cancer stage, was collected from the medical records.

### Statistical Analysis

Population characteristics were presented as mean  $\pm$  standard deviation (SD) or numbers and percentages. Normality of the data was checked by visual inspection of the QQ-plots. In case of data not following a normal distribution, median and interquartile ranges (IQR) were presented. Changes in 25(OH)D<sub>3</sub> levels over time were compared for patients and the comparison group using a linear mixed model analysis with three time points (T1–T3). Serum 25(OH)D<sub>3</sub> levels were considered as dependent variable in these analyses. Time, group and their interaction (time\*group) were included as fixed factors and subjects were defined as random factors in the model. A random intercept was included to take into account the individual variation in levels between participants and the correlation between observations in the same participant. The variance of components (VC) was used as covariance structure. The final model was adjusted for age at time of blood withdrawal (continuous) and season of each individual blood withdrawal (spring, summer, autumn, winter) as potential confounders, which were both included as fixed factors. Results of the mixed model analyses were presented as

estimated marginal means and 95% confidence intervals (95% CI), which were compared in a post-hoc analysis with Sidak correction for multiple comparisons (30). Explorative stratified analyses for timing of chemotherapy (neoadjuvant versus adjuvant) and menopausal status (pre-menopausal versus post-menopausal) were conducted. Moreover, a sensitivity analysis excluding the inconsistent users, i.e., those who reported changes in dietary supplement containing vitamin D or with incomplete data on supplement use, was performed. IBM SPSS Statistics version 23 was used for all analyses and the values of  $P < 0.05$  were considered statistically significant.

### Results

Data on circulating 25(OH)D<sub>3</sub> levels at all time points were available for 95 breast cancer patients undergoing chemotherapy and 52 women without cancer (Table 1). Age of the participants was on average 52.0 (SD 8.8, range 25–69) and 53.4 (SD 8.9, range 31–71) years (women with and without breast cancer, respectively). Menopausal status was comparable for the two groups. Women with breast cancer tended to smoke more often (19%) at the time of assessment as compared to the women without cancer (8%). At baseline, the majority of the participants adhered to the guideline for physical activity (60–63% at T1). Six months after chemotherapy, a decline in adherence to this guideline was observed for the patients with breast cancer (48% versus 63% for women without cancer) (Table 1).

The majority of the patients ( $n = 62$ , 65%) received chemotherapy in the adjuvant setting. A regimen with docetaxel, doxorubicin and cyclophosphamide (TAC) or with 5-fluorouracil, epirubicin, cyclophosphamide and docetaxel (FEC/DOC) was most commonly prescribed (37% and 33%, respectively). The median time between breast cancer diagnosis and start of chemotherapy was 25 days (IQR 19–37) for patients receiving neoadjuvant chemotherapy and 91 days (IQR 71–111) for patients receiving adjuvant chemotherapy. The participants of the current study were representative for the total study population of the COBRA study with regard to the studied variables ( $n = 181$  patients and  $n = 180$  women without cancer, data not shown).

In this study, 79% of the women with breast cancer showed an insufficient vitamin D status ( $<75$  nmol/L) before the start of chemotherapy and 50% of the women even showed a vitamin D deficiency ( $<50$  nmol/L). In the comparison group, insufficient and deficient levels of 25(OH)D<sub>3</sub> at start of the study

