Histol Histopathol (2012) 27: 1315-1325
DOI: 10.14670/HH-27.1315

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Expression of cyclooxygenase-2 in invasive breast carcinomas and its prognostic impact

Hari Prasad Dhakal[1], Bjørn Naume[2,6], Marit Synnestvedt[2], Elin Borgen[1], Rolf Kaaresen[3,6], Ellen Schlichting[3], Gro Wiedswang[4], Assia Bassarova[1], Ruth Holm[1], Karl-Erik Giercksky[5,6] and Jahn M. Nesland[1,6]

[1]Department of Pathology, Oslo University Hospital, The Norwegian Radium Hospital, Oslo, Norway, [2]Division of Surgery and Cancer Medicine, Department of Oncology, Oslo University Hospital, The Norwegian Radium Hospital, Oslo, Norway, [3]Department of Breast and Endocrine Surgery, Oslo University Hospital, Oslo, Norway, [4]Department of Gastrointestinal Surgery, Oslo University Hospital, Oslo, Norway, [5]Division of Surgery and Cancer Medicine, Department of Gastrointestinal Surgery, Oslo University Hospital, The Norwegian Radium Hospital, Oslo, Norway and [6]Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Norway

Summary. Representative tumour sections from 468 patients with invasive breast cancer were immunostained for cyclooxygenase-2 (COX-2) and evaluated. The relationships between COX-2 expression, clinical outcome and various clinicopathological variables, including tumour vascularity and disseminated tumour cells (DTC) in the bone marrow were examined. COX-2 expression in invasive breast carcinoma cells was positively associated with oestrogen receptor and/or progesterone receptor positivity ($p<0.001$). Triple-negative tumours showed no/low COX-2 expression more frequently than other tumour types ($p<0.001$). Expression of COX-2 was not associated with breast cancer-specific survival ($p=0.49$, log-rank) or distant disease-free survival ($p=0.67$, log-rank) for all patients, including lymph node-negative, untreated patients ($p>0.14$, log-rank). There was also no significant association between COX-2 expression and histological grade, tumour size, nodal status, DTC in bone marrow, p53, HER2, or tumour vascularity. In conclusion, COX-2 expression in this series was associated with the presence of hormone receptors. Low COX-2 expression was observed in triple-negative breast carcinomas.

Key words: COX-2, DTC, Breast cancer, Prognosis, Vascularity

Offprint requests to: Hari Prasad Dhakal, MD, Department of Pathology, Oslo University Hospital, The Norwegian Radium Hospital, Montebello 0310 Oslo, Norway. e-mail: aastha2@yahoo.com

Introduction

The cyclooxygenase (COX) isoenzymes COX-1 and COX-2 are involved in the conversion of free arachidonic acid into prostaglandins (Howe, 2007). Prostaglandin E2 (PGE2) promotes tumour growth through enhanced tumour cell proliferation, invasion and angiogenesis (Mann et al., 2005). Prostaglandin expression is higher in invasive breast cancer cells compared to benign or normal breast epithelial cells (Bennett et al., 1977) and increased PGE2 expression in breast cancer cells has been associated with cellular motility, invasiveness and angiogenesis (Rozic et al., 2001). In breast tumours, constitutively expressed COX-1 is localized mostly in stromal cells, whereas inducible COX-2 is found mainly in carcinoma cells (Hwang et al., 1998). COX-2 expression has also been reported in normal breast epithelial cells, adenosis, ductal carcinoma in situ and infiltrating ductal carcinomas (Half et al., 2002; Boland et al., 2004; Cho et al, 2006; Leo et al, 2006) and its expression has been associated with cell proliferation, migration, invasion and angiogenesis (Rozic et al., 2001; Boland et al., 2004; Chang et al., 2004; Kuwano et al., 2004). However, in human breast cancer, conflicting results have been reported on the relationship between COX-2 expression and angiogenesis (Costa et al., 2002; Davies et al., 2003; Thorat et al., 2009).

