We correlated serum 25-hydroxyvitamin D$_3$ (25OHD) levels with tumor characteristics and clinical disease outcome in breast cancer patients and assessed the impact of genetic determinants of vitamin D insufficiency. We collected serum from 1800 early breast cancer patients at diagnosis, measured 25OHD by radioimmunoassay (RIA), and determined genetic variants in vitamin D-related genes by Sequenom. Multivariable regression models were used to correlate 25OHD levels with tumor characteristics. Cox proportional hazard models were used to assess overall survival (OS), disease-specific survival (DSS), and disease-free interval (DFI). Lower 25OHD serum levels significantly correlated with larger tumor size at diagnosis ($P = 0.0063$) but not with lymph node invasion, receptor status, or tumor grade. Genetic variants in 25-hydroxylase (CYP2R1) and vitamin D-binding (DBP) protein significantly determined serum 25OHD levels but did not affect the observed association between serum 25OHD and tumor size. High serum 25OHD ($>30$ ng/mL) at diagnosis significantly correlated with improved OS ($P = 0.0101$) and DSS ($P = 0.0192$) and additionally had a modest effect on DFI, which only became apparent after at least 3 years of follow-up. When considering menopausal status, serum 25OHD had a strong impact on breast cancer-specific outcome in postmenopausal patients [hazards ratios for 25OHD $>30$ ng/mL versus $\leq 30$ ng/mL were $0.15$ ($P = 0.0097$) and $0.43$ ($P = 0.0172$) for DSS and DFI, respectively], whereas no association could be demonstrated in premenopausal patients. In conclusion, high vitamin D levels at early breast cancer diagnosis correlate with lower tumor size and better OS, and improve breast cancer-specific outcome, especially in postmenopausal patients.

**Introduction**

Deficiency of vitamin D is common and represents a major health problem. Early in life, vitamin D deficiency causes growth retardation and rickets, whereas in adults, it contributes to osteopenia/osteoporosis and to various chronic illnesses, including autoimmune diseases, infectious diseases, and cardiovascular diseases (1–3). Remarkably, an association between the risk of developing cancer, latitude, low sun exposure, and poor vitamin D status has been observed (4–6). Additional studies investigating the link between circulating 25-hydroxyvitamin D$_3$ (25OHD) levels, dietary vitamin D intake, and cancer risk also observed a consistent inverse relationship between 25OHD levels and colorectal cancer incidence (7–9), whereas studies related to breast cancer risk were less conclusive. A recent meta-analysis, which correlated serum 25OHD with breast cancer risk, confirmed an inverse correlation in case-control studies sampling 25OHD immediately after breast cancer diagnosis, but not in prospective studies when 25OHD measurement was done years before diagnosis (10). A second independent meta-analysis also revealed a slight decrease in breast cancer risk following preventive vitamin D intake (11). Moreover, the Women’s Health Initiative (WHI), a large randomized, placebo-controlled calcium/vitamin D trial, showed that calcium/vitamin D supplementation significantly decreased the risk of total, breast, and colorectal cancers (12). Despite these promising observations, vitamin D supplementation has not yet been implemented in dietary cancer prevention programs (13,14).

Interestingly, inverse correlations between serum 25OHD levels measured at diagnosis and subsequent breast cancer recurrence and mortality have also been reported (15,16). Similar observations were made in other cancer types, such as early-stage non-small cell lung cancer (17), colorectal cancer (8,18), melanoma (19), and non-Hodgkin’s lymphoma (20). These findings suggest that preventive vitamin D supplementation might not only be beneficial to reduce the risk of cancer development, but may also prevent tumor recurrence. Conversely, the hypothesis that 25OHD is inversely associated with overall cancer mortality was not confirmed in a follow-up report (21) on the NHANES III (Third National Health and Nutritional Survey) cohort, which included >16 000 subjects. In fact, this study even demonstrated a positive correlation between 25OHD levels and lung cancer mortality among males (21), suggesting that caution is warranted before increasing 25OHD levels for cancer protection purposes.

The controversy on how vitamin D influences the natural course of certain malignancies and whether its supplementation should be encouraged is particularly relevant in breast cancer, as >60% of cases occur in postmenopausal women, a population showing high prevalence of vitamin D deficiency and osteoporosis (22). Moreover, due to hormonal therapy, women surviving breast cancer have a markedly higher fracture risk than women without a history of breast cancer (23,24). Consequently, current breast cancer guidelines encourage daily supplementation with calcium and vitamin D, but the benefit of these supplements remains unclear and, not surprisingly, their routine application is still debated (25).

In general, it has been difficult to interpret the role of vitamin D in the context of clinical and epidemiological studies, due to the many confounding factors that are known to influence circulating 25OHD levels, including dietary intake, seasonal variation, outdoor activity, and weight – particularly because several of these confounding factors are difficult to assess on a routine basis. Furthermore, a recent genome-wide association study by Wang et al. reported that genetic variants in key genes of the vitamin D metabolism pathway act as important determinants of circulating 25OHD levels (26,27). In humans, the major route of 25OHD formation involves the skin, where 7-dehydrocholesterol is photoconverted into vitamin D$_3$ (cholecalciferol), which is transported to the liver to undergo 25-hydroxylation by cytochrome P450 2R1 (CYP2R1) into the circulating 25OHD form (28). In the kidney, cytochrome P450 27B1 (CYP27B1) subsequently converts 25OHD into the biologically active form 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), which acts on various target tissues.