**Table 1.** Description of the study population.

	Breast cancer patient group (n = 95)	Comparison group (n = 52)	P-value <sup>g</sup>
General characteristics			
Age (years), mean (SD)	52.0 (8.8)	53.4 (8.9)	0.355
Body mass index (kg/m <sup>2</sup> ), median (IQR) <sup>a</sup>	25.2 (22.5–27.9)	23.3 (21.7–27.1)	0.075
Smoking status, n (%) <sup>b</sup>			0.048
Current	18 (19%)	4 (8%)	
Former	40 (42%)	18 (35%)	
Never	35 (37%)	29 (56%)	
Menopausal status, n (%) <sup>b</sup>			0.988
Pre-menopausal	53 (56%)	29 (56%)	
Post-menopausal	40 (42%)	22 (42%)	
Educational level, n (%)			0.154
Low	7 (7%)	3 (6%)	
Medium	36 (38%)	13 (25%)	
High	49 (52%)	36 (69%)	
Unknown	3 (3%)	–	
Use of dietary supplements containing vitamin D at start of the study (T1) <sup>c</sup> (yes), n (%)	30 (32%)	16 (31%)	0.802
Season of drawing first blood sample (T1), n (%)			0.927
Spring (21 March–21 May)	21 (22%)	11 (21%)	
Summer (21 June–21 August)	16 (17%)	11 (21%)	
Autumn (21 September–21 November)	18 (19%)	10 (19%)	
Winter (21 December–21 February)	40 (42%)	20 (39%)	
Season of drawing last blood sample (T3), n (%)			0.023
Spring (21 March–21 May)	17 (18%)	8 (15%)	
Summer (21 June–21 August)	12 (13%)	14 (27%)	
Autumn (21 September–21 November)	29 (31%)	6 (12%)	
Winter (21 December–21 February)	37 (39%)	24 (46%)	
Adherence to recommendation for physical activity (T1) <sup>d</sup> (yes), n (%)	57 (60%)	33 (64%)	0.686
Adherence to recommendation for physical activity (T3) <sup>e</sup> (yes), n (%)	46 (48%)	33 (64%)	0.075
Clinical characteristics			
Tumor stage, n (%)			–
I	32 (34%)	NA	
II	52 (55%)	NA	
III	11 (12%)	NA	
Timing of chemotherapy, n (%)			–
Neoadjuvant	33 (35%)	NA	
Adjuvant	62 (65%)	NA	
Type of chemotherapy, n (%)			–
TAC	35 (37%)	NA	
FEC/DOC	31 (33%)	NA	
ACPT	11 (12%)	NA	
AC/DOC/T	6 (6%)	NA	
Other	12 (13%)	NA	
Circulating vitamin D levels			
Serum 25OHD at baseline (T1) (nmol/L), mean (SD)	54.1 (22.8)	66.1 (23.5)	0.003
Serum 25OHD directly after CTx (T2) <sup>f</sup> (nmol/L), mean (SD)	52.4 (21.4)	70.5 (25.5)	<0.001
Serum 25OHD 6 months after CTx (T3) <sup>f</sup> (nmol/L), mean (SD)	64.9 (24.5)	69.0 (21.2)	0.304