Although higher levels of COX-2 expression are reported in breast cancer compared to normal or benign tissue (Hwang et al., 1998; Half et al., 2002; Boland et al., 2004; Cho et al., 2006) and COX-2 expression has

been implicated in breast cancer development and progression (Perrone et al., 2005, 2007), the prognostic significance of COX-2 expression in human breast cancer remains to be definitively established (Costa et al., 2002; Ristimaki et al., 2002; Wulfing et al., 2003; Witton et al., 2004; Nakopoulou et al., 2005; Nassar et al., 2007; Kim et al., 2012). The present study evaluated COX-2 expression in 468 invasive primary human breast cancers and its relationship with clinical outcome, tumour vascularisation, disseminated tumour cells (DTC) in bone marrow, a known prognostic factor in early breast carcinoma (Wiedswang et al., 2003; Braun et al., 2005) and other clinicopathological parameters.

Materials and methods

Patients and tumours

Primary breast cancer vascularisation has been previously reported for tumours from 498 patients with invasive breast cancer out of the 920 total patients enrolled in the Oslo Breast Cancer Micrometastasis Study between 1995 and 1998 (Dhakal et al., 2008). For the present COX-2 immunohistochemical study, 468 of 498 cases were available for possible evaluation. Thirty of these cases could not be included in the present study due to a lack of sufficient tumour tissue. The presence of disseminated tumour cells (DTC) in the bone marrow and primary tumour vascularisation, their relationships with various clinicopathological variables, and their prognostic significance have been reported (Wiedswang et al., 2003; Dhakal et al., 2008).

The protocol for bone marrow aspiration from the iliac crests, sample processing and clinical evaluation has been described previously (Naume et al., 2001). Briefly, a total of 40 mL of bone marrow was aspirated from anterior and posterior iliac crests bilaterally. Bone marrow mononuclear cells (MNCs) were separated ($5x10^5$ MNCs per slide) using density centrifugation, collected, and then cytospin slides were prepared using the isolated cells.

Anti-cytokeratin monoclonal antibodies against AE1 and AE3 (Sanbio) were applied to four slides ($2x10^6$ MNCs) per sample. An equal number of slides was incubated with an irrelevant isotype-specific monoclonal antibody as a negative control. Alkaline phosphatase/anti-alkaline phosphatase labelling was used for visualisation of bound antibodies. Nuclear morphology was evaluated based on haematoxylin counter staining. Immunocytochemically stained cytospin preparations were manually screened for disseminated tumour cells (DTC) using a simple microscope at low power (x10 objective lens). Immunostained cells observed in clusters or that contained enlarged nuclei compared to haematopoietic cells in the surrounding area were scored as DTC. Additionally, cells with strong and/or irregular cytoplasmic staining partially covering the nucleus that did not show haematopoietic cell features were also scored as DTC. Samples were excluded from diagnostic consideration if positive immunostained tumour cells were present on both AE1/AE3 stained slides and the corresponding negative control slides.

Relevant clinicopathological information was collected from the Oslo Breast Cancer Micrometastasis Study database. One hundred and eight out of 461 patients with information available on systemic relapses had a recorded systemic relapse. The median follow up time was 84 months (range 1-125). Eighty-four of 468 patients died of breast cancer during the follow up period. Following national guidelines, patients received postoperative systemic adjuvant therapy (chemotherapy and/or tamoxifen) and/or radiotherapy (Wiedswang et al., 2003). Information on postoperative therapy was available for 449 patients. All patients provided written informed consent and the study was approved by the Regional Ethical Committee.

