Prostaglandin Metabolising Enzymes and PGE_2 are Inversely Correlated with Vitamin D Receptor and $25(OH)_2D_3$ in Breast Cancer

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Abstract. *Background: Breast cancer is associated with inflammatory processes based on an up-regulation of cyclooxygenase-2 (COX-2) expression. The antiproliferative effects of calcitriol (1,25$(OH)_2D_3$) mediated via the vitamin D receptor (VDR) render vitamin D a promising target in breast cancer therapy. First data suggest a correlation between vitamin D and prostaglandin metabolism. Materials and Methods: We determined the expression of VDR, COX-2, 15-PGDH and the prostaglandin receptors EP_2/EP_4 in normal and malignant breast tissue by real-time PCR and Western blot analysis, as well as 25$(OH)_2D_3$ and PGE_2 plasma levels from healthy and breast cancer patients. Results: Significantly higher COX-2, lower VDR and lower EP_2 and EP_4 receptor protein levels in the malignant tissue and a significantly lower 15-PGDH protein level in normal breast tissue were detected. Breast cancer patients older than 45 years, diagnosed and sampled in the wintertime had significantly lower 25$(OH)_2D_3$ and higher PGE_2 serum levels. Conclusion: The inverse correlation between VDR and both COX-2 and 15-PGDH, as well as between PGE_2 and 25$(OH)_2D_3$ levels, suggests a possible link between VDR-associated target genes and prostaglandin metabolism.*

Besides classical molecular prognostic parameters such as the estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER-2) expression, recent studies have defined new biomarkers that have correlated with a poor prognosis such as prostaglandin E2 (PGE_2) and the associated proteins in the cyclooxygenase (COX) system (1-5).

The similarity of pathophysiological reactions in inflammation and in cancer is based on several molecular factors: An elevation of cytokines, chemokines and proteases is observed in both malignancies and inflammation (6). Various prostaglandins (PGs) are found in different types of tissues, synthesised by COX-1 and -2 from free arachidonic acid. In contradiction to the ubiquitously expressed COX-1 isoenzyme, COX-2 expression is induced in specific tissues by growth factors, cytokines and PGs (7). Overexpression of COX-2 in epithelial cancer has been detected in several types of tissue, and it has been associated with carcinogenesis, particularly neoangiogenesis, and with tumour progression (1-5, 8). The hypothesis of COX-2-promoted carcinogenesis has been corroborated by a transgenic mouse model (9). Although the data for correlation of COX-2 expression and clinicopathological parameters in breast cancer tissues are inconclusive, there is evidence of a tendency towards a positive correlation with defined parameters of poor prognosis (8, 10). COX-2 apparently promotes the transcription of aromatase and thus promotes enlargement of tumour cells in estrogen-responsive breast cancer (11). Furthermore, a meta-analysis of 14 epidemiological studies indicated that the continued intake of COX inhibitors (non-steroidal anti-inflammatory drugs, NSAIDs) reduces the risk for breast cancer by 18% (12).

PGE_2 is one of the most prevalent PGs and acts as a ligand of the G-protein-coupled receptors (GPCR) EP1-4 (13). The pivotal role of the EP1 receptor in colon carcinogenesis was demonstrated by receptor knockout mice and selective EP1 receptor antagonists (14). The signalling pathway *via* Gs/cAMP/protein kinase, which follows the binding of the ligand to the EP_2 receptor, enhances an increased vascular

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endothelial growth factor (VEGF) expression which is associated with tumour-neoangiogenesis (15). 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) inactivates PGE_2 and consequently constitutes a tumour-suppressing enzyme. Low levels of 15-PGDH are associated with poor prognosis while an increase of 15-PGDH has been observed in the comparatively well-differentiated ER-positive MCF-7 breast cancer cell line (16).