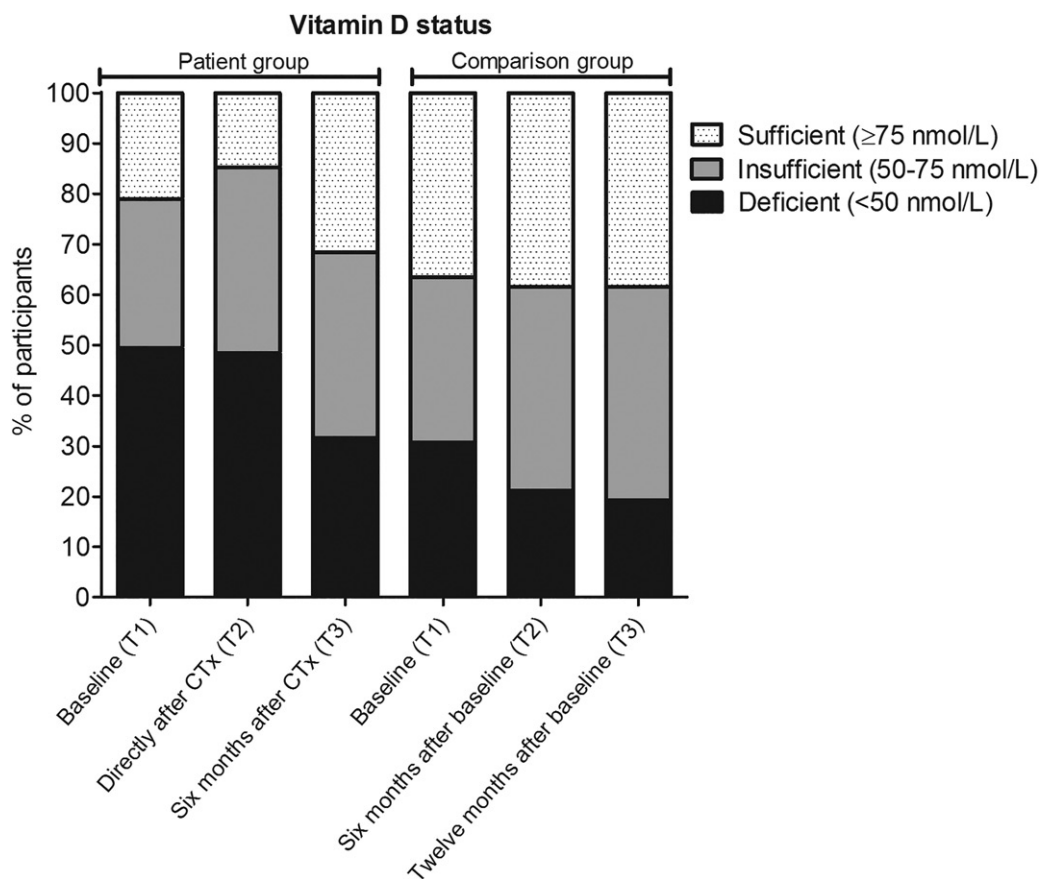
Data are presented as mean (standard deviation), median (interquartile range) or numbers (percentage). <sup>a</sup>Data missing for one patient. <sup>b</sup>Data missing for one participant from the comparison group and two patients. <sup>c</sup>Multivitamins or specific dietary supplements containing vitamin D. Data missing for two participants from the comparison group and seven patients. <sup>d</sup>Dutch recommendations for physical activity referring to  $\geq 150$  min moderate intensive physical activity per week. Data missing for one participant from the comparison group and two patients. <sup>e</sup>Data missing for three participants from the comparison group and six patients. <sup>f</sup>Comparable time points for women without cancer refer to 6 months (T2) and 12 months after baseline (T3). <sup>g</sup>Calculated through Mann–Whitney *U* tests, Student's *t*-tests or chi-square tests. CTx, chemotherapy; TAC, docetaxel, doxorubicin, cyclophosphamide; FEC/DOC, 5-fluorouracil, epirubicin, cyclophosphamide and docetaxel; ACPT, doxorubicin, cyclophosphamide, paclitaxel and trastuzumab; AC/DOC/T, doxorubicin, cyclophosphamide, docetaxel and trastuzumab; NA, not applicable.

were found in 63% and 31% of the women, respectively (Fig. 1). It should be noted, however, that these descriptive data did not take the season of blood sampling into account, which slightly differed between the groups. Therefore, we continued with linear mixed model analyses to confirm these findings while considering season of blood sampling as well as age at time of blood withdrawal.

The estimated marginal means (95% CI) retrieved from the linear mixed model analysis are presented in Fig. 2. A statistically significant interaction for time

and participant group was found ( $P = 0.001$ ). Post-hoc analyses demonstrated that before start of chemotherapy, 25(OH)D<sub>3</sub> levels were lower in the patients with breast cancer (55.8, 95% CI 51.2–60.4 nmol/L) as compared to the comparison group (67.2, 95% CI 61.1–73.3 nmol/L) ( $P = 0.003$ ). Also directly after chemotherapy, patients had lower 25(OH)D<sub>3</sub> levels (50.7, 95% CI 46.1–55.2 nmol/L) as compared to women without cancer (68.7, 95% CI 62.6–74.9 nmol/L) ( $P < 0.001$ ), whereas 6 months after chemotherapy these differences between the groups disappeared