Histopathological evaluation, immunohistochemistry procedure including CD34 immunohistochemical staining and vascular quantification have been described previously (Dhakal et al, 2008). Briefly, for CD34 immunohistochemical staining, primary monoclonal antibody CD34, QBend-10 (Monoson) and Dako Envision+ System Peroxidase (DAB, K4007, Dako Corporation) were used. The Chalkley method was used to quantify tumour vascularity on CD34 stained slides. Specifically, after identification and selection of three vascular hot spots at low power, a 25-point Chalkley eye piece graticule was applied to each hotspot at x200 magnification, which creates a grid area of 0.1886 mm^2 at this magnification, using a Nikon Eclipse E400 microscope. The grid was oriented to allow the maximum number of black dots in the graticule to fall on or within immunostained microvessels, and then these dots on immunostained microvessels were counted as Chalkley count. The patients were categorised into high and low vascular groups using a Chalkley count of 7 as the cut-off point.

Tumours were histologically classified using WHO recommendations (Ellis et al., 2003) and tumour grading was performed as per Elston and Ellis (Elston and Ellis, 1991). Vascular invasion, inflammatory cell infiltrate and necrosis, including relation of tumour cells/tumour stroma, were evaluated on slides stained with hematoxylin and eosin. Using a simple microscope, subjective categorisation of inflammatory cell infiltrate into the categories of 'minimal/mild' and 'moderate/marked' was performed based on the frequency of mononuclear inflammatory cell infiltration observed in invasive tumour. Tumour necrosis was classified as present or absent based on necrotic tumour cells in the invasive portion of the tumour.

Hormone receptor (HR) status includes both oestrogen receptor (ER) and progesterone receptor (PgR) status, where HR positivity represents positivity for either ER or PgR or both, while HR negativity represents negative expression of both receptors. Based on tumour

expression of HRs and human epidermal growth factor receptor 2 (HER2), tumours were classified as HR+/HER2- (Lunimal A), HR+/HER2+ (Luminal B), HER2+/HR- and HR-/HER2- (triple negative breast carcinoma- TNBC) as described (Gruver et al., 2011).

Immunohistochemistry

Four-micrometer-thick sections of representative formalin-fixed, paraffin-embedded tumour tissue were immunohistochemically stained using the Dako EnVision™ + System (K4011, Dako, USA) and a Dako Autostainer. Briefly, slide-mounted tissue sections were deparaffinized and microwaved in 10mM citrate buffer, pH 6 for antigen retrieval. Slides were incubated in 0.03% hydrogen peroxide (H_2O_2) treatment for 5 minutes to block the endogenous peroxidase. COX-2 expression was detected using a rabbit monoclonal COX-2 antibody (Clone SP-21, cat.#RM-9121, diluted 1:50, Thermo Fisher Scientific, USA) which is reported to detect COX-2 expression in various tumours, including breast cancer (Dias Pereira et al., 2009; Galamb et al., 2010), with incubation for 30 minutes at room temperature. The slides were then incubated with a peroxidase-labelled polymer conjugated to goat anti-rabbit IgG for 30 minutes at room temperature and then slides were stained for 10 minutes with 3,3'-diaminobenzidine tetrahydrochloride (DAB). Finally, the slides were counterstained with haematoxylin and mounted in Diatex. All staining series included positive controls. As a negative control, primary antibody was substituted with diluent. All controls gave satisfactory results.

Immunoscoring of COX-2 slides

COX-2 immunohistochemical assessments were performed by two pathologists (JMN and HPD) using a two-headed microscope and a consensus staining score was recorded. Scoring of COX-2 immunostaining was based on both staining intensity and the fraction of COX-2-positive tumour cells as reported previously (Soslow et al., 2000; Perrone et al., 2005) with some modification, including scoring the percentage of COX-2-positive tumour cells. Semi-quantitative categorisation of cytoplasmic COX-2 immunohistochemical expression was based on the percentage of positive tumour cells and was recorded as 0= no COX-2-positive cells; 1= <10% positive tumour cells; 2= 10% to 50% positive tumour cells; and 3= >50% positive tumour cells. Staining intensity was further graded subjectively and was recorded as 0= no staining; 1= weak staining in tumour cells; 2= moderate staining in tumour cells and 3= strong staining in tumour cells. The product of the score for fraction of positive tumour cells multiplied by the score for staining intensity (range, 0 to 9) was calculated as a composite immunohistochemical score. As a basis for further analyses, patients were categorised into the following three groups: no/low expression (composite score 0-3), moderate expression (composite score 4-6), and high expression (composite score >6).