Vitamin D status at breast cancer diagnosis: correlation with tumor characteristics, disease outcome, and genetic determinants of vitamin D insufficiency

**Abbreviations:** 25OHD, 25-hydroxyvitamin D$_3$; BMI, body mass index; CI, confidence interval; CYP2R1, cytochrome P450 2R1 (25-hydroxylase); DBP, D-binding protein; DDFI, distant disease-free interval; DFI, disease-free interval; DSS, disease-specific survival; ER, estrogen receptor; OS, overall survival; RIA, radioimmunoassay; SNP, single nucleotide polymorphism; VDR, vitamin D receptor.

† These authors contributed equally to this study.
active hormone 1α,25-dihydroxyvitamin D₃, which binds to the nuclear vitamin D receptor (VDR) (28). This results in transcriptional regulation of numerous target genes. A strong correlation between 25OHD serum levels and genetic variants in CYP2R1 and 7-dehydrocholesterol reductase (DHCR7) was identified in the study by Wang et al. Additionally, genetic variants in VDR and the vitamin D-binding protein (DBP, also called GC), a plasma protein that binds vitamin D and its metabolites and transports them to target tissues, also affect the vitamin D signaling pathway (27,29). The relevance of these genetic changes with respect to 25OHD levels in cancer patients, and in particular their association with tumor characteristics and disease outcome, is unknown.

In this study, we have used a clinically well-documented and prospectively recruited cohort of early breast cancer patients to explore the complex interplay between serum 25OHD levels, genetic variability in the vitamin D pathway, and breast cancer biology. More specifically, we examined whether 25OHD serum levels at the time of breast cancer diagnosis correlate with various tumor characteristics and/or affect breast cancer relapse and survival, and whether this is influenced by genetic variability, in particular vitamin D-related genes.

Methodology

Study population

Since 2003, the Leuven Multidisciplinary Breast Center (University Hospitals Leuven) has systematically collected plasma and serum from all consenting (i.e. ≥75%) breast cancer patients at the time of diagnosis. Since 2003, the Leuven Multidisciplinary Breast Center (University Hospitals Leuven) has systematically collected plasma and serum from all consenting (i.e. ≥75%) breast cancer patients at the time of diagnosis. From 2007 for all newly diagnosed breast cancer patients as well as for patients in follow-up. All breast cancer patients are also included in a clinical database, containing extensive general and tumor-related information, as well as clinical follow-up such as relapse and cause of death.

For this study, eligible subjects were selected from this database based on the following inclusion criteria: (i) patients were diagnosed with primary (i.e. non-relapse), early (i.e. non-locally advanced, non-metastatic), invasive (i.e. non-in situ) breast cancer between June 2003 and February 2010, (ii) patients received primary surgery and pathological confirmation in our institution, and (iii) serum had been collected at the time of diagnosis, before initiation of any treatment. For each eligible patient, the following clinical data were collected: age at diagnosis, date and season of serum collection, body mass index (BMI), menopausal status, largest pathological tumor diameter and pT staging, worst tumor grade, number of tumor foci, histological subtype, estrogen receptor (ER), progesterone receptor (PR) and HER2/neu status, and axillary lymph node status, assessed as both (i) the number of positive lymph nodes and (ii) pN staging according to six ordered categories [pN0(i): no lymph node metastasis detected; pN0(i+): only isolated tumor cells detected; pN1(mi): micrometastases >0.2 mm and <2.0 mm detected; pN1: 1–3 positive lymph nodes; pN2: 4–9 positive lymph nodes; pN3: 10 or more positive lymph nodes]. Determination of tumor grading and ER, PR, and HER2/neu status was done according to established procedures (30). ER and PR were considered positive if >1% of cells stained positive on immunohistochemistry (IHC). HER2 was considered positive if the FISH test, systematically performed in all IHC 2+ tumors, showed HER2 genomic amplification or, in the absence of FISH, if IHC was 3+. In case of a multifocal (number of tumor foci >1) and/or bilateral breast tumor, only the characteristics of the dominant focus and/or tumor [i.e. with the highest Nottingham Prognostic Index (NPI)] were included in the data set. Furthermore, different types of administered adjuvant therapy (i.e. radiotherapy, hormonal therapy, and chemotherapy) were also recorded, as well as follow-up information (date of local/locoregional recurrence and/or distant metastasis, date and cause of death, date of last follow-up, i.e. last clinical visit). The exact cause of death was documented in 119 out of 134 cases (87%). This study is in compliance with the Helsinki Declaration. Blood sampling, collection of patient data, and genetic analysis were approved by the ethics committee of our institution (University Hospitals Leuven). All patients included in the study gave written informed consent.