First references suggest a link between PG and vitamin D metabolism in tumour cells (17-19). Based on the antiproliferative effects in tumour cells, the biologically active form of vitamin D (calcitriol, 1,25-dihydroxycholecalciferol, $1,25(OH)_2D_3$) is a relevant factor in tumour prevention and therapy. Individual vitamin D balance, which is quantified by the calcediol (25-hydroxyvitamin D3, $25(OH)_2D_3$) serum level, depends on nutritional intake and on endogenous production, which is enhanced by sunlight exposure (20). There is strong evidence supporting the important impact of $1,25(OH)_2D_3$ on cell cycle arrest, apoptosis, cell differentiation, suppression of cell proliferation and immune response modulation (21, 22). In invasive breast cancer cell lines, $1,25(OH)_2D_3$ and synthetic vitamin D analogues promote apoptosis and reduce cell proliferation (23). $1,25(OH)_2D_3$ activates signalling pathways by ligand binding to the vitamin D receptor (VDR), a nuclear hormone receptor, which regulates the transcriptional processing of its target genes (24). For instance, the VDR is associated with p21, a cyclin-dependent kinase inhibitor, which is responsive to p53, the guardian of the genome (25). The expression of VDR has been detected in normal breast tissues, as well as in breast cancer tissues to the same extent, however patients with ER-positive tumours had significantly longer disease-free survival than those with ER-negative tumours (26).

There is strong evidence supporting an interaction of calcitriol and prostaglandin metabolism in cancer. Moreno *et al*. observed a significant growth inhibition of cancer cells by the combination of calcitriol and NSAIDs in prostate cancer cell lines *in vitro* (17). Calcitriol induces a PGE_2 decrease which is due to an inhibition of COX-2 expression and an up-regulation of the PG catabolising enzyme 15-PGDH. Summarising the above, a link between VDR-associated target genes and PG metabolism in breast cancer can be hypothesised and still requires further investigation in order to develop new molecular targeted therapies.

Materials and Methods

Tissue samples. We analysed a non-consecutive series of primary breast carcinomas (n=22) and normal-appearing breast epithelia (n=20) that were obtained from our institutional tumour bank. From the normal breast tissue group, tissue samples from women with chronic diseases such as diabetes, rheumatoid arthritis, renal insufficiency, liver diseases and endometriosis were excluded. Tissue samples were frozen in liquid nitrogen and represented different tumour stages (Table I), according to the TNM classification.

Blood samples. Blood samples were taken from both healthy (n=19) and tumour patients (n=48) in order to evaluate their $25(OH)_2D_3$ plasma levels. They were also taken from healthy (n=32) and tumour patients (n=42) for PGE_2 plasma level evaluation. Blood samples were then centrifuged at 4°C, 4000 rpm for 10 min and frozen at −80°C until measurement. Blood samples from patients with any of the chronic diseases mentioned above were excluded.

RNA isolation. The total RNA from harvested cells and homogenised healthy and malignant breast tissue samples were extracted with Qiazol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity of isolated RNA was verified by 1% agarose gel electrophoresis and the amount of total RNA was spectrophotometrically quantified at OD260/280 nm.

Reverse transcription and real-time PCR. Prior to utilization, RNA was treated with DNAse I (Invitrogen, Karlsruhe, Germany). The synthesis of cDNA from 1 μg total RNA of tissue samples and cells was performed using Super Script-II reverse transcriptase (Invitrogen) and oligo-d(T)15 primer (Invitrogen). Reverse transcription reaction mixtures were diluted 1:10 and quantitative real-time PCR was accomplished using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), primers for human COX-2, 15-PGDH (HPGD) and VDR genes (Qiagen, Hilden, Germany) and 2 μl of RT reaction mixture as template. A melting curve was generated after 50 cycles for the final PCR product of all the investigated genes by reducing the temperature to 65°C for 15 s followed by a slow increase in temperature up to 95°C. The fluorescence was measured at 0.2°C increments during the slow heating process. The obtained threshold cycles (Ct) were normalised to TATA-binding protein (TBP) and porphobilinogen deaminase (PBDG) as housekeeping genes and MCF-7, an epithelial breast cancer cell line, as calibrator. The fold change was determined by the formula: efficiency target gene^(investigated calibrator gene–sample gene)/efficiency housekeeping gene^(calibrator housekeeping gene–sample housekeeping gene) (27). The experiments were performed in triplicates for each gene and then were repeated twice.