Statistical analysis

The Pearson's Chi square test or a linear-to-linear test was applied to evaluate possible associations between COX-2 expression and various clinico-pathological variables. Since necrosis/inflammatory cell infiltrates are known to occur more frequently in infiltrating ductal carcinomas, association between COX-2 expression and necrosis/inflammatory cell infiltrate was further tested in multivariate models (linear regression) with COX-2 expression as a dependent variable and tumour histological subtypes and necrosis or inflammatory cell infiltrate as independent variables. Two separate models were developed to determine if association between tumour COX-2 expression and necrosis or inflammatory cell infiltrate were associated with tumour histological subtypes. The Kaplan-Meier method was used for univariate survival analysis and a p value was computed using the log-rank test. The end points for the survival analyses were breast cancer-specific survival (BCSS) and distant disease-free survival (DDFS). These endpoints were measured from the date of surgery to the date of breast cancer-related death, systemic relapse or otherwise censured at the time of the last follow up visit, or non cancer-related death. A p value ≤ 0.05 was accepted as significant. Statistical analyses were performed using the statistical software SPSS, Version 16.

Results

COX-2 expression and its relation with clinicopathological parameters, including tumour vascularity and DTC

Representative images of carcinoma cell COX-2 immunoreactivity are presented in Figure 1. Out of 468 cases, 37.6% (176) had no/low COX-2 expression, 28.8% (135) showed moderate expression and 33.5% (157) had high expression. The relationships between COX-2 expression and different clinicopathological parameters are reported in Table 1. High COX-2 expression was significantly associated with HR-positive tumours ($p<0.001$), an absence of necrosis ($p=0.047$) and minimal/mild inflammatory cell infiltrate ($p=0.049$). Evaluation of histological subtypes showed a higher percentage of infiltrating ductal carcinomas expressing no/low COX-2 than was observed for lobular or other subtypes ($p=0.05$) (Table 1). Although there was a significant negative association of necrosis and inflammatory cell infiltrate with COX-2 expression in a univariate analysis, negative trends for necrosis ($p=0.058$) and inflammatory cell infiltrate ($p=0.086$) were also seen in a multivariate analyses, but results did not reach statistical significance.

A significant negative association was seen between

Table 1. COX-2 expression and various clinicopathological parameters.