Serum collection and determination of 25OHD serum levels

Peripheral blood was sampled in 4 mL BD Vacutainer SST II Advance tubes, and the samples were incubated at room temperature for 20–60 min. After centrifugation, the supernatant (serum) was isolated and stored protected from light at ~80°C. 25OHD levels were subsequently measured as follows: 25OHD and other hydroxylated metabolites were first extracted from serum with acetonitrile. After extraction, an equilibrium RIA procedure was performed to assess the amount of 25OHD in the sample, and subsequently, 25OHD was measured by RIA (using the 25OHD 125I RIA kit of Diasorin, Stillwater, MN). Levels are expressed in ng/mL (conversion factor 2.5 for nmol/L). The detection limit of the kit was 1.5 ng/mL 25OHD. The assay range within this patient population was 2.6–86.9 ng/mL. Between- and within-run variances were 9.5% and 7.4%, respectively. Patients were arbitrarily classified in three categories, according to the frequently used 25OHD cut-off values of 20 ng/mL (31,32) and 30 ng/mL (31,33): the ‘low,’ ‘intermediate,’ and ‘high’ vitamin D groups comprised patients with 25OHD <20 ng/mL, 25OHD between 20 and 30 ng/mL, and 25OHD >30 ng/mL, respectively. The seasonal categories that were used in the analysis were (i) summer (21 June–20 September), (ii) winter (21 December–20 March), and (iii) spring/autumn (21 March–20 June and 21 September–20 December).

DNA analysis

Peripheral blood was sampled in 4 mL BD Vacutainer K2E-EDTA tubes, and after centrifugation, germ-line DNA was extracted from the precipitated leukocyte cell fraction according to standard procedures. Genotyping for the single nucleotide polymorphisms (SNPs) rs7041 and rs4588 in DBP, rs731236, rs739837, and rs10735810 (known as Taq1, Apal, and Fok1, respectively) in VDR; rs10741657 in 25-hydroxylase (CYP2R1); rs12785878 in DHCR7, and rs6013897 in the degradation enzyme 24-hydroxylase (CYP24A1) was performed in a blinded manner using iPLEX technology on a MassARRAY Compact Analyzer (Sequenom Inc., San Diego, CA) at the Vesalius Research Center. Quality control was performed by genotyping 56 samples in duplicate, with a duplicate concordance of 100%. No significant deviations from Hardy–Weinberg were observed for any of the SNPs.

Statistical analysis

Multivariable linear regression models were used to study the association between 25OHD serum levels and tumor characteristics. In an initial exploratory analysis, separate models were fitted for each of the tumor characteristics while correcting for age at diagnosis, BMI, and seasonal variation. Multiple imputation was applied to account for missing BMI values (about 12% of cases) (34,35). The following tumor characteristics were considered: tumor size, tumor grade, nodal stage, ER, and HER2 status. Non-linear relationships between 25OHD and continuously measured tumor parameters were explored graphically. An interaction between tumor size and a binary variable indicating the presence of multiple foci was included to test whether the effect of tumor size was different for patients with one tumor focus compared with patients with multiple foci. In the second stage, the multivariable model was built including all clinical variables plus corrections for BMI, age, and seasonal variation. In the third stage, we considered interactions between tumor characteristics and SNPs. Given the large number of interactions, we corrected for multiple testing using a false discovery rate (FDR) correction of 5% (36). Linear regression models were used to assess the effect of SNPs on 25OHD serum levels, while correcting for age at diagnosis, seasonal variation, and BMI. To evaluate genotypic effects, separate models were fitted for each SNP.
were fitted for each of the SNPs. Correction for multiple testing was performed using a FDR of 5%. In the second stage, the pairwise difference between the alternative genotypes was tested. A Bonferroni correction was applied to correct for multiple testing.

To investigate possible associations between SNP genotypes and tumor/patient characteristics, models were built for each separate SNP with genotype as explanatory variable and the tumor or patient parameter as response variable. The continuous variables tumor size (log-transformed) and age were modeled by linear regression models. pN was considered as an ordinal categorical variable (six ordered categories) and analyzed by means of a proportional odds model. Finally, the number of positive lymph nodes was analyzed using a Poisson log-linear model. Correction for multiple testing was performed using a FDR of 5%.

A Cox proportional hazard model was used for follow-up data analysis, where the time to the event was modeled as a function of 25OHD levels. The disease-specific survival (DSS) was determined as the time between breast cancer diagnosis and breast cancer-related death. DFI was calculated as the time elapsing between breast cancer diagnosis and local recurrence and/or lymph node metastasis and/or distant metastasis; distant disease-free interval (DDFI) was defined as the time between diagnosis and metastasis at distant sites. Deceased patients with undocumented cause of death were censored at last follow-up and patients who died from a non-breast cancer-related cause were censored at death. For the analysis of DSS, surviving patients were censored at last follow-up; for the analysis of DFI, non-relapsing patients were censored at last follow-up and for the analysis of DDFI, patients without distant metastasis were censored at last follow-up. Correction for a number of known prognostic factors was performed by including them as explanatory variables in the model. In an initial exploratory phase, we modeled each of the explanatory variables separately to find out whether or not they are related to the event risk and to explore the functional shape of this relationship for continuous variables. To explore the functional form, we considered models with linear and quadratic terms as well as models using smoothing splines with 4 and 5 knots. The proportional hazard assumption was checked for all covariates. In cases where this assumption was violated, the model was adapted by including an interaction term with time (37). In the second phase, we built the final multivariable model, including 25OHD, and each of the prognostic factors significantly correlated to the event risk. Finally, we repeated the multivariable analysis with stratification for menopausal status by including an interaction term between 25OHD and menopausal status. The effect of 25OHD on the event risk was then estimated separately for premenopausal and postmenopausal patients. Patients with a perimenopausal (n = 45) or unknown menopausal status (n = 105) were excluded from this analysis.