Immunoblotting. To determine COX-2 and 15-PGDH protein expression, cytosolic and membrane proteins of deep frozen tissue samples were isolated with Qproteome cell compartment kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The detection of VDR, ERβ and EP receptors 2 and 4 was performed with whole cell lysates of deep frozen tissue samples lysed in sample buffer (125 mM Tris, 30% Glycerine, % SDS, pH 6.8). Proteins were subjected to 10% SDS PAGE under reducing conditions and blotted onto nitrocellulose and PVDF membranes (Schleicher Schuell, Dassel, Germany), respectively. After blocking in 5% non fat dry milk in PBST (1 × PBS, 0.2% Tween) for 1 h at room temperature, membranes were incubated with the primary antibodies for human COX-2 and 15-PGDH (Cayman Chemicals, Ann Arbor, Michigan, USA), and EP receptors 2 and 4 (Biozol, Munich, Germany) at a dilution of 1:1000 in blocking reagent overnight at 4°C with gentle shaking. The VDR antibody (Dianova, Hamburg, Germany) was used at a dilution of 1:10000. After several washing steps, membranes were incubated for 1 h at room temperature with the secondary antibodies conjugated to horseradish peroxidase (New England Biolabs, Frankfurt (Main), Germany) at a dilution of 1:2000 (COX-2, 15-PGDH) 1:5000 (VDR, EP_2 receptor) and 1:20000 (EP_4 receptor) in PBST. The signals obtained were visualised using the enhanced chemiluminescence (ECL) detection system (Millipore GmbH, Schwalbach, Germany) and compared to β-actin as loading control.

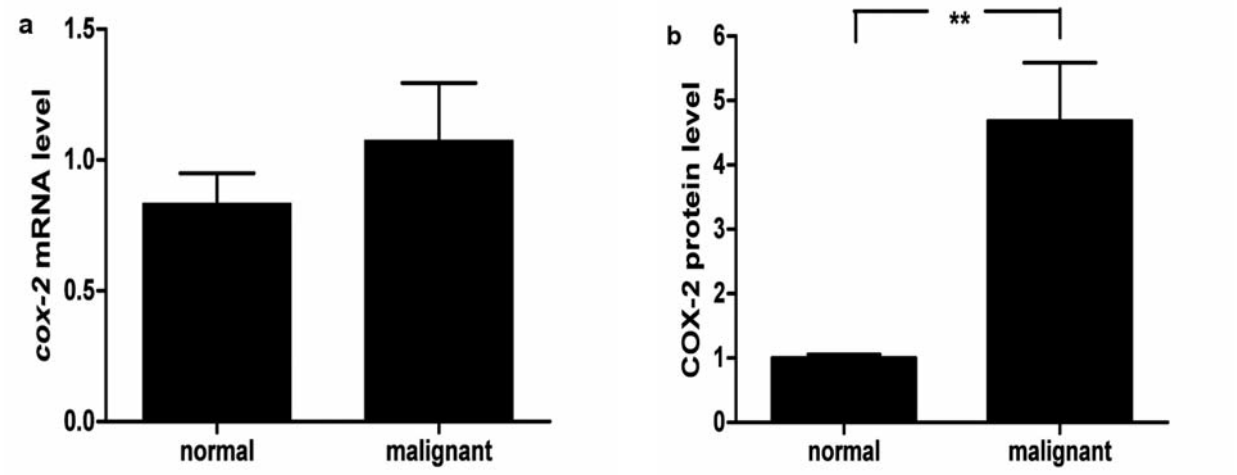

Figure 1. *Increased COX-2 expression in malignant breast tissues compared to normal tissue samples. Expression of COX-2 mRNA expression of was estimated using real-time PCR (a). Protein expression of COX-2 from normal (n=20) and malignant (n=22) tissue samples was densitometrically analysed and normalised to β-actin as loading control (b); **p<0.01.*