Characteristics	COX-2 expression			
	Low	Moderate	High	P*
All patients (n=468)	176 (37.6%)	135 (28.8%)	157 (33.5%)	
Histological grade				
I (n=100)	44 (44%)	25 (25%)	31 (31%)	
II (n=239)	65 (27.2%)	77 (32.2%)	97 (40.6%)	
III (n=129)	67 (51.9%)	33 (25.6%)	29 (22.5%)	0.064
Histological types				
IDC (n=339)	139 (41%)	98 (28.9%)	102 (30.1%)	
ILC (n=83)	21 (25.3%)	26 (31.3%)	36 (43.4%)	
Others (n=68)	16 (34.8%)	11 (23.9%)	19 (41.3%)	0.05‡
pTumour status				
pT1 (n=255)	106 (41.6%)	68 (26.7%)	81 (31.8%)	
pT2 (n=177)	61 (34.5%)	52 (29.4%)	64 (36.2%)	
pT3-4 (n=25)†	5 (20%)	12 (48%)	8 (32%)	
pTx (n=11)	4 (36.4%)	3(27.2%)	4(36.4.%)	0.096
LN status				
N0 (n=284)	118 (41.5%)	73 (25.7%)	93 (32.7%)	
N+ (n=175)§	57 (32.6%)	59 (33.7%)	59 (33.7%)	0.23
HER2				
- (n=435)	160 (36.8%)	128 (29.4%)	147 (33.8%)	
+ (n=30)	15 (50%)	6 (20%)	9 (30%)	0.32
P53				
- (n=362)	131 (36.2%)	106 (29.3%)	125 (34.5%)	
+ (n=105)	45 (42.9%)	28 (26.7%)	32 (30.5%)	0.27
Necrosis				
- (n=429)	155 (36.1%)	126 (29.4%)	148 (34.5%)	
+ (n=39)	21 (53.8%)	9 (23.1%)	9 (23.1%)	0.047
Inflammatory cell infiltrate				
Minimal/mild (n=380)	134 (35.3%)	113 (29.7%)	133 (35%)	
Moderate/marked (n=88)	42 (47.7%)	22 (25%)	24 (27.3%)	0.049
Tumour stroma relation				
Tumour<stroma (n=346)	129 (37.3%)	103 (29.8%)	114 (32.9%)	
Tumour>stroma (n=122)	47 (38.5%)	32 (26.2%)	43 (35.2%)	0.95
DTC in BM				
- (n=384)	143 (37.2%)	115 (29.9%)	126 (32.8%)	
+ (n=58)	22 (37.9%)	12 (20.7%)	24 (41.4%)	0.56
Vascular invasion				
Absence (n=366)	137 (37.4%)	106 (29%)	123 (33.6%)	
Presence (n=102)	39 (38.2%)	29 (28.4%)	34 (33.3%)	0.95
HR status				
- (n=86)	50 (58.1%)	24 (27.9%)	12 (14%)	
+ (n=382)	126 (33%)	111(29%)	145 (38%)	<0.001
Molecular types				
Luminal A- (n=364)	118 (32.4%)	107 (29.4%)	139 (38.2%)	
Luminal B- (n=15)	7 (46.7%)	3 (20%)	5 (33.3%)	
HER2+/HR- (n=15)	8 (53.3%)	3 (20%)	4 (26.7%)	
TNBC(n=71)	42 (59.2%)	21 (29.6%)	8 (11.35)	<0.001‡
CD34 Chalkley count				
Low (n=275)	99 (36%)	82 (29.8%)	94 (34.2%)	
High (n=193)	77 (39.9%)	53 (27.5%)	63 (32.6%)	0.5

BM, bone marow; IDC, infiltrating ductal carcinoma; ILC, invasive lobular carcinoma: HR, hormone receptor (HR+ denotes positivity for oestrogen receptor and/or progesterone receptor and HR– denotes negativity for both oestrogen and progesterone receptors); HER2, human epidermal growth factor receptor-2; LN, lymph node; Low, no/low expression; Moderate, moderate expression; High, high expression; COX-2, cyclooxygenase-2; TNBC, triple negative breast carcinoma; DTC, disseminated tumour cell. *: p value computed by linear by linear test; ‡: p value computed by Pearson's Chi square test; §: Number of pN1=111, pN2=45 and pN3=19; **: Other subtypes include carcinomas of histological subtypes different from IDC and ILC such as mucinous, neuroendocrine, medullary and mixed carcinomas. †: T3=23 and T4=2.

COX-2 expression and triple negative tumours (p<0.001), with a large percentage of triple negative tumours expressing no/low COX-2 (Table 1). Among the 15.2% (n=71) of patients with triple negative tumours in the present study cohort, 72% (n=51) of tumours were histological grade III, 24% (n=17) were grade II and only 4% (n=3) were grade I. In triple negative tumours, low COX-2 expression was significantly associated with histological grade III (p=0.006), with only 5.9% of grade III tumours showing high COX-2 expression.

In this patient series, we did not observe any association between DTC in bone marrow and COX-2 expression in invasive breast carcinoma cells (Table 1).

