Prostaglandin Metabolizing Enzymes in Correlation with Vitamin D Receptor in Benign and Malignant Breast Cell Lines

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Abstract. *Background: The antiproliferative effects of calcitriol (1,25(OH)$_2$D$_3$) mediated via the vitamin D receptor (VDR), render the biologically active form of vitamin D a promising target in breast cancer therapy. Furthermore, breast cancer is associated with inflammatory processes based on an up-regulation of cyclooxygenase-2 (COX-2) expression, the prostaglandin E$_2$ (PGE$_2$) synthesizing enzyme. The PGE$_2$ metabolizing enzyme, 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is described as a tumor suppressor in cancer. First references suggest a correlation between vitamin D and prostaglandin metabolism through the impact of 1,25(OH)$_2$D$_3$ on the expression of COX-2 and 15-PGDH. Materials and Methods: The expression of VDR, COX-2 and 15-PGDH in benign MCF-10F and malignant MCF-7 breast cells was determined by real-time PCR (RT-PCR) and Western blot analysis. Results: Although the RT-PCR data were divergent from those obtained from the Western blot analysis, the COX-2 protein expression was MCF-7 2-fold higher in the MCF-7 compared to the MCF-10F cells. Moreover, a correlation of 15-PGDH to VDR by RT-PCR was found in both cell lines. The VDR protein levels were inversely correlated to the 15-PGDH protein levels and revealed that the MCF-10F cells had the highest VDR expression. Conclusion: A possible link between VDR-associated target genes and prostaglandin metabolism is suggested.*

Besides the clinicopathological parameters, it is possible to determine molecular factors in order to estimate the prognosis of malignancies by the expression of proteins or mRNA in tumor tissue. One of these potential molecular prognosis factors might be cyclooxygenase-2 (COX-2). New therapeutic approaches with drugs that attack definitely defined proteins in tumor tissue need proof of the expression of these targets in the tumor tissue.

Prostaglandins (PGs) are produced in many tissues and are responsible for a wide variety of biological responses. They are potent mediators and affect several signal transduction pathways that modulate cellular adhesion, growth, and differentiation (1, 2). PGs are synthesized from free arachidonic acid by the cyclooxygenases (COXs). COXs catalyse the conversion of arachidonic acid to prostaglandin E$_2$ (PGE$_2$). This is the rate-limiting step in prostaglandin and thromboxane biosynthesis. Two isoforms of prostaglandin G (PGG) and H (PGH) synthases have been identified, COX-1 and COX-2 (3). COX-1 is constitutively expressed in most tissues and is thought to be involved in maintaining cellular homeostasis (4). It has no prognostic relevance in tumorigenesis (5). In contrast, COX-2 is frequently undetectable at baseline in normal tissues but can undergo rapid induction in response to a variety of stimuli, including cytokines, growth factors, mitogens and hormones (6). These effects are mediated *via* tyrosine kinase, protein kinase C and protein kinase A signal transduction pathways (6). It is also involved in inflammatory reactions and pathological processes. The overexpression of COX-2 and elevated concentrations of PGs have been associated with chronic inflammatory disease such as rheumatoid arthritis (7, 8) and several types of human cancer. PGs are implicated in the initiation and progression of many malignancies, including head and neck (9), colon (10, 11), breast (5), ovary (12), prostate (13), lung (14), liver, pancreas (15), stomach (16) and esophagus (17). COX-2 overexpression is known to correlate with the aggressive and invasive potential of tumor cells by several mechanisms (18). Tumor cells with elevated COX-2 levels are highly resistant to apoptosis (19), show increased proliferation (5), invasion and

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migration (5, 20, 21) and even chemoresistance (22). Furthermore, they have an increased angiogenic potential presumably through an increased production of proangiogenic factors such as VEGF (vascular endothelial growth factor) and IL-8 (interleukin-8) (23-25). Moreover COX-2 overexpression leads to increased estrogen (estradiol) production and an up-regulation of aromatase activity: an enzyme complex responsible for the local production of estrogens in breast cancer (26, 27). The substrate for COXs, arachidonic acid, and the product prostaglandin E_2 (PGE_2) also stimulate the proliferation of cancer cells causing increases in the expression of immediate early genes including *c-fos* (a transcription factor) that are involved in growth regulation (28).

