Correlation of Prostaglandin Metabolizing Enzymes and Serum PGE\textsubscript{2} Levels with Vitamin D Receptor and Serum 25(OH)\textsubscript{2}D\textsubscript{3} Levels in Breast and Ovarian Cancer

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Abstract. Background: Vitamin D and its active form calcitriol have multiple effects in cancer cells, such as anti-proliferative effects, induction of apoptosis and cell cycle arrest. There is a link between vitamin D metabolism and inflammatory processes, which should be considered in cancer therapy. An association between these two types of metabolism is also observed in breast and ovarian cancer. These inflammatory processes are based on an increase of cyclooxygenase-2 (COX-2) activity. The current study aimed to evaluate the expression of prostaglandin-metabolising enzymes COX-2 and 15-hydroxyprostaglandin-dehydrogenase (15-PGDH) along with the vitamin D receptor (VDR) in benign and malignant breast and ovarian tissues.

Patients and Methods: VDR, COX-2, 15-PGDH and prostanoid receptor E2/E4 expression were measured in tissues by western blot analysis. Additionally, plasma 25(OH)\textsubscript{2}D\textsubscript{3} and PGE\textsubscript{2} levels were measured in healthy patients and cancer patients. Results: We detected an elevated COX-2 and inversely a lowered VDR expression in cancer patients compared to healthy women. Breast cancer patients diagnosed during wintertime had a significantly lower serum level of 25(OH)\textsubscript{2}D\textsubscript{3}; PGE\textsubscript{2} serum levels were higher in both types of cancer. Conclusion: These results support the idea of a link between prostaglandin and vitamin D metabolism in regards to their influences on breast and ovarian cancer.

Cancer causes 20% of deaths in the European region. Causing about 1.7 million deaths each year in Europe, cancer is the second most frequent reason for death and morbidity. Breast and ovarian cancer are the most common cancer entities in gynaecology (1). Several hypotheses have been proposed to explain metabolism and pathways inducing carcinogenesis in both these entities. Vitamin D metabolism is known to have an effect on several cancer entities, such as breast, endometrial, cervical and ovarian cancer (2-4).

In the microenvironment of cancer cells, a linkage between vitamin D metabolism, the expression of the metabolizing enzymes of vitamin D, the vitamin D receptor and inflammatory processes is evident (5). The two isoenzymes cyclooxygenase-1 (COX-1) and COX-2 are involved in control of all these inflammatory processes by mediating prostaglandin (PG) synthesis from arachidonic acid. COX-1 enzyme is expressed in almost every tissue and seems to be linked to homeostatic processes, COX-2 expression is stimulated by different growth factors, cytokines and PGs and is seen as a prognostic factor for malignancy (6). COX-2 overexpression seems to have an effect on ovarian carcinogenesis by increasing proliferation, reducing apoptosis and mediating neoangiogenesis (7-9). PGE\textsubscript{2}, one of the end products of PG synthesis, acts as a ligand of the G-protein coupled prostaglandin E2 receptor subtypes (EP\textsubscript{1-4}) and regulates several key processes of tumor growth in different carcinomas (45). Additionally, COX-2 expression in breast cancer tissues shows a tendency towards a positive correlation with defined parameters of poor prognosis (10, 11); COX-2 apparently promotes the transcription of aromatase and thus promotes enlargement of tumor cells in estrogen-responsive breast cancer (12). Furthermore, a meta-analysis of 14 epidemiological studies indicated that continued intake of COX inhibitors (non-steroidal anti-inflammatory drugs, NSAIDs) reduces the risk for breast cancer by 18% (13).
The link between 25(OH) vitamin D3 levels and cancer has lead to the issue of cancer prevention and the role of vitamin D metabolites in cellular growth and carcinogenesis. Many factors have been related to breast cancer risks, including lack of vitamin D3 synthesis in the skin due to limited sunlight exposure or dietary intake (14). Several studies show that vitamin D3 status might be inversely correlated with breast exposure or dietary intake (14). Several studies show that 25(OH)D3 and PGE2 in benign and malignant breast and additionally, serum levels of 25-hydroxycholecalciferol (25(OH)2D3) and PGE2 in prostate cancer (20) and breast cancer tumor cells (5). Evidence for an interaction of VDR, associated target genes and processing of its target genes (19). There seems to be strong binding to specific DNA sequences and regulates the transcriptional binding to its ligand, the VDR interacts by dimerisation, binds to specific DNA sequences and regulates the transcriptional processing of its target genes (19). There seems to be strong evidence for an interaction of VDR, associated target genes and PG in prostate cancer (20) and breast cancer tumor cells (5).