Analyses of tumour COX-2 expression and patient survival

In univariate survival analyses for the entire patient cohort, COX-2 expression was not significantly associated with either BCSS (p=0.49, log-rank) or DDFS (p=0.67, log-rank) for the whole cohort of patients (Fig. 2). In addition, a separate analysis of patients with negative lymph nodes who did not receive systemic therapy did not show an association with patient outcome (p>0.14, log-rank) (Fig. 2).

The relationships between COX-2 expression, HR status (positive for ER and/or PgR) and survival were also evaluated. In patients with HR-positive tumours, high COX-2 expression group had significantly reduced DDFS (p=0.029, log rank) compared to the no/low expression group, despite the lack of overall significance in this group (Fig. 3D). In HR-negative patients and triple negative breast carcinoma patients, reduced survival was observed in the moderate COX-2 expression group compared to the high expression or no/low expression groups (Fig. 3). The impact of DTC

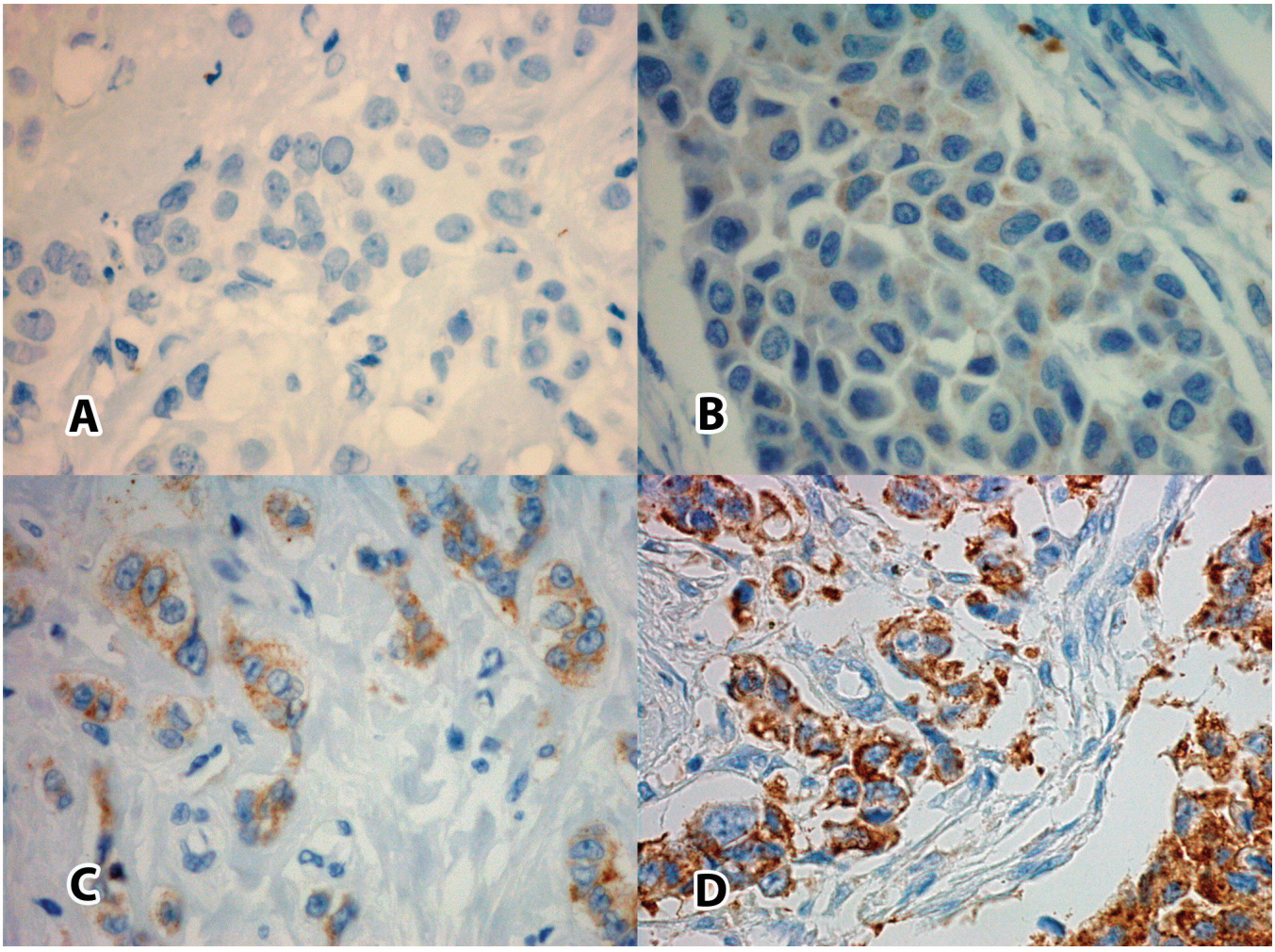

Fig. 1. Representaitive images of COX-2 immunoreactivity in invasive breast carcinoma cells. **(A)** negative staining (no expression), **(B)** weak (low expression), **(C)** moderate (moderate expression) and **(D)** strong staining (high expression). Images were taken by a Leica DFC 320 digital camera with a Plan-neofluar 40x objective lens in Axiophot microscope (Zeiss Germany) at magnification x 400

on survival among patients with different levels of COX-2 expression was also examined (Fig. 4). From these analyses, DTC status was found to be a significant or borderline significant prognostic indicator in all COX-2 expression subgroups.

Discussion