COX-1 and COX-2 are the targets for nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs vary in selectivity for the COX-1 or COX-2 isoform. Typical COX-2 inhibitors are celecoxib and rofecoxib, and nonselective COX inhibitors that inhibit both isoforms are indomethacin, acetylsalicylic acid and ibuprofen (20, 29).

15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the key enzyme that regulates PG levels by converting them to the corresponding 15-keto derivatives and has been found to be down-regulated in some carcinomas (21, 30-33). 15-PGDH is responsible for the biological inactivation of PGs (34), is widely expressed in many mammalian tissues and has been shown to be modulated by several hormones and factors (35-37). Elevated 15-PGDH levels are associated with well-differentiated cells, whereas decreased levels or even a loss of 15-PGDH is found in poorly differentiated subtypes (31, 38). Low levels of 15-PGDH are often associated with estrogen receptor (ER)-negative tumors with a highly invasive potential. 15-PGDH is known to have tumor suppressor activity as it antagonizes the effects of COX-2 and also inhibits angiogenesis *in vivo*, in colon (32, 39), lung (30) and bladder cancer (40), and as shown recently in breast cancer as well (31). Furthermore, 15-PGDH expression is associated with the ER pathway in breast cancer. 15-PGDH is down-regulated when estradiol levels are increased, whereas down-regulation of 15-PGDH increases the ERE (estrogen responsive element) activity and aromatase levels. The inhibition of the ER pathway also diminishes the effects of 15-PGDH on the cell cycle (31).

In addition to its role in calcium homeostasis, epidemiological studies have suggested that vitamin D has a protective effect against the formation and progression of several types of carcinoma (41-43). Calcitriol [1,25$(OH)_2D_3$], the hormonally active form of vitamin D, is known to inhibit the growth and development of many carcinomas through multiple mechanisms. Calcitriol interacts with the vitamin D receptor (VDR) to directly inhibit the growth of colon and breast cancer cells (44, 45) and a number of studies have demonstrated the antiproliferative and pro-differentiating effects of calcitriol in prostate cancer cells as well (46-52). Both normal and malignant breast tissue express the VDR (41, 53) and polymorphisms in the *VDR* have been proposed as risk markers for breast cancer (54).

In the current study, the expression of COX-2, 15-PDGH and VDR was investigated in breast cell lines, the benign MCF-10F and the malignant MCF-7 cell line.

Materials and Methods

Cell culture. The human benign breast cell line MCF-10F (ATCC No. CRL-10318) and breast cancer cell line MCF-7 (ATCC No. HTB-22) were purchased from the European Collection of Cell Culture (Wiltshire, UK). The MCF-7 cells were maintained in RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 25 mM HEPES, 1% L-glutamine and 10% fetal bovine serum (FBS) and the MCF-10F cells in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone 0.04 mM final calcium concentration and 5% horse serum (Invitrogen, Karlsruhe, Germany). The cells were grown subconfluently and washed twice with phosphate-buffered saline (pH 7.4). The total RNA and proteins were isolated as stated below.

RNA isolation. The total RNA from the harvested cells was extracted with Qiazol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The integrity of the 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, the RNA was DNase I (Invitrogen) treated. The synthesis of cDNA from 1 μg total cellular RNA was performed using Super Script-II reverse transcriptase and oligo-$d(T)_{15}$ primer (Invitrogen). The reverse transcription reaction mixtures were diluted 1:10 and quantitative real-time PCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), primers for human *COX-2, 15-PGDH* and *VDR* (Qiagen) and 2 μl of RT reaction mixture as a 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 to 95˚C. The fluorescence was measured at 0.2˚C increments during the slow heating process. The obtained threshold cycles (Ct) were normalized to *TBP* (TATA-binding protein) and *PBDG* (porphobilinogen deaminase) as housekeeping genes and MCF-7 cDNA as calibrator. The fold change was determined with the formula: efficiency target gene^ (investigated calibrator gene–sample gene)/efficiency housekeeping gene^(calibrator housekeeping gene–sample housekeeping gene) (55). The experiments were performed in triplicate for each gene and were repeated twice.