Quantification of prostaglandin E2 and 25(OH)$_2$D$_3$. PGE_2 levels of serum samples were determined by PGE_2 monoclonal enzyme immunoassay (Biozol, Munich, Germany) according to the protocol of the manufacturer. Serum concentration of $25(OH)_2D_3$ was assessed with Elecsys vitamin D3 chemiluminescent immunoassay (Roche Diagnostics, Mannheim, Germany) by a photomultiplier (Elecsys 2010, Hitachi, Tokyo, Japan).

Statistical significance. Statistical analysis of real-time PCR, Western blots results and $25(OH)_2D_3$ and PGE_2 measurements were performed using Student's *t*-test. All experiments were performed in duplicates and repeated twice. Data are shown as mean values relative to healthy individuals as the control group.

Results

In our recent analyses, we investigated the expression of COX-2, 15-PDGH and VDR in the benign MCF-10F and the malignant MCF-7 cell line and observed a basic inverse correlation between the protein levels of VDR and COX-2 (23). These findings suggest a possible link between the VDR-associated target genes and PG metabolism. Therefore, we wanted to go further to confirm the possible link between PG and vitamin D metabolism and analyse the expression of COX-2, 15-PDGH, PG receptors (EP_2 and EP_4) and VDR in normal and malignant breast tissue, as well as 25-$(OH)_2D_3$ and PGE_2 serum level in healthy and breast cancer.

Patient characteristics. We evaluated archival tissue specimens from 22 women with invasive breast cancer, ranging in age from 40 to 77 years (median age, 59.9 years), as shown in Table I. Furthermore, we analysed tissue from 20 healthy women from the same institutional tumour bank.

COX-2 expression in normal and malignant breast tissue. The *COX-2* mRNA was non-significantly higher in the malignant tissue (1.075±0.2202) in the RT-PCR as compared to the normal tissue samples (0.8332±0.1162) (Figure 1a). In the Western blot analysis, a more than 4-fold significantly higher ($p<0.01$) COX-2 protein level was shown in the malignant tissue (4.68±0.90) as compared to the normal tissue samples (1.00 ± 0.05) (Figure 1b).

15-PGDH expression in normal and malignant breast tissue. In the normal breast tissue, a non-significant lower level of HPGDH mRNA was detected by RT-PCR analysis (1.00±0.29) as compared to the malignant tissue samples (2.17±0.74) (Figure 2a). Western blot analysis presented a similar result. In the normal breast tissue samples, the protein level of 15-PGDH (1.03±0.06) was significantly ($p<0.05$) lower than that in the malignant tissue (1.40±0.15) (Figure 2b).

VDR expression in normal and malignant breast tissue. In the RT-PCR, the benign tissue showed a similar VDR gene expression (1.14±0.13) to the malignant tissue sample (1.10±0.09). In the Western blot analysis, a highly significantly lower VDR expression ($p<0.01$) was found in the malignant tissue samples (0.38±0.16) compared to the normal tissue (1.00±0.14) and therefore an inverse correlation to the COX-2 and 15-PGDH protein expression was found.

25-$(OH)_2D_3$ and PGE_2 serum levels in healthy women and breast cancer patients. We only observed significant differences in women older than 45 years, both with and without breast cancer who were diagnosed during wintertime