Results

Study population
In total, 1800 eligible patients were included in this study, based on the inclusion criteria described in Methodology. Demographics and baseline characteristics of the study population are summarized in Tables I and II. Bilateral and multifocal breast tumors were present in 57 (3.2%) and 200 (11.1%) cases, respectively. Circulating 25OHD levels at diagnosis were measured for all participants (Table I). ‘High’ 25OHD levels (>30 ng/mL) were observed in 647 patients (35.9%), while 570 patients (31.7%) showed ‘intermediate’ 25OHD levels (20–30 ng/mL) and 583 patients (32.4%) were classified in the ‘low’ 25OHD group (<20 ng/mL).

As expected, age, BMI, and seasonal variation significantly correlated with vitamin D status. In particular, 25OHD levels strongly decreased with increasing age (quadratic relation with P < 0.0001) and BMI (P < 0.0001), and mean serum 25OHD concentrations were lower in the winter compared with summer (22.6 ± 10.5 versus 31.6 ± 12.9 ng/mL, P < 0.001). Accordingly, as shown in Table I, patients belonging to the ‘low 25OHD’ group exhibited the highest mean age and BMI and were markedly more represented in the winter (48.1%) than in the summer (17.8%). These findings confirm well-established correlations of vitamin D status with aging, weight, and seasonal variation.

Circulating 25OHD levels at breast cancer diagnosis correlate with tumor size
First, we assessed whether 25OHD levels correlate with breast tumor-related characteristics. Separate models were fitted for tumor size, tumor grade, ER, HER2, and nodal status (considering either pN stage or number of positive lymph nodes), while systematically correcting for age at diagnosis, seasonal variation, and BMI. Interestingly, serum 25OHD levels correlated significantly with tumor size at diagnosis (P = 0.0197). It should be noted that tumor size of multifocal tumors was determined as the size of the tumor focus with the worst NPI, which in most but not all of the cases also represents the largest focus. To verify, however, that the correlation between 25OHD levels and tumor size was independent of the number of tumor foci, we tested for an interaction effect between tumor

### Table I. Demographics and serum 25OHD levels

<table>
<thead>
<tr>
<th>Age</th>
<th>Total population (n = 1800)</th>
<th>Low 25OHD (&lt;20 ng/mL) (n = 583)</th>
<th>Intermediate 25OHD (20–30 ng/mL) (n = 570)</th>
<th>High 25OHD (&gt;30 ng/mL) (n = 647)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (years) and range (min – max)</td>
<td>57.7 (22.0 – 94.0)</td>
<td>61.4 (28.0 – 94.0)</td>
<td>56.9 (22.0 – 93.0)</td>
<td>55.0 (26.0 – 88.0)</td>
</tr>
<tr>
<td>Mean (kg/m²) and range (min – max)</td>
<td>25.5 (14.9 – 48.1)</td>
<td>26.9 (16.3 – 48.1)</td>
<td>25.7 (14.9 – 47.9)</td>
<td>24.2 (16.1 – 40.4)</td>
</tr>
<tr>
<td>Season of sampling</td>
<td>Winter</td>
<td>Spring–Autumn</td>
<td>Summer</td>
<td>Winter</td>
</tr>
<tr>
<td>Low</td>
<td>393 (21.8%)</td>
<td>189 (48.1%)</td>
<td>76 (17.8%)</td>
<td>93 (23.7%)</td>
</tr>
<tr>
<td>Intermediate</td>
<td>318 (32.5%)</td>
<td>318 (32.5%)</td>
<td>141 (32.9%)</td>
<td>343 (35.0%)</td>
</tr>
<tr>
<td>High</td>
<td>318 (32.5%)</td>
<td>318 (32.5%)</td>
<td>141 (32.9%)</td>
<td>343 (35.0%)</td>
</tr>
</tbody>
</table>

[1] Percentages refer to the total population (n = 1800).
[2] Percentages are row percentages and refer to the total number of patients in the corresponding season category or menopausal status group.
[3] The percentages of missing values were 12.7% for BMI and 5.8% for menopausal status. Missing values for BMI were imputed using various imputation techniques. None of the other variables contained missing values.
Table II. Tumor characteristics and treatment of study subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>−0.0402</td>
<td>0.0063</td>
</tr>
<tr>
<td>Number of positive lymph nodes</td>
<td>0.1406</td>
<td>0.1019</td>
</tr>
<tr>
<td>Grade</td>
<td>0.0006</td>
<td>0.9989</td>
</tr>
<tr>
<td>ER status</td>
<td>−0.1290</td>
<td>0.8745</td>
</tr>
<tr>
<td>HER2 status</td>
<td>−0.4668</td>
<td>0.6052</td>
</tr>
</tbody>
</table>

The model additionally included the covariates age (quadratic effect), season, and BMI.