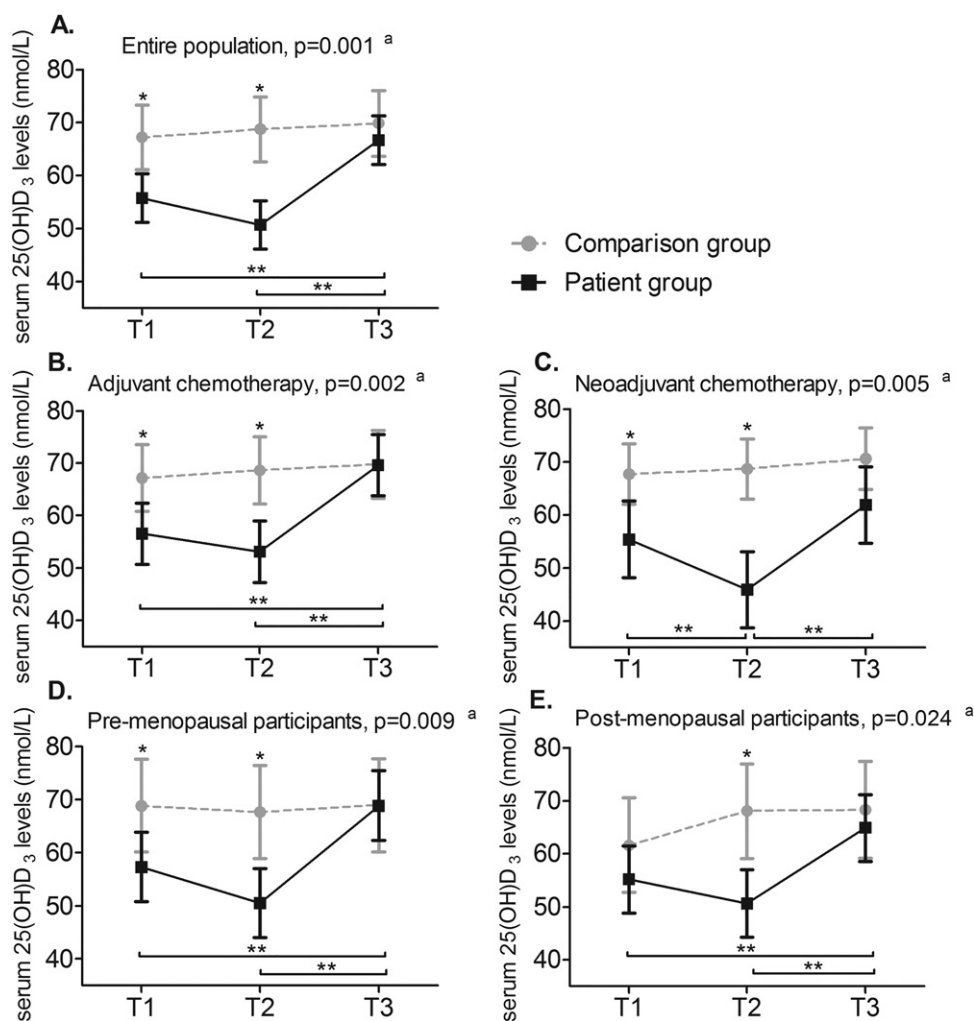


**Figure 1.** Percentage of participants with a sufficient, insufficient or deficient vitamin D status. Vitamin D status in breast cancer patients ( $n=95$ ) and women without cancer ( $n=52$ ) according to the time of sample collection. Baseline samples were collected before start of chemotherapy (CTx) for patients with breast cancer.

(66.7, 95% CI 62.1–71.2 and 69.8, 95% CI 63.6–76.0 nmol/L, respectively,  $P=0.421$ ) (Fig. 2A). Directly after chemotherapy, 25(OH) $D_3$  levels were on average 5.1 (95% CI  $-10.7$ – $0.5$ ) nmol/L lower as compared to pre-chemotherapy values (T2 versus T1,  $P=0.082$ ) in the patients. In this group, 25(OH) $D_3$  levels increased again 6 months after chemotherapy (T3 versus T2 = 16.0, 95% CI 10.4–21.7 nmol/L,  $P<0.001$ ) and ended up higher as compared to pre-chemotherapy values (T3 versus T1 = 10.9, 95% CI 5.5–16.4 nmol/L,  $P<0.001$ ), whereas 25(OH) $D_3$  levels of the women without cancer did not statistically significantly change over time (Table 2). Stratified analyses for patients receiving neoadjuvant versus adjuvant chemotherapy or for pre-menopausal versus post-menopausal participants showed similar findings (Fig. 2B–E).