Western blotting. Cells were lysed in sample buffer (125 mM Tris, 30% glycerine, 8% SDS, pH 6.8). Then 40 μg of the isolated proteins were subjected to 10% SDS PAGE under reducing conditions and the nitrocellulose membranes (Schleicher Schuell, Dassel, Germany) were blocked after transfer in 5% non-fat dry milk in PBST for 1 h at room temperature. The membranes were incubated with the primary antibodies for human COX-2 and 15-PGDH (IBL International GmbH, Hamburg, Germany) in a dilution

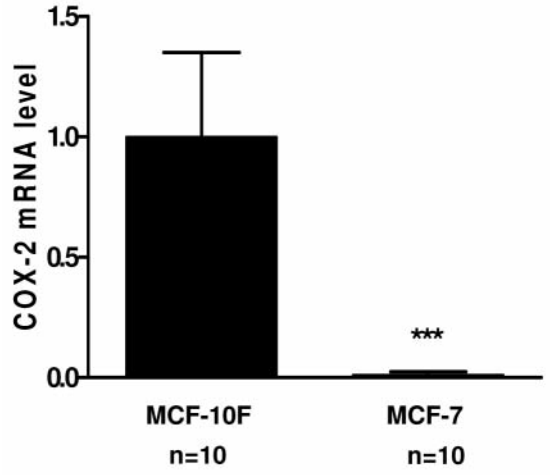

Figure 1. *Real-time PCR analysis of COX-2 mRNA expression in MCF-10F and MCF-7 cells, ***p<0.001.*

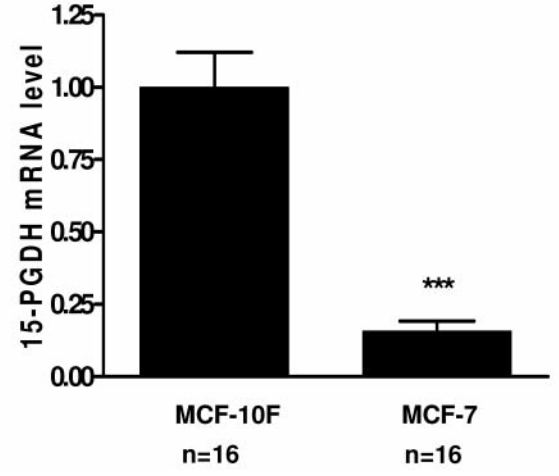

Figure 3. *Real-time PCR analysis of 15-PGDH mRNA expression in MCF-10F and MCF-7 cells, ***p<0.001.*

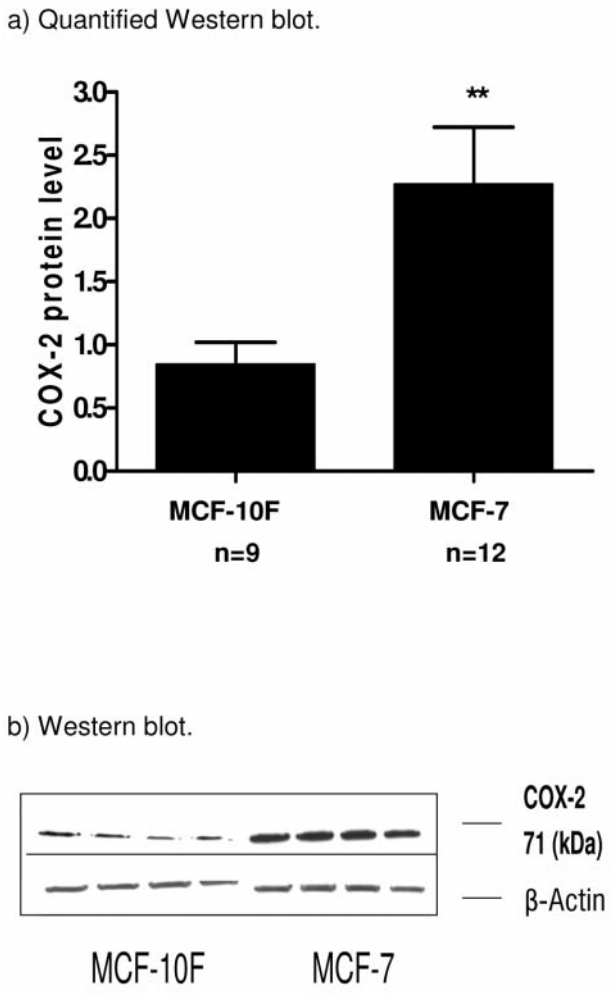

Figure 2. *Western blot analysis of COX-2 protein level in MCF-10F and MCF-7 cells, **p<0.01, ***p<0.001.*