The aim of this study was to analyse the link between PG-metabolising enzymes in correlation with VDR, and additionally, serum levels of 25-hydroxycholecalciferol (25(OH)2D3) and PGE2 in benign and malignant breast and ovarian tissues.

**Patients and Methods**

**Tissue samples.** Samples from patients with primary breast or ovarian cancer and normal-appearing breast or ovarian epithelia were collected in the Department of Gynaecology and Obstetrics at the University Medical Center Schleswig-Holstein, Campus Lübeck. Tissues were frozen and stored in liquid nitrogen. Women with chronic diseases, such as diabetes, rheumatoid arthritis, renal insufficiency, liver diseases and endometriosis were excluded.

**Blood samples.** For the measurement of plasma levels of 25(OH)D3 and PGE2 from healthy women as well as from cancer patients with breast or ovarian carcinomas, blood samples were centrifuged at 4000 rpm for 10 min at 4°C and stored at −80°C.

**Western blotting.** Total protein lysates were extracted with sample buffer (125 mM Tris, 30% glycerine, 10% SDS, pH 6.8) to determine VDR and EP receptors 2 and 4. Cytosolic and membranous proteins from samples were extracted using the Q-proteome cell compartment-kit (Qiagen, Hilden, Germany) to detect COX-2 and 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The proteins were electrophoresed on 10% PAGE and blotted onto nitrocellulose membranes. The membranes were incubated in 5% non-fat dry milk in PBST (1x PBS, 0.2% Tween-20) for 1 h at room temperature before the primary antibodies were added at the following dilutions: human COX-2 and 15-PGDH (both Cayman Chemicals, Ann Arbor, USA), and EP receptors 2 and 4 (both Biozol, Munich, Germany) at 1:1,000; the VDR antibody (Dianova, Hamburg, Germany) at 1:10,000. After three washings, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (New England Biolabs, Frankfurt-Main, Germany) for 1 h at room temperature at dilutions of 1:2,000 for COX-2 and 15-PGDH, 1:5,000 for VDR and EP2 receptor, and at 1:20,000 for EP4 receptor in PBST. The enhanced chemiluminescence (ECL) detection system (Millipore GmbH, Schwalbach, Germany) was used for visualization of detected protein bands. The quantification of proteins was performed densitometrically with β-actin as reference protein using Easy Win 32 (Herolab, Wiesloch, Germany).

**Serum levels of PGE2 and 25(OH)2D3.** The serum levels of PGE2 and 25(OH)2D3 were measured in a PGE2-monoclonal enzyme immunoassay (Biozol, Munich, Germany) and a 25(OH)2D3-Elecsys vitamin D3 chemiluminescent immunoassay (Roche Diagnostics, Mannheim, Germany) by a photomultiplier (Elecsys 2010; Hitachi, Tokyo, Japan).

**Statistics.** The experiments were performed in duplicate and repeated twice. For statistical analysis, Student’s t-test was used.

**Results**

**Patient characteristics for samples of benign and malignant breast tissues.** Samples from 22 women with invasive breast cancer were evaluated, ranging in age from 40 to 77 years (median age, 59.9 years). As controls, samples from 20 healthy women were analysed.

**Patient characteristics for samples of benign and malignant ovarian tissues.** Tissue specimens from 13 women with primary ovarian cancer, ranging in age from 38 to 80 years (median age, 63.6 years) were investigated. Furthermore, tissues from 13 healthy women were analysed.