The present analyses did not demonstrate an overall prognostic significance for COX-2 expression in invasive breast carcinomas for the entire patient cohort or for node negative, untreated patients. Although these findings are similar to those from earlier reports (Kelly et al., 2003; Wulfing et al., 2003; Nakopoulou et al., 2005; Nassar et al., 2007; Barisik et al., 2010), several other studies have demonstrated that high COX-2 expression has prognostic significance in breast cancer (Costa et al., 2002; Ristimaki et al., 2002; Denkert et al., 2003; Spizzo et al., 2003; Witton et al., 2004; Cho et al., 2006; Zerkowski et al., 2007; Haffty et al., 2008; Kim et al., 2012).

Witton and coworkers reported a reduced survival in ER-negative patients with high COX-2 (Witton et al., 2004). Furthermore, Kim et al described reduced survival in patients with stage III, ER-negative breast cancer (Kim et al., 2012). In the present study, a separate analysis of HR-negative subgroup revealed a poorer survival for patients with tumours showing moderate COX-2 expression compared to patients with tumours showing high or no/low COX-2 expression. On the other hand, high COX-2 expression in HR-positive tumours was associated with significantly reduced DDFS on pair-wise comparison with tumours showing no/low expression, despite the lack of overall significance in the group. This trend of reduced survival in the high COX-2 expression group among patients with HR-positive tumours is similar to the findings reported in other studies with poor prognosis in high COX-2-expressing breast cancer patients with HR positivity (Ristimaki et al., 2002; Haffty et al., 2008; van Nes et al., 2011). Moreover, there was a significant association between high COX-2 expression and HR positivity (ER and/or PgR) which is similar to earlier reports (Singh-Ranger et al., 2003; Nakopoulou et al., 2005). However, other investigators have shown a significant negative association (Ristimaki et al., 2002; Denkert et al., 2003; Wulfing et al., 2003; Zerkowski et al., 2007) or no relationship (Davies et al., 2003; Spizzo et al., 2003;