Before start of chemotherapy (T1), 32% ( $n=30$ ) of the patients and 31% ( $n=16$ ) of the women without cancer reported use of dietary supplements containing vitamin D in the past month. Six months after chemotherapy (T3), this percentage increased to 47%

( $n=45$ ) of the patients and 29% ( $n=15$ ) of the women without cancer. Consistent vitamin D supplement use was reported in 23% ( $n=22$ ) of the patients and 23% ( $n=12$ ) of the women without cancer (Fig. 3). Throughout the study, 21% ( $n=20$ ) of the patients reported that they started taking dietary supplements with vitamin D, whereas 6% ( $n=6$ ) patients stopped using supplements with vitamin D. For the group of women without cancer, 6% ( $n=3$ ) started and 6% ( $n=3$ ) stopped use of dietary supplements with vitamin D. Given the dynamics of supplement use in this study population, we explored to what extent changes in supplement use may have accounted for the observed changes in 25(OH) $D_3$  levels. A sensitivity analyses excluding participants who reported changes in dietary supplement use ( $n=26$  breast cancer patients and  $n=6$  women without cancer) or with unavailable data on supplement use ( $n=13$  breast cancer patients and  $n=6$  women without cancer) showed similar findings (Table 2). Thus, also for this population of consistent users and nonusers of dietary supplements containing vitamin D, 25(OH) $D_3$  levels



**Figure 2.** Changes in circulating 25(OH)D<sub>3</sub> levels. Estimated marginal means (95% confidence intervals) of the circulating 25(OH)D<sub>3</sub> levels throughout the course of chemotherapy (CTx) in patients with breast cancer and at comparable time points for women from the comparison group based on a linear mixed model analysis. The analyses were adjusted for age and season of each blood withdrawal. <sup>a</sup>Statistically significant interaction for time\*group in the linear mixed model analysis. \*Indicates a statistically significant difference (Sidak-adjusted  $P < 0.05$ ) for the comparison between patients and women without cancer identified through a pairwise post-hoc analysis. \*\*Indicates a statistically significant change over time in the patients with breast cancer (Sidak-adjusted  $P < 0.05$ ). A] All patients with breast cancer ( $n = 95$ ) and women without cancer ( $n = 52$ ), B] Patients receiving adjuvant chemotherapy ( $n = 62$ ) and women without cancer ( $n = 52$ ), C] Patients receiving neoadjuvant chemotherapy ( $n = 33$ ) and women without cancer ( $n = 52$ ), D] Pre-menopausal patients ( $n = 53$ ) and women without cancer ( $n = 29$ ), E] Post-menopausal patients ( $n = 40$ ) and women without cancer ( $n = 22$ ).

declined directly after chemotherapy and increased again 6 months after chemotherapy in the breast cancer patients ( $n = 56$ ), but remained stable in the women without cancer ( $n = 40$ ) (Table 2).

## Discussion

The aim of this study was to assess changes in circulating 25(OH)D<sub>3</sub> levels throughout the course of chemotherapy in patients with stage I–III breast cancer as compared to a group of women without cancer. We have shown that suboptimal levels of 25(OH)D<sub>3</sub> are common among breast cancer patients as well as

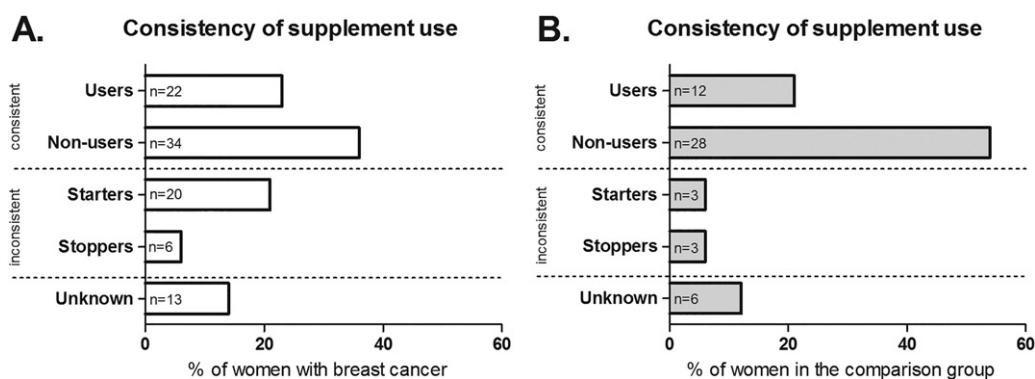
women without cancer. Importantly, 25(OH)D<sub>3</sub> levels before start of chemotherapy were lower in patients with breast cancer as compared to women without cancer and further declined during chemotherapy. The proportion of patients with a sufficient vitamin D status (15%) became alarmingly small directly after chemotherapy. Six months after the end of chemotherapy, 25(OH)D<sub>3</sub> levels in breast cancer patients returned to levels observed in the comparison group independent of the season of blood sampling or changes in dietary supplement use. Circulating levels of 25(OH)D<sub>3</sub> remained stable throughout the study in women without cancer.