**COX-2, 15-PGDH and VDR expression.** COX-2 expression detected by Western blot analysis revealed a more than 4-fold increase in malignant breast tissue (4.68±0.90; p<0.01) compared to normal breast tissue (1.00±0.05) (Figure 1A). Malignant ovarian tissue had a more than 2-fold COX-2 protein level (2.71±0.44; p<0.01) compared to normal ovarian tissue (1.09±0.11) (Figure 2A). The protein levels in ovarian tissue samples of 15-PGDH were 80-fold higher (80.73±12.11; p<0.001) compared to normal tissue samples (5.49±3.05). The protein levels of 15-PGDH (1.40±0.15) were significantly higher (p<0.05) in malignant breast tissue compared to healthy tissue (1.03±0.06). The VDR expression was reduced in breast cancer tissues (0.38±0.16, p<0.01) compared to normal tissues (1.00±0.14) (Figure 1B) and was also reduced in ovarian cancer tissues (0.37±0.13; p<0.01) compared to normal tissue (1.00±0.18) (Figure 2B). These results demonstrated an inverse correlation of the COX-2 and 15-PGDH with VDR protein expression changes in cancer tissues.
25-(OH)\textsubscript{2}D\textsubscript{3} and PGE\textsubscript{2} serum levels in healthy women, and breast and ovarian cancer patients. In breast tissue, we observed significant differences in women older than 45 years, among those with and those without breast cancer, diagnosed during wintertime (October – February). Healthy women had significantly higher serum levels of 25-(OH)\textsubscript{2}D\textsubscript{3} (29.2±7.8 ng/ml) than women with breast cancer (20.6±6.2 ng/ml; \(p<0.05\)) (Figure 3A). We also detected a reduction of the serum levels of 25-(OH)\textsubscript{2}D\textsubscript{3} in ovarian cancer tissues (25.32±1.57 ng/ml) compared to healthy women (29.15±2.74 ng/ml) but it was not statistically different (Figure 4A).

PGE\textsubscript{2} levels in breast cancer patients were higher (1499.0±283.3 pg/ml) compared to healthy women (587.0±82.28 pg/ml; \(p<0.01\)) (Figure 3B). The PGE\textsubscript{2} levels were also significantly higher \((p<0.01)\) in ovarian cancer patients older than 45 years (1132.0±210.1 pg/ml) than in healthy women of the same age (587.0±82.28 pg/ml) (Figure 4B). We did not find any significant difference in PGE\textsubscript{2} levels in women younger than 45 years.

PGE\textsubscript{2} receptor EP\textsubscript{2} and EP\textsubscript{4} expression. A significant reduction of EP\textsubscript{2} protein level \((p<0.01)\) was found in malignant tissue samples (breast 0.49±0.03, \(n=12\); ovary 0.48±0.05, \(n=8\)) as compared to healthy tissue samples (breast 1.00±0.12, \(n=12\); ovary 1.00±0.04, \(n=11\)). Moreover, we also detected a significantly lower \((p<0.05)\) level of EP\textsubscript{4} protein in malignant breast tissue (0.81±0.05, \(n=16\)) compared to healthy breast tissue (1.00±0.81, \(n=10\)). These findings were similar for ovarian cancer cases, but without statistical significance (malignant 0.87±0.05, \(n=13\); benign 1.00±0.03, \(n=14\)).
Discussion