**Effect of circulating 25OHD on overall survival**

During the follow-up period of this study (median follow-up of 4.7 years), 134 deaths of any cause occurred, i.e. 64 breast cancer-related deaths, 55 deaths due to other causes, and 15 deaths with undocumented cause. In initial exploratory univariable analyses, significant effects on overall survival (OS) were observed for age (P < 0.0001), tumor size (logarithmic effect, P = 0.0003), lymph node involvement (pN: quadratic effect with P < 0.0001; number of positive lymph nodes: logarithmic effect with P < 0.0001), tumor grade (P < 0.0001), and ER status (P < 0.0001). A significant effect of serum 25OHD, both as continuous variable (P < 0.0001) or dichotomized with cut-off at either 20 ng/mL, i.e. upper limit for the ‘low’ vitamin D patient group (P = 0.0002), or 30 ng/mL, i.e. lower limit for the ‘high’ vitamin D patient group (P = 0.0802) was also found, with patients exhibiting lower vitamin D levels having an increased risk of death. In contrast, no association with OS was observed for BMI (P = 0.5406), season of diagnosis (P = 0.6551), HER2 status (P = 0.6225), or any of the SNPs (all P > 0.2). In the subsequent multivariable model, including correction for those variables that correlated significantly with OS plus correction for BMI, the inverse correlation between vitamin D level and risk of death was confirmed when considering 25OHD as a continuous variable [hazard ratio 0.79, 95% confidence interval (CI) = 0.65–0.95, P = 0.0104, per 10 ng/mL increase interval in serum 25OHD] or as dichotomized variable with cut-off at 30 ng/mL (hazard ratio 0.53, 95% CI = 0.33–0.86, P = 0.0101). This association was not dependent on menopausal status because the interaction between serum 25OHD and menopausal status was not significant.

**Effect of circulating 25OHD on DFS**

In the course of this study, 64 breast cancer-related deaths and 55 cases of non-breast cancer-related death occurred. The well-known prognostic value of tumor size (logarithmic effect, P < 0.0001), lymph node involvement (pN: linear effect with P < 0.0001; number of positive lymph nodes: logarithmic effect with P < 0.0001) tumor grade (P < 0.0001), and ER status (P < 0.0001) with regard to breast cancer-related death was confirmed in exploratory univariable analyses. Age at diagnosis also correlated significantly (quadratic effect, P = 0.0462), whereas no association was found for HER2 (P = 0.2241), BMI (P = 0.9185), or any of the eight evaluated SNPs (all P > 0.15). Subsequently, we observed a significant inverse correlation between serum 25OHD and risk of breast cancer-related death in a multivariable model correcting for age, BMI, tumor size, grade, and ER when considering serum 25OHD either as continuous variable [hazard ratio 0.79 per 10 ng/mL increase interval in serum 25OHD, 95% CI = 0.62–1.00, P = 0.0490] or as dichotomized variable [hazard ratio 0.49 for 25OHD >30 ng/mL versus ≤30 ng/mL, 95% CI = 0.27–0.89, P = 0.0192]. Interestingly, when stratifying for menopausal status, we found that high 25OHD levels (considered either continuous or dichotomized with cut-off at 30 ng/mL) were significantly associated with improved DSS in postmenopausal patients [hazard ratio 0.15 for 25OHD >30 ng/mL versus ≤30 ng/mL, 95% CI = 0.03–0.63, P = 0.0097] but not in premenopausal women [hazard ratio 0.93, 95% CI = 0.43–2.02, P = 0.8527]. Figure 1 shows Kaplan–Meier plots for DSS in premenopausal (panel A) versus postmenopausal women (panel B).