of 1:1,000 in blocking reagent overnight at 4˚C with gentle shaking. The VDR antibody (Dianova, Hamburg, Germany) was used at a dilution of 1:10,000. The secondary antibodies conjugated to horseradish peroxidase (New England Biolabs, Frankfurt Main, Germany) were added at a dilution of 1:2,000 (COX-2 and 15-PGDH) and 1:5,000 (VDR) and the membranes were incubated 1 h at room temperature. The obtained signals were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Freiburg, Germany) and compared to β-actin as loading control.

Statistical significance. The statistical analysis of real-time PCR (RT-PCR) and Western blots results was performed using Student's *t*-test.

Results

COX-2 expression in MCF-10F and MCF-7 cells. *COX-2* mRNA was significantly lower ($p<0.001$) by 98.8% in the MCF-7 cells as compared to the MCF-10F cells (Figure 1). In the Western blot analysis, a significantly higher ($p<0.01$) COX-2 protein level was shown in the MCF-7 cells compared to the MCF-10F cells: COX-2 protein in MCF-10F cells was 41% of that in the MCF-7 cells (Figure 2a, b).

15-PGDH expression in MCF-10F and MCF-7 cells. In the MCF-7 cells, a significantly lower (84.4%) ($p<0.001$) level of *15-PGDH* mRNA was detected by RT-PCR analysis as compared to the MCF-10F cells (Figure 3). Western blot analysis presented a different result. In the MCF-7 cells, the protein level of 15-PGDH was 24% (non-significantly) higher as compared to the MCF-10F cells (Figure 4a, b).

VDR expression in MCF-10F and MCF-7 cells. In the RT-PCR, the MCF-7 cells also showed significantly lower (77.4%) *VDR* gene expression ($p<0.001$) compared to the MCF-10F cells

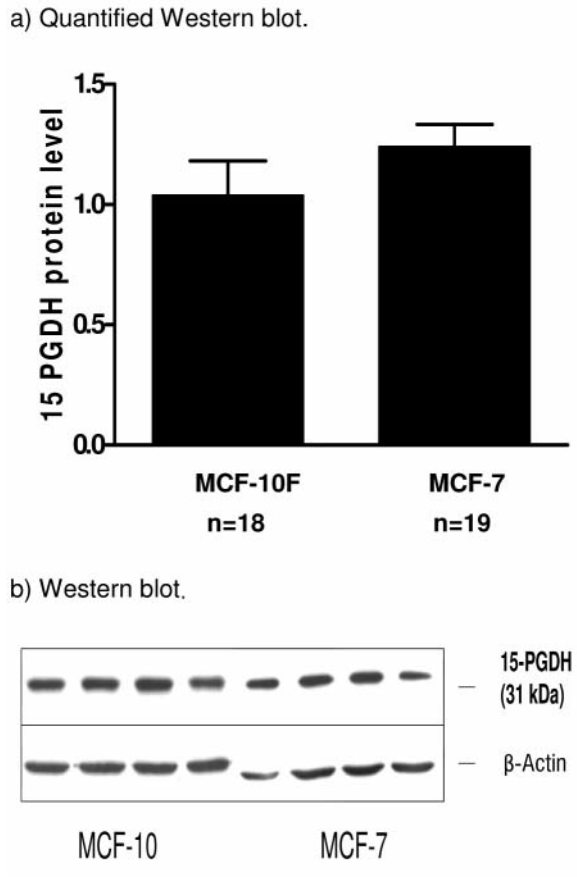

Figure 4. *Western blot analysis of 15-PGDH protein level in MCF-10F and MCF-7 cells.*

(Figure 5). In the Western blot analysis, a highly significantly (95.9%) lower VDR expression ($p<0.001$) was found in the MCF-7 cells as compared to the MCF-10F cells (Figure 6a, b).