Calcitriol, as shown in numerous previous studies, plays an important role in carcinoma cell growth, as well as in inflammatory pathways. Vitamin D and its metabolites inhibit cancer cell growth in cell cultures and suppress proliferation in xenografts of human tumours (21-23). In our present data, an inverse correlation between the VDR expression, COX-2 and 15-PGDH expressions was detectable, as well as between 25(OH)2D3 and PGE2 serum levels. Higher levels of COX-2 expression were observed by western blotting in both types of malignant tissues compared to benign samples. Our data are consistent with several other studies and might give evidence that COX-2 is overexpressed in cancer patients due to increased proliferation, reduced apoptosis, or enhanced neoangiogenesis (24, 25). In our present study, COX-2 expression was detected by Western blot. Other studies mainly used immunohistochemistry (11, 26, 27); however, these immunohistochemical methods were not quantitative and would strongly depend on the quality of the antibody and the staining protocol, as well as the selection of the analysed region. Therefore, the variations in findings for COX-2 expression among different studies may partly be attributed to different scoring systems and cut-off values used for COX-2 immunoreactivity (28, 29). In a literature review, we found a detection rate of COX-2 protein expression in malignant breast tissue to be 40% on average by immunohistochemistry and of COX-2 mRNA expression on an average of 90%. Thus it seems likely that COX-2 may undergo complex post-transcriptional and post-translational modifications to yield the active enzyme (30).

A significant difference between tumor and normal breast tissue samples was found in western blot analysis for 15-PGDH levels. A study by Wolf et al. presented low 15-PGDH expression in estrogen receptor (ER)-negative breast cancer samples in contrast to high expression in ER-positive tumours (31). This is contradictory to our results; low levels of 15-PGDH are often associated with ER-negative tumors that exhibit a metastatic potential and correlate with unfavourable prognostic factors (32). An ER-negative status was found in 5/22 of the analysed breast cancer samples and 3/5 of the ER-negative samples were triple negative (i.e. ER-negative, PR-negative and HER2-negative). This might be an explanation for significantly higher 15-PGDH expression in ER-negative samples. Our data are consistent with several other studies observed a more frequent COX-1 expression (45, 46). Some studies detected COX-2 expression (10, 45) other studies observed a more frequent COX-1 expression (45, 46). There are also results that suggest COX-1 overexpression to be a stimulus in ovarian carcinoma tumor growth (47). But these findings seem not to have any prognostic relevance (48). Thus, our analysis of tumor samples from 13 patients is

in MCF-7 and MDA-MB 231 cells; greater invasiveness (observed in MDA-MB 231 cells) resulted in lower receptor levels (35). This might be in line with our results, as we found lower EP2 and EP4 expression in malignant tissue. As a possible consequence, COX-2 is highly expressed in a subset of breast carcinomas and is associated with poor prognosis (36). Recent results indicate that antagonism of EP4 may be as effective as COX inhibition (37). EP2 and EP4 may be critical determinants in cancer cell behaviour in breast cancer.

Experiments with prostate cancer cells have shown that calcitriol acts in multiple pathways to inhibit cell proliferation (38-40). This leads to a possible regulation of PG levels and PG actions by calcitriol and inhibition of the stimulation of prostate cancer cell proliferation by endogenously derived PGs. The three following mechanisms are involved: suppression of COX-2 expression, up-regulation of 15-PGDH expression, and reduction of mRNA expression of the PGE2 receptor subtype EP2 and the PGF2 α (prostaglandin F2α) receptor FP (20).

VDR expression is found in healthy breast tissues and in more than 80% of breast cancer tissues (41). The natural ligand of the VDR, 1,25(OH)2D3, and its analogues inhibit cell proliferation and induce apoptosis in breast cancer cell lines (23, 42). This was also shown for animal models (41). A significantly lower VDR expression (nearly 3-fold) in western blot analysis was observed in our breast cancer tissue samples. This is in line with our own published data for breast cell lines (5). Compared to other groups, an inconsistency in results is reported for studies evaluating cell lines (42) and tissues (43). Townsend et al. detected a 7-fold increase of VDR mRNA level by RT-PCR in breast cancer (p<0.003) (43). This might be due to post-transcriptional modifications of VDR mRNA on its way to functional protein (44). Our results show an inverse correlation of COX-2 and 15-PGDH protein levels, as well as the VDR protein level. This is in line with our findings in MCF-10F and MCF-7 cell lines where we also detected an inverse correlation of COX-2 and VDR protein expressions. These findings suggest a possible link between the VDR, associated target genes and PG metabolism (5). Thus, we suggest that a growing body of evidence exists regarding a possible link between PG and the vitamin D metabolism in cancer.