**Table 2.** Changes in 25OHD levels over time.

Overall analyses						
Breast cancer patient group (n = 95)			Comparison group (n = 52)			
	Estimated mean change (nmol/L)	95% confidence interval	P-value <sup>a</sup>	Estimated mean change (nmol/L)	95% confidence interval	P-value <sup>a</sup>
T2–T1	–5.1	–10.7 ; 0.5	0.082	1.5	–5.8 ; 8.9	0.945
T3–T2	16.0	10.4 ; 21.7	<0.001	1.1	–6.3 ; 8.5	0.978
Stratified analysis for menopausal status: pre-menopausal <sup>b</sup>						
Breast cancer patient group (n = 53)			Comparison group (n = 29)			
T2–T1	–6.8	–14.9 ; 1.3	0.125	–1.2	–11.7 ; 9.3	0.990
T3–T2	18.3	10.1 ; 26.6	<0.001	1.3	–9.2 ; 11.9	0.987
Stratified analysis for menopausal status: post-menopausal <sup>b</sup>						
Breast cancer patient group (n = 40)			Comparison group (n = 22)			
T2–T1	–4.5	–12.1 ; 3.0	0.386	6.4	–4.2 ; 17.1	0.377
T3–T2	14.3	6.5 ; 22.0	<0.001	0.3	–10.4 ; 10.9	1.000
Sensitivity analysis <sup>c</sup>						
Breast cancer patient group (n = 56)			Comparison group (n = 40)			
T2–T1	–7.9	–13.8 ; –2.1	0.004	0.8	–6.0 ; 7.6	0.987
T3–T2	12.8	6.9 ; 18.6	<0.001	0.7	–6.2 ; 7.5	0.994

Estimated mean changes in 25(OH)D<sub>3</sub> levels over time calculated using a linear mixed model analysis adjusted for age and season of blood withdrawal. Time points indicate T1: before start of chemotherapy, T2: directly after chemotherapy and T3: 6 months after chemotherapy for patients and T1: baseline, T2: 6 months after baseline and T3: 12 months after chemotherapy for women without cancer in the comparison group. <sup>a</sup>Adjusted for multiple comparisons using the Sidak procedure in the pairwise post-hoc analysis. <sup>b</sup>Analyses stratified for menopausal status. Menopausal status unknown for two patients and one participant from the comparison group. <sup>c</sup>Sensitivity analysis excluding participants reporting inconsistent use of dietary supplements (starters and stoppers) and the participants for whom it is unknown whether they used dietary supplements at the indicated time points.



**Figure 3.** Use of dietary supplements containing vitamin D. Dietary supplements with vitamin D as well as multivitamins containing vitamin D were considered. Use of these supplements was reported by the participants at start of the study (T1) and 6 months after the end of chemotherapy for patients (i.e. 12 months after start of the study for the comparison group) (T3). Consistency is reflecting use or nonuse of these supplements at the indicated time points, whereas inconsistency refers to changes in dietary supplement use (i.e., starting of stopping use of dietary supplements containing vitamin D). A] Patients with breast cancer (n = 95), B] Women without cancer from the comparison group (n = 52).