Discussion

In the present study the COX-2 results were contradictory, as in the Western blot analysis of the malignant breast cell line MCF-7, a significantly higher COX-2 protein expression was detected, and in the RT-PCR a significantly lower mRNA level in the MCF-7 cells than in the MCF-10F cells. Other authors have also reported inconsistent data. Half *et al.* (56) analyzed two breast cancer cell lines, the estrogen-dependent, poorly invasive and non-metastatic MCF-7, and the estrogen-independent, highly invasive and metastatic MDA-MB-231 cells for COX-2 expression by immunoblotting. The MCF-7 cells were found to lack COX-2 protein and the MDA-MB-231 cells displayed a low level of COX-2 (56). This was in line with the data by Denkert *et al.* (5), who were barely able to identify basal COX-2 expression in MCF-7 cells. Basu *et al.*

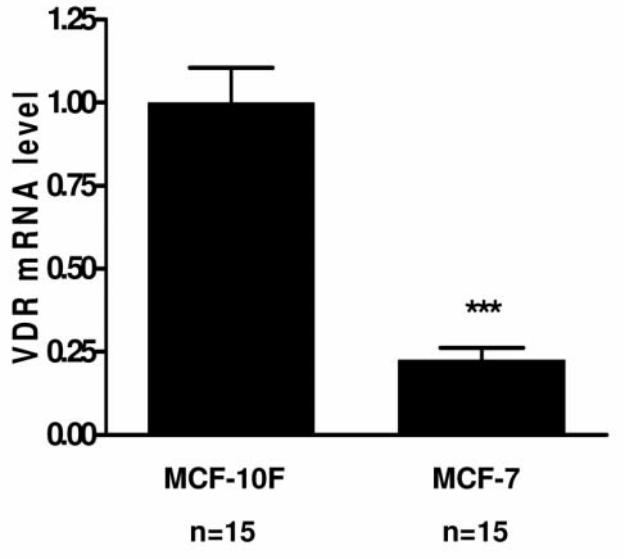

Figure 5. *Real-time PCR analysis of VDR mRNA expression in MCF-10F and MCF-7 cells, ***p<0.001.*

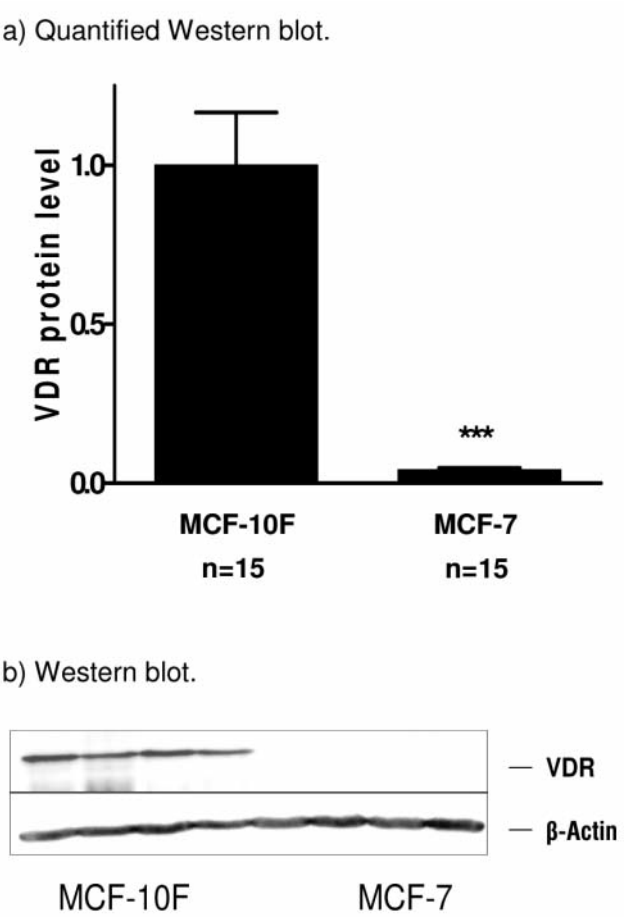

Figure 6. *Western blot analysis of VDR protein level in MCF-10F and MCF-7 cells, ***p<0.001.*

(19) presented an overexpression of COX-2 in the invasive MDA-MB-231 cells, a moderate expression in the moderately invasive MDA-MB-435 cells and a very low expression or lack of expression in the MCF-7 cell line using Western blot analysis. In a review of the literature, a detection rate of COX-2 protein expression in malignant breast tissue on average of 40% by immunohistochemistry (IHC) and *COX-2* mRNA expression on average of 90% was found. Thus it seems likely that COX-2 may undergo complex post-transcriptional and post-translational modification to yield the active enzyme (7). Nevertheless, in comparison to the benign MCF-10F cells, overexpression was found in the malignant cell line.