**Effect of circulating 25OHD on DFI**

Next, we assessed whether serum 25OHD levels measured at diagnosis influence DFI. In total, 116 of 1800 patients showed relapse, defined as local invasive breast tumor recurrence (n = 28) and/or locoregional lymph node metastasis (n = 17) and/or distant metastasis (n = 94). Initial exploratory univariable analyses revealed significant associations of relapse risk with age at diagnosis (P = 0.0055), tumor size (logarithmic effect, P < 0.0001), pN (quadratic effect, P < 0.0001), the number of positive lymph nodes (logarithmic effect, P < 0.0001), and tumor grade (P < 0.0001). The effect of ER status on relapse risk proved not constant over time (non-proportional hazards):
initially, patients with ER-positive tumors exhibited reduced relapse risk compared with patients with ER-negative tumors (P < 0.0001 at 1 and 3 years), but this difference disappeared over time (P = 0.8963 at 6 years). To account for this, the Cox model was extended with an ER-by-time interaction (37). Patients with HER2-positive tumors did not show a significant increase in risk of relapse relative to patients with HER2-negative tumors (P = 0.1124). There was no evidence for a link between BMI and risk of relapse (P = 0.4617) neither for an effect of any of the SNPs. When considered as a continuous measure, serum 25OHD failed to correlate with DFI in uni- or multivariable analysis, except for the postmenopausal group, where the hazard ratio for a 10 ng/mL increase of 25OHD was 0.74 (95% CI = 0.57–0.96, P = 0.0225). However, when dichotomizing 25OHD levels with cut-off at 30 ng/mL and allowing for an interaction with time to account for non-proportional hazards, a decreased relapse risk was noticed within the entire cohort at 3 or 6 years after diagnosis for patients exhibiting serum 25OHD levels >30 ng/mL. In a multivariable analysis, implementing corrections for age, BMI, tumor size, pN, grade, and ER and an interaction with time, the hazard ratios for relapse were 1.20 (95% CI = 0.63–2.28, P = 0.5852), 0.50 (95% CI = 0.29–0.85, P = 0.0103), and 0.25 (95% CI = 0.09–0.70, P = 0.0082) at 1, 3, and 6 years, respectively. The observed relapse rates at median follow-up (i.e. 4.7 years) were 7.8% (95% CI = 6.01–9.51) for patients with 25OHD levels ≤30 ng/mL versus 5.6% (95% CI = 3.67–7.52) for patients with 25OHD levels >30 ng/mL. Stratification for menopausal status revealed that, similar to DSS, the effect of vitamin D on DFI was confined to postmenopausal women. Figure 1 shows Kaplan–Meier plots for DFI in premenopausal (panel C) versus postmenopausal (panel D) patients with serum 25OHD levels ≤30 ng/mL (solid line) versus >30 ng/mL (dotted line).

Finally, we also considered DDFI. Distant metastasis occurred in 94 of 1800 cases. As expected, tumor size, grade, lymph node status, ER status (all P < 0.0001), and age (P = 0.0054) significantly affected event risk. When correcting for these parameters plus BMI, a border-line non-significant relationship (P = 0.0575) between DDFI and serum 25OHD, considered as continuous variable, was found. This association was significant when considering 25OHD as a dichotomized variable with cut-off at 30 ng/mL (P = 0.0413). Stratified analysis showed that only postmenopausal and not premenopausal women have a significantly higher risk of metastasis if they are vitamin D deprived; the hazard ratio for postmenopausal women with serum 25OHD >30 ng/mL versus ≤30 ng/mL was 0.34 (95% CI = 0.15–0.77, P = 0.0092).

**Genetic determinants of vitamin D deficiency do not affect tumor characteristics or survival**

Since a recent genome-wide association study reported significant correlations between vitamin D status and genetic variants in DBP (rs2282679 or the synonymous variants rs222040 and rs7041), the 25-hydroxylase gene CYP2R1 (rs10741657), the 24-hydroxylase gene CYP24A1 (rs6013897), and DHCR7 (rs12785878) (27), these four loci were included in this study. For DBP, we genotyped rs7041 and rs4588, which is in strong linkage disequilibrium with rs7041 (29). Furthermore, three well-described functional variants in the VDR gene (rs10735810 or Fok1, rs731236 or Taq1, and rs739837 or Apa1) (38,39) were selected. Genotyping for the selected SNPs was carried out on the study cohort subpopulation of 1338 patients (74%) with available germ-line DNA. Genotype success rates for all the SNPs were >98%.

When correlating these SNPs with circulating 25OHD, several highly significant associations were observed while correcting for age, seasonal variation, and BMI (Table IV). In particular,
the functional SNPs located in the DBP locus (rs7041, rs4588) and CYP2R1 locus (rs10741657) significantly affected 25OHD serum levels. Surprisingly, we failed to identify any significant correlation between SNP genotypes and tumor characteristics (i.e. tumor size, pN, number of positive lymph nodes, and age at diagnosis) or survival (i.e. DSS, DFI, DDFI) in separate univariable models (data not shown). The association between serum 25OHD and tumor characteristics was independent of the SNPs; for none of the tested SNPs (rs7041, rs4588, rs10741657, rs6013897, rs12785878, rs10735810, rs739837, and rs731236), a significant interaction effect between SNP genotypes and tumor characteristics was observed (data not shown).

### Discussion

In this study, we measured 25OHD levels in serum collected from 1800 patients at the time of diagnosis of primary non-metastasized breast cancer and correlated their vitamin D status with tumor characteristics and disease outcome. Our most remarkable finding was that reduced 25OHD levels correlate with increased tumor size. This observation is intriguing and has not previously been reported.