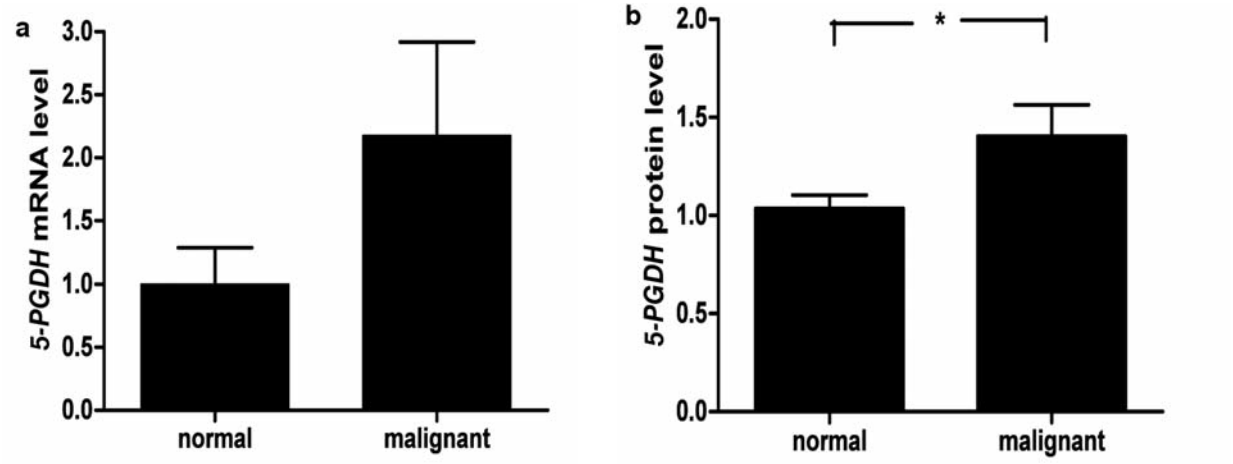

Figure 2. *Increased 15-PGDH expression in malignant breast tissues compared to normal tissue samples. Expression of 5-PGDH mRNA expression was estimated using real-time PCR (a). Protein expression of 5-PGDH of normal (n=21) and malignant (n=19) tissue samples was densitometrically analysed and normalised to β-actin as loading control (b); *p<0.05.*

(October – February). In healthy women, we found significantly higher 25-$(OH)_2D_3$ serum levels (p<0.01) (29.2±7.8 ng/ml) as compared to the women with breast cancer (20.6±6.2 ng/ml). The PGE_2 levels were inversely correlated to the 25-$(OH)_2D_3$ level as we detected significantly higher (p<0.01) PGE_2 levels in breast cancer patients (1499.0±283.3 pg/ml) than in the healthy women (587.0±82.28 pg/ml).

PGE_2 receptor (PTGERs) EP_2 and EP_4 expression in normal and malignant breast tissue. A significantly lower (p<0.01) EP_2 protein level was found in malignant tissue samples (0.49±0.03, n=12) as compared to the normal breast tissue samples (1.00±0.12, n=12). Moreover, we also detected a significantly lower (p<0.05) EP_4 protein level in malignant tissue (0.81±0.05, n=16) compared to the normal breast tissue (1.00±0.81, n=10). Thus, both PG receptors were lower in the malignant breast tissue.

Discussion

In the present study, we report an inverse correlation between the VDR and both the COX-2 and 15-PGDH expressions, as well as between 25$(OH)_2D_3$ and PGE_2 serum levels. This is the first study that reports a possible link between the vitamin D and prostaglandin metabolism in breast cancer.

In our analysis, we detected *COX-2* mRNA expression in both tumour and normal breast tissue by RT-PCR in all patients. By RT-PCR we observed a non-significantly higher COX-2 expression and by Western blot, a significantly higher COX-2 expression in malignant tissue. This is consistent with several other studies and is again evidence that COX-2 is frequently overexpressed in breast cancer (8, 28). In the present study, COX-2 expression was analysed by RT-PCR and by Western blot. Studies that compared COX-2 expression in malignant and normal breast tissue used mainly immunohistochemistry (10, 29, 30), however, these immunohistochemical methods were not quantitative and would strongly depend on the quality of the antibody and the staining protocol, as well as on the selection of the analysed region. Therefore, the variations in findings for COX-2 expression between different studies could partly be attributed to different scoring systems and cut-offs used for COX-2 immunoreactivity (31, 32). Only a few studies analysed the expression of COX-2 by RT-PCR (10, 29, 30). In a review of the literature, we found a detection rate of COX-2 protein expression in malignant breast tissue on average of 40% by immunohistochemistry and *COX-2* mRNA expression on an average of 90%. Thus it seems likely that COX-2 may undergo complex posttranscriptional and posttranslational modifications to yield the active enzyme (33).