Vitamin D deficiencies are commonly reported in patients with breast cancer (10,14–21). Also in our study, half of the breast cancer patients (50%) had a vitamin D deficiency at start of the study. Various, but not all, studies agreed that higher vitamin D levels were associated with better breast cancer outcomes and survival (7–11). Most of these studies assessed serum 25OHD levels up to one year prior to or shortly after breast cancer diagnosis. It should be noted that various factors, including changes in diet and lifestyle after cancer diagnosis and cancer treatments may impact circulating 25OHD levels. This

raises the clinically relevant question whether 25OHD levels are subjective to changes after cancer diagnosis.

Only few studies have examined 25OHD levels throughout the course of chemotherapy in breast cancer patients. A small study among nine women with breast cancer suggested that serum 25OHD levels remained stable during chemotherapy with FEC (25). Others, however, have reported an increased prevalence of vitamin D deficiencies after neoadjuvant chemotherapy among 77 women with locally advanced breast cancer (44% before treatment versus 73% around the last cycle) (17). Similarly, 73 patients

with breast cancer from Iran showed declined levels of serum 25(OH)D<sub>3</sub> (minus 13%) 8 months after the end of adjuvant chemotherapy as compared to pre-chemotherapy values (23). Charehbili et al. have studied the effects of neoadjuvant chemotherapy, with or without the bisphosphonate zoledronic acid, on 25(OH)D<sub>3</sub> levels in 73 stage II–III breast cancer patients with HER2-negative tumors participating in the NEOZOTAC trial (22). For patients receiving neoadjuvant TAC without zoledronic acid and vitamin D/calcium ( $n=34$ ), the decline in 25(OH)D<sub>3</sub> levels was  $-16$  nmol/L (SD 25.2) (22). It should be noted, however, that these analyses were not adjusted for season of blood sampling. In our study, an adjusted decrease of 5 nmol/L (95% CI  $-10.7$ – $0.5$ ) was found 1–3 weeks after the last cycle of chemotherapy compared to pre-chemotherapy values ( $P=0.082$ ). Hence, our findings are consistent with the results of the NEOZOTAC trial (22), which was conducted among a comparable group of breast cancer patients in the Netherlands. However, the magnitude of the decrease in 25(OH)D<sub>3</sub> levels directly after chemotherapy was modest in our study compared to the NEOZOTAC trial. Potential explanations for this discrepancy may be the timing of blood sampling which was conducted before the last cycle of chemotherapy in the NEOZOTAC trial and 1–3 weeks after chemotherapy in our study. Possibly, levels of 25(OH)D<sub>3</sub> already started to recover shortly after chemotherapy. Also baseline 25(OH)D<sub>3</sub> levels, which were higher for the NEOZOTAC participants as compared to our population (deficiency in 38% and 50% of the patients, respectively), or different chemotherapeutic settings (neoadjuvant TAC and various neoadjuvant or adjuvant regimens, respectively) may explain these findings.

Based on our data, we also had the opportunity to study changes in 25(OH)D<sub>3</sub> levels over a prolonged time period and to compare trajectories in 25(OH)D<sub>3</sub> levels with those of women without cancer. Interestingly, 6 months after the end of chemotherapy, serum 25(OH)D<sub>3</sub> levels returned to values observed in women without cancer. As a consequence, also the prevalence of vitamin D deficiencies among the breast cancer patients decreased from 50% before start of chemotherapy to 32% 6 months after the end of chemotherapy. These results are in agreement with the findings of Kim et al. who demonstrated that serum 25OHD levels in 93 Korean women with breast cancer receiving adjuvant chemotherapy decreased during the first 6 months after diagnosis, but recovered 12 months after diagnosis (24). We have now added further evidence suggesting that serum

25(OH)D<sub>3</sub> levels even returned to 25(OH)D<sub>3</sub> levels observed in women without cancer. Various mechanisms may explain the recovery of 25(OH)D<sub>3</sub> levels 6 months after the end of chemotherapy. Our first thought was that patients, who may become more health conscious during and after cancer treatment (31), could have changed their use of dietary supplements containing vitamin D. Dietary supplements containing vitamin D substantially contribute to 25(OH)D<sub>3</sub> levels (32,33). At the start of our study, 32% of the patients (and 31% of the women without cancer) reported use of dietary supplements containing vitamin D in the past month. At the end of the study, the percentage of patients who reported use of dietary supplements containing vitamin D in the previous month increased to 47%, which is in line with previous studies showing that dietary supplement use is common among cancer survivors in general, and breast cancer survivors in particular (34–36). However, our sensitivity analysis for which the participants who changed their use of dietary supplements containing vitamin D were excluded, showed similar results and provided compelling evidence that changes in dietary supplement use were not primarily responsible for the observed increases in serum 25(OH)D<sub>3</sub> levels after chemotherapy.