Goodwin et al. reported that vitamin D levels were significantly lower in women with high-grade breast tumors (16), while Yao et al. recently found that reduced 25OHD levels were correlated with higher tumor grade and ER negative tumors among premenopausal women only; but not when pre- and postmenopausal women were considered together (40). We did not observe a correlation with tumor grade or hormone receptor status. It should be noted, however, that both studies were conducted on smaller cohorts, involving 512 and 579 breast cancer cases, respectively. Our own observations, indicating that low 25OHD levels correlate with larger breast tumors, clearly point toward a role of vitamin D in breast tumor biology. In particular, vitamin D could be involved in local growth-inhibitory effects in the microenvironment of the breast tumor. Indeed, although vitamin D is primarily involved in elevating calcium and phosphorus plasma levels for skeleton mineralization (3), vitamin D is also known to modulate cellular growth, differentiation, and apoptosis of mammary epithelial cells (3,41,42). For instance, 25OHD exerts both antiproliferative and pro-apoptotic functions in transformed mammary cells by suppressing growth stimulatory signals and potentiating growth inhibitory signals (41). Our observations that high serum 25OHD levels are associated with smaller breast tumors, together with earlier reports that vitamin D deficiency may increase (breast) cancer risk (10,11), thus seem to confirm that vitamin D exerts a growth-inhibitory effect in the early phase of tumor initiation and outgrowth. As such, our data could also favor preventive supplementation of vitamin D. Additional research, including prospective interventional trials, is needed, however, to assess whether preventive vitamin D supplementation is indeed capable of directly affecting tumor size at diagnosis. Moreover, the observed effect of vitamin D status on tumor size, although highly significant, was rather modest and hence, its clinical relevance may be questioned. Nevertheless, our finding is most intriguing from a biological point of view, as it confirms an interplay between vitamin D physiology and breast tumor biology in vivo.

In agreement with previous reports describing the effect of vitamin D status on mortality in the general population (43,44), we found that patients exhibiting low serum 25OHD levels at breast cancer diagnosis exhibited an increased risk of death from any (breast cancer-related or -unrelated) cause. With regard to breast cancer-specific outcome, Goodwin et al. previously reported that women with vitamin D deficiency had an increased risk of distant recurrence and death (16). In this study, this correlation was confirmed, although the effect was less pronounced. There was indeed a weak but significant correlation of 25OHD with DSS. A significant association with DFI was observed after at least 3 years of follow-up, whereas the association with distant recurrence (DDFI) was border-line significant. The shorter follow-up period (~4.7 years) and, consequently, the relatively lower number of relapse events in our study (6.4% relapses and 3.6% breast cancer-related deaths), as compared with the Goodwin study (23% distant recurrences and 17% breast cancer-related deaths during a mean follow-up of 11.6 years), may at least partly account for the weaker effects of serum 25OHD on breast cancer outcome in this study. As a matter of fact, the observed effect of vitamin D status on DFI clearly increased with time of follow-up in our breast cancer cohort, suggesting that stronger correlations with outcome may

### Table IV. Pairwise comparison between genotypes and 25OHD serum level

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genetic variant</th>
<th>Genotypes</th>
<th>Number of patients (%)</th>
<th>25OHD levels (Mean ± standard deviation)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2R1</td>
<td>rs10741657</td>
<td>GG</td>
<td>508 (38.1)</td>
<td>25.9±12.0</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>616 (46.1)</td>
<td>28.8±12.4</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>211 (15.8)</td>
<td>29.1±13.7</td>
<td></td>
</tr>
<tr>
<td>CYP24A1</td>
<td>rs6013897</td>
<td>TT</td>
<td>783 (60.8)</td>
<td>28.4±12.9</td>
<td>0.1564</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TA</td>
<td>450 (34.9)</td>
<td>27.1±12.3</td>
<td>0.3009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>55 (4.3)</td>
<td>26.5±11.5</td>
<td></td>
</tr>
<tr>
<td>DHCR7</td>
<td>rs12785878</td>
<td>TT</td>
<td>756 (58.7)</td>
<td>28.5±12.7</td>
<td>0.1426</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG</td>
<td>459 (35.6)</td>
<td>27.0±12.4</td>
<td>0.3810</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>74 (5.7)</td>
<td>26.8±13.7</td>
<td></td>
</tr>
<tr>
<td>DBP</td>
<td>rs7041</td>
<td>GG</td>
<td>445 (33.5)</td>
<td>28.3±12.9</td>
<td>0.1624</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GT</td>
<td>637 (47.9)</td>
<td>28.1±12.5</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>248 (18.6)</td>
<td>25.7±11.8</td>
<td></td>
</tr>
<tr>
<td>DBP</td>
<td>rs4588</td>
<td>CC</td>
<td>696 (52.1)</td>
<td>28.9±13.1</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA</td>
<td>537 (40.2)</td>
<td>26.7±11.7</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>102 (7.6)</td>
<td>24.4±11.9</td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>rs10735810 (Fok1)</td>
<td>CC</td>
<td>481 (37.3)</td>
<td>27.1±12.1</td>
<td>0.4551</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>616 (47.8)</td>
<td>28.1±13.1</td>
<td>0.5313</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>191 (14.8)</td>
<td>29.2±12.7</td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>rs731236 (Taq1)</td>
<td>TT</td>
<td>457 (34.6)</td>
<td>27.7±12.5</td>
<td>0.8942</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TC</td>
<td>651 (49.4)</td>
<td>27.9±12.6</td>
<td>0.7748</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>211 (16.0)</td>
<td>27.5±12.6</td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>rs739837 (Apal)</td>
<td>TT</td>
<td>364 (28.2)</td>
<td>27.5±12.4</td>
<td>0.2503</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG</td>
<td>638 (49.5)</td>
<td>28.4±12.8</td>
<td>0.8325</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>288 (22.3)</td>
<td>27.2±12.9</td>
<td></td>
</tr>
</tbody>
</table>