By RT-PCR, a non-significantly higher 15-PGDH level in the tumour tissue was observed and a significant difference between tumour and normal breast tissue samples was found in Western blot analysis. In view of the study by Wolf *et al.,* who presented a low 15-PGDH expression in ER-negative breast cancer samples and high expression in ER-positive tumour samples (16), we rate our results as follows: Low levels of 15-PGDH are often associated with ER-negative tumours that exhibit a metastatic potential and correlate with unfavourable prognostic factors (34). In our study, just 22.7% (5/22) of the analysed breast cancer samples were from ER-negative tumours and 60% (3/5) of the ER-negative samples were triple negative (ER-negative, PR-negative and HER2-negative). This might be the reason for the significantly higher 15-PGDH expression in breast cancer tissue in the Western blot analysis.

One alternative approach to targeting COX-2 enzyme activity is to block binding of PGE_2 ligand to the EP receptors. However, the role of PGE_2 receptors EP_{1-4} is still under investigation. EP_1, EP_2 and EP_4 all appear to have pro-tumourigenic activity in at least one breast cancer model (35); EP_2 and EP_4 are important for regulating aromatase expression and activity in breast cancer cells (36). A recently published study investigated different breast cancer cells, such as MCF-7 and MDA-MB 231, and found both EP_2 and EP_4 receptor expression; however, in the more invasive MDA-MB 231 cells, the receptors were produced at lower levels (37). This might be in line with our results as we found both EP_2 and EP_4 expression, but in the malignant tissue, significantly lower protein levels were observed compared to the normal tissue. COX-2 is highly expressed in a subset of breast cancer and is associated with poor prognosis (2). Recent results indicate that antagonism of EP_4 may be as effective as COX inhibition (13). Therefore, evidence is building that EP_2 and EP_4 are critical determinants of cancer cell behaviour in breast cancer.

Some studies have shown that calcitriol acts in multiple pathways to inhibit the proliferation of prostate cancer cells (38-40). In light of these studies, it can be concluded that calcitriol regulates PG levels and PG actions and inhibits the stimulation of prostate cancer cell proliferation by endogenously derived PGs by the following three mechanisms: suppression of the COX-2 expression, up-regulation of 15-PGDH expression, and reduction of mRNA expression of the PGE_2 receptor subtype EP_2 and the PGF2 α (prostaglandin $F_{2\alpha}$) receptor FP (prostaglandin F receptor) (17).

VDR expression has been shown in healthy breast tissues and in more than 80% of breast cancer tissues (41). The natural ligand of the VDR, $1,25(OH)_2D_3$, and many new developed synthetic vitamin D analogues inhibit cell proliferation and induce apoptosis in breast cancer cell lines (23, 42). Furthermore, in animal models, vitamin D analogues retard tumour growth and lead to a regression of breast tumours (41). The *VDR* mRNA expression in the present study shows no difference between normal and breast cancer tissue in RT-PCR. However, a significantly lower VDR expression (nearly 3-fold) in the Western blot analysis was observed in the breast cancer tissue samples. This is in line with our own published data evaluating breast cell lines (19). In the literature, inconsistent data are reported in studies evaluating cell lines (43) and tissue (44). Townsend *et al*. observed a 7-fold increased *VDR* mRNA level by RT-PCR in breast cancer tissue as compared to normal breast tissue ($p<0.003$) (44). One possible reason for the inconsistent data and the significantly lower VDR protein level that we observed in breast cancer tissue might be the posttranscriptional modification of *VDR* mRNA on its way to the functional protein (45). In correlation with the VDR protein level, a basic inverse correlation with COX-2 and 15-PGDH protein levels was found in the present study. This concurs with our findings evaluating MCF-10F and MCF-7 breast cell lines where we detected an inverse correlation between COX-2 and VDR protein expressions in Western blot analysis as well. These findings suggest a possible link between the VDR, associated target genes and PG metabolism (19).