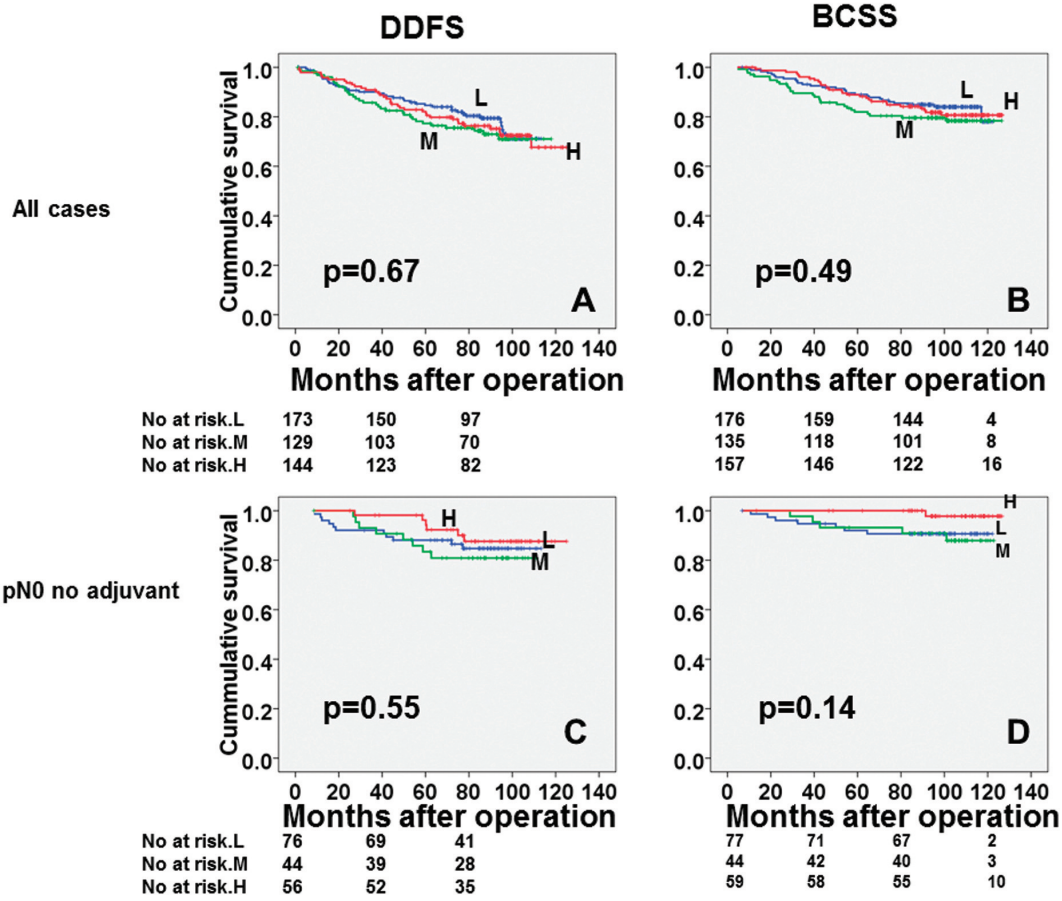

Fig. 2. Kaplan-Meier survival plottings for COX-2 expression in invasive breast carcinomas. **(A)** distant disease free survival (DDFS) and **(B)** breast cancer specific survival (BCSS) for all patients. **(C)** DDFS and **(D)** BCSS in node negative patients not treated with systemic adjuvant therapy. In the survival plotting; L, M and H represent no/low, moderate and high expression of COX-2 respectively.

Ranger et al., 2004; Witton et al., 2004; Leo et al., 2006; Nassar et al., 2007; Lucci et al., 2009) between tumour COX-2 expression and HR status. Nakopoulou and coworkers demonstrated a positive association between COX-2 and PgR expression in breast cancer (Nakopoulou et al., 2005). Furthermore, although statistically insignificant, these investigators also found that ER was expressed in a higher percentage of COX-2-positive tumours.

These conflicting results may stem from the use of different assessment methods and different cut-offs for statistical analyses. There is currently no consistent and uniform system for classifying tumours based on COX-2 expression (Ristimaki et al., 2002; Davies et al., 2003; Singh-Ranger et al., 2003; Spizzo et al., 2003; Witton et al., 2004; Nakopoulou et al., 2005; Leo et al., 2006; Zerkowsky et al., 2007; Kim et al., 2012; van Nes et al., 2011).