In the present study, the 15-PGDH expression by RT-PCR and Western blot analysis was inconsistent. In the Western blot analysis, just a small difference between MCF-10F (100%) and MCF-7 (124%) cells was found but a significantly (84.4%) lower 15-PGDH level was found in the MCF-7 cell line by RT-PCR. In the light of the study by Wolf *et al*. (31) who showed low expression in the estrogen independent MDA-MB-231 cells and high expression in the MCF-7 cells, we interpret our results as follows. Low levels of 15-PGDH are often associated with ER-negative tumors that exhibit a metastatic potential and correlate with unfavourable prognostic factors. Elevated 15-PGDH levels are associated with good nuclear grading and well-differentiated cells (31). These results are in line with breast cancer microarray data and suggest that the loss of 15-PGDH expression may play a role in the pathogenesis of less differentiated subtypes of breast cancer (38). The MCF-7 cell is estrogen receptor dependent hence associated with high 15-PGDH levels. This might be the reason for the slightly elevated 15-PGDH level in the Western blot analysis. No previous data compared breast cancer cells to benign breast cells. In our prospective research, we have to compare the MCF-10F with the MCF-7 and a more invasive cell line to prove our results. The significantly lower 15-PGDH level in the MCF-7 cell line in the RT-PCR analysis might correlate with the grade of invasiveness of the cell line and we suggest that the level of 15-PGDH in a more invasive cell line might be even lower.

David Feldman and co-workers have shown that calcitriol acts by multiple pathways to inhibit the proliferation of prostate cancer cells (46, 49, 57-59) and concluded that calcitriol regulates PG levels and actions and inhibits the stimulation of prostate cancer cell proliferation by endogenously derived PGs by the following three mechanisms. First, it suppresses the COX-2 expression, second, it up-regulates the 15-PGDH expression, and third, it reduces the mRNA expression of the PGE receptor subtype EP2 and the $PGF_{2\alpha}$ receptor FP (49).

The VDR expression in the present study showed significant low levels in the MCF-7 cells both in Western blot and in RT-PCR analysis. In the literature, inconsistent data are reported. Kemmis *et al*. (60) examined different cell lines (MCF-7, HKC-8, HMEC) and found VDR expression was highest in the noncarcinogenic HMEC cell line. In contrast to these data, Townsend *et al*. (61) found a 7-fold increased *VDR* mRNA level in breast cancer tissue as compared to normal breast tissue ($p<0.003$). In correlation with the VDR protein level, a basically inverse correlation with the COX-2 protein level was found in the present study. These findings suggested a possible link between the VDR, associated target genes and PG metabolism.

As COX-2 plays an important role in tumorigenesis, COX inhibitors might be indicated to avoid tumor growth. COX-2 inhibitors have been shown to suppress cancer cell growth both *in vivo* and *in vitro* (62, 63). The possibility of a synergistic action with the combination of calcitriol and NSAIDs for treating cancer cells should be considered. Moreno *et al*. (49) proposed that a combination of calcitriol and NSAIDs might be a useful therapeutic strategy in prostate cancer and possibly in other carcinomas as well. Several studies have investigated the benefit of selective and non-selective COX-2 inhibitors in combination with endocrine therapy (64), chemotherapy (65, 66), tyrosine kinase inhibitors (67) and other new approaches (68). Some of them are encouraging, such as the results of the ASCENT trial (65), and some disappointing. Further work is required to establish how NSAIDs can be best applied for therapeutic benefit.

It can be concluded that VDR and COX-2 expression are inversely correlated, which is encouraging and suggests a possible link between the VDR, associated target genes and PG metabolism. Further research is needed to determine if the combination of calcitriol and NSAIDs is a useful therapeutic and/or chemopreventive strategy in breast cancer treatment and if the combination of calcitriol and NSAIDs influence angiogenesis

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Conflict of interest statement

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

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