Several studies suggest overexpression of COX-2 due to increased proliferation, reduced apoptosis and enhanced mediation of neoangiogenesis (7-9) and chemoresistance in ovarian cancer (7). But the data is inconsistent. Although some studies detected COX-2 expression (10, 45) other studies observed a more frequent COX-1 expression (45, 46). There are also results that suggest COX-1 overexpression to be a stimulus in ovarian carcinoma tumor growth (47). But these findings seem not to have any prognostic relevance (48). Thus, our analysis of tumor samples from 13 patients is
in line with the literature, as we detected a significantly higher COX-2 expression in the malignant tissues.

Measurement of circulating 25(OH)2D3 levels in serum is considered to be an excellent measurement of the availability of vitamin D from the diet and supplements, and from synthesis in the skin (49). It is of potential importance in breast carcinogenesis due to the fact that 25(OH)2D3 can be metabolised to 1,25(OH)2D3 by 1-α-hydroxylase in benign and malignant breast tissue (18).

Data in epidemiologic studies are inconsistent regarding a possible association between vitamin D levels and breast cancer risk. Some data suggest an association between plasma levels of vitamin D and breast cancer incidence (50, 51). Our data show significantly lower 25(OH)2D3 plasma levels in breast cancer patients older than 45 years during the wintertime. This difference was not detected in women under 45 years of age nor when serum levels were diagnosed during summer. As COX-2 is responsible for the conversion of arachidonic acid into PGE2, our aim was to compare the serum levels of PGE2 from breast cancer patients with healthy individuals. Interestingly, we found the serum levels of PGE2 increased while 25(OH)2D3 was decreased in breast cancer patients compared to healthy women. Because of this inverse correlation a link between the vitamin D- and the PGE2-metabolism could be proposed.

To date, there have also been several epidemiologic studies about the association between vitamin D and ovarian cancer risk (52). In two recently published studies, Tworoger et al. and Toriola et al. observed no clear associations of serum vitamin D levels and ovarian cancer risk (52, 53). However, Tworoger et al. found an association of serum vitamin D levels and ovarian cancer risk only in the subgroup of serous carcinoma of obese women. Our investigation did not show any association, neither in summer- nor in wintertime, but we did not find significantly serum levels of PGE2 (2-fold) in the tumor patients, but only in the subgroup of patients older than 45 years and only during wintertime; but the mechanism and reason remain unclear.

COX inhibitors suppress cancer cell growth both in vivo and in vitro (50, 51). The possibility of a synergistic effect in combination with calcitriol and NSAIDs for cancer treatment should be considered but needs further investigation. Based on the elevated synthesis of PGs in cells that express COX-2, aromatase expression and activity appears to be increased in breast cells. This leads to an elevation of estrogens which might induce steroid hormone receptor expression. In order to test this theory, several studies investigated the benefit of selective and non-selective COX-2 inhibitors in combination with endocrine therapy (54, 55). Encouraging results for the adoption of calcitriol in combination with docetaxel were reported for prostate cancer in 2008 (56). But recent results from Scher et al. investigating this combination of docetaxel and calcitriol in prostate cancer therapy did not present an expected benefit and led to a discontinuation of the treatment arm due to a shorter patient survival (57). Further investigations are clearly required to establish the use of NSAIDs.

In summary an inverse correlation of VDR and COX-2 and 15-PGDH expression was shown in our results. This suggests a possible link between VDR, associated target genes and PG metabolism in both ovarian and breast cancer. These findings are consistent with previously published data for breast cancer cell lines (58). In months with low UV radiation during wintertime, tumor associated changes of serum levels of 25(OH)2D3 and PGE2 are inversely correlated. Past results of a combination of calcitriol and NSAIDs need further investigation in cancer treatment. The use of calcitriol with or without NSAIDs in combination with cytotoxic therapies is still not evaluated. Further randomized controlled trials are needed in order to elucidate complex actions in cancer treatment.

Conflict of Interest Statement

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

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References


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