Alternative explanations for the observed recovery of 25(OH)D<sub>3</sub> levels may refer to other behavioral changes or biological aspects. Potential diet and lifestyle behaviors that the patients adopted after cancer diagnosis (37,38), such as changed dietary habits, increased physical activity, more outdoor activities and sunlight exposure, may have contributed to increased 25(OH)D<sub>3</sub> levels. In the current study, data on sun exposure were not available. Our data on adherence to the national recommendations for physical activity did not provide evidence that women with breast cancer increased their physical activity after the end of chemotherapy, although changes in type of activities or outdoor behaviors cannot be excluded. From a tumor biology point of view, removal of the tumor by surgical resection and chemotherapy may have also resulted in recovery of circulating 25(OH)D<sub>3</sub> levels. Previous studies suggested that regulation of the vitamin D pathway may be altered in breast cancer cells (39,40). For example, more stable *CYP24* mRNA profiles, responsible for clearing of the active metabolite 1,25(OH)<sub>2</sub>D, were found in human breast cancer cells as compared to normal human mammary epithelial cells (41). Also (epigenetic) deregulation and altered expression levels of the vitamin D receptor (*VDR*) gene during breast

carcinogenesis have been described (39,40,42,43). Moreover, exogenous 25OHD exposure resulted in local accumulation of 25OHD and the active metabolite 1,25(OH)<sub>2</sub>D in tumor tissue of a breast cancer mouse model (44). Altogether, these findings may point toward altered metabolism of vitamin D during carcinogenesis and potentially increased demands of breast cancer cells. This hypothesis remains, however, speculative and needs to be confirmed, since the exact role of uptake, storage and metabolism of vitamin D in breast cancer tissue is not fully understood (39) and cannot be addressed in our study. Also alternative mechanisms or personal or clinical factors determining vitamin D levels in patients receiving chemotherapy, need to be explored in future studies.

Potential limitations of our study include the relatively small sample size and our heterogeneous population in terms of tumor characteristics, timing of treatment, and cytotoxic regimens. This can, however, also be considered a strength, since the population of breast cancer patients in the Netherlands is characterized by heterogeneity as well. It should be recognized that patients and women from the comparison group slightly differed with regard to some characteristics (e.g., smoking status, BMI and physical activity). These differences may have potentially impacted comparisons between the groups, but are not likely to affect analyses dedicated to changes in 25(OH)D<sub>3</sub> levels within the respective groups. Strengths of our study are the measurements of 25(OH)D<sub>3</sub> levels at multiple time points after diagnosis and the assessment of dietary supplement use throughout the course of cancer therapy. Moreover, the consideration of a comparison group of women without cancer can be considered a major strength of the current study.

In conclusion, our results confirmed that vitamin D deficiencies are common among Dutch patients with breast cancer. After the end of chemotherapy, circulating 25(OH)D<sub>3</sub> levels recovered and within 6 months even returned to levels observed in women without cancer. However, suboptimal levels of vitamin D were still observed in the majority of the women participating in this study, which may have important clinical implications. This finding underpins the relevance of awareness and potentially careful monitoring of vitamin D levels in this vulnerable population of women approaching menopausal age with an increased risk of declined bone health. In addition, our findings may provide interesting leads for further studies dedicated to the hypothesis that low serum 25(OH)D<sub>3</sub> levels are, at least partly, resulting from breast tumor biology.

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## ORCID

Dieuwertje E. Kok  <http://orcid.org/0000-0001-7154-8207>

Hanneke W. M. van Laarhoven  <http://orcid.org/0000-0003-3546-9709>

Renate M. Winkels  <http://orcid.org/0000-0002-0376-8811>

Ellen Kampman  <http://orcid.org/0000-0002-8606-7075>

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