*P-values are based on pairwise comparison between wild-type (genotype in upper row) and heterozygous (middle row) or homozygous (lower row) variant.
become apparent with longer follow-up. On the other hand, it should also be noted that the conclusions reported by Goodwin et al. are based on univariable analyses and were less pronounced in multivariable analyses including corrections for prognostic factors such as age, nodal status, grade, and hormone receptor status, which were all included in our multivariable survival analysis. A remarkable finding was that in a stratified analysis, serum 25OHD levels at diagnosis were significantly associated with all three outcome parameters DSSF, DFI, and DDFI within the subgroup of postmenopausal but not premenopausal women. Although the clinical relevance of this discrepancy between both patient groups is not entirely clear at this moment, it may possibly be related to the favorable effect of vitamin D on bone density and metabolism, particularly in postmenopausal women with low estrogen environment in the bone. In premenopausal women, the bone-protecting effect of vitamin D is probably largely overruled by the dominant action of estrogen. Increasing evidence indeed seems to link bone turnover to the development of bone metastases, and thus disease outcome in breast cancer patients. Interestingly, similar observations were made in the Adjuvant Zoledronic Acid to Reduce Recurrence (AZURE) trial (45), wherein the bone-stabilizing bisphosphonate zoledronic acid markedly improved disease-free survival solely in post- but not premenopausal patients.

A particular strength of this survival analysis is that breast cancer-specific survival was used as an endpoint and that the cause of death was well documented in the vast majority of our cases. Previous studies on vitamin D in relation to cancer outcome often considered OS as a measure for disease outcome, which is less adequate for breast cancer, as a comparable proportion of patients (~3%) die of breast cancer-unrelated causes. In fact, our patients were very well characterized for all parameters that were used in this study, which allowed accurate analyses on homogeneous groups. For instance, in the stratified analysis for menopausal status, only purely pre- and postmenopausal women were included, and perimenopausal women were omitted.

Recent insights into vitamin D physiology indicate that 25OHD serum concentrations of 20 ng/mL or higher are required to maintain bone homeostasis (13, 32). Our current data imply that, with respect to breast cancer outcome, serum 25OHD levels >30 ng/mL might be more optimal. Another recent study even suggested that 25OHD levels of >40 ng/mL, although difficult to achieve, would be desirable for the prevention of treatment-associated arthralgia in breast cancer patients (46). The question remains, however, whether vitamin D supplementation would actually be beneficial after diagnosis of the breast cancer. Previous studies in several types of cancer, including breast cancer, suggest that vitamin D supplementation might reduce the risk of recurrence and mortality (8,47–49). However, caution is warranted when interpreting correlations between vitamin D status and cancer survival, as they do not necessarily imply that vitamin D deficiency causally affects tumor biology and/or relapse. In fact, vitamin D status, which is influenced by sunlight exposure and outdoor activity, could merely be a reflection of the patient’s physical fitness—a well-recognized cancer prognostic marker (50). In this regard, it should also be noted that vitamin D levels can change over time, while this study only considered serum 25OHD concentration at one time point, i.e. at tumor diagnosis. Furthermore, given the immunosuppressive effects of vitamin D (i.e. inhibition of dendritic cells and down regulation of the T-helper cell 1 response) (3), vitamin D intake might actually reduce immune surveillance and thereby enhance tumor progression and dissemination.

To the best of our knowledge, this study is the first to correlate genetic variants, which affected 25OHD levels in a genome-wide screen of several epidemiological cohorts (27), with serum 25OHD levels collected from breast cancer patients at diagnosis. As in healthy individuals, genetic variations in the plasma protein DBP and 25-hydroxylase CYP2R1 significantly affected serum 25OHD levels in our breast cancer cohort. Surprisingly, however, we did not observe any correlation between these variants and breast tumor size or disease outcome. Moreover, the relationship between serum 25OHD levels and tumor characteristics and survival proved independent of vitamin D-related genotypes. This can probably be explained by the fact that the associations between serum 25OHD levels and SNP genotypes are relatively weak. Genotypes explain at maximum a 1.5% of the total variability observed in serum 25OHD, which is insufficient for serum 25OHD levels to function as a confounding factor in the association between genotypes and tumor size.

Conclusion

In conclusion, we observed a significant inverse correlation between serum 25OHD concentration and breast tumor size in a large cohort of newly diagnosed breast cancer patients. Furthermore, an association of favorable vitamin D status with improved outcome was noticed, especially in the subgroup of postmenopausal women. Additional investigations will be mandatory to unequivocally resolve the issue whether or not vitamin D supplementation would be beneficial with regard to breast cancer prevention and/or should be systematically incorporated into standard (breast) cancer treatment regimens.

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References


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