The circulating concentration of $25(OH)_2D_3$ is considered to be an excellent measure of the availability of vitamin D from diet and supplements and from synthesis in the skin (46). Its potential importance in breast carcinogenesis is due to the fact that $25(OH)_2D_3$ can be metabolised to $1,25(OH)_2D_3$ by 1-α-hydroxylase in breast tissue (47). Thus, $25(OH)_2D_3$ levels may be more representative of intracellular levels of $1,25(OH)_2D_3$ than circulating levels of $1,25(OH)_2D_3$ (48). To date, there have been several epidemiological studies about the association between vitamin D and breast cancer risk, however, their results have not been consistent. Some studies observed an association between plasma levels and breast cancer incidence (48, 49). In this study, we observed significantly lower $25(OH)_2D_3$ plasma levels in breast cancer patients older than 45 years during the wintertime. The $25(OH)_2D_3$ plasma levels of patients younger than 45 years showed no significant difference. It was no surprise not to find a difference in $25(OH)_2D_3$ levels in plasma sampled during summertime as sunlight exposure leads to vitamin D synthesis in the skin. These results suggested an association between breast cancer and the $25(OH)_2D_3$ levels. As COX-2 is responsible for the conversion of arachidonic acid into (PGE_2), our aim was to measure the serum level of PGE_2 in patients with breast cancer, as well as in healthy patients. Interestingly, we found an inverse correlation to the $25(OH)_2D_3$ serum level as PGE_2 levels in breast cancer patients were significantly higher (nearly 3-fold) compared with that of the healthy women. All the examined women were older than 45 years. This significant difference was detected only during wintertime. These findings even more strongly suggest a link between the PGE_2 and vitamin D metabolism in breast cancer.

COX inhibitors have been shown to suppress cancer cell growth both *in vivo* and *in vitro* (50, 51). The possibility of a synergistic action with the combination of calcitriol and NSAIDs for treating cancer cells should be considered. In prostate cancer, Moreno *et al*. proposed that a combination of calcitriol and NSAIDs might be a useful therapeutic strategy (17). In the light of our findings, this might also be true for breast cancer as well. Based on the elevated synthesis of PGs in cells that express COX-2, the aromatase expression and activity is increased in breast cells. Expression of aromatase leads to estrogen production and from cell line studies, we know that hormone receptor expression can be induced by sex steroid hormones. This supports the close correlation between COX-2 and hormone receptors. Therefore, several studies have investigated the benefit of selective and non-selective COX-2 inhibitors in combination with endocrine therapy (52, 53). Furthermore,

we do have encouraging results for the adoption of calcitriol in combination with docetaxel in prostate cancer (54). Further work is required to establish how NSAIDs can be best applied for therapeutic benefit.

We conclude that VDR and both COX-2 and 15-PGDH expression are inversely correlated, which suggests a possible link between VDR, associated target genes and PG metabolism. These findings are in line with our previously published data evaluating breast cell lines (23). Furthermore, serum levels of $25(OH)_2D_3$ and PGE_2 were inverse correlated when sampled and measured during wintertime. This is even more evidence for a link between vitamin D and PG metabolism. In addition, we found a positive correlation between the VDR and the EP_2/EP_4 receptor protein levels. We think these results are encouraging and further research is needed to determine if the use of an EP_2/EP_4 agonist will lead to increased proliferation and angiogenesis, as well as if the combination of calcitriol and NSAIDs is a useful therapeutic strategy in breast cancer treatment. Moreover, the combination of calcitriol and NSAIDs and their possible influence on angiogenesis need to be elucidated.

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Conflict of Interest Statement

The Authors declare no conflict of interest relevant to this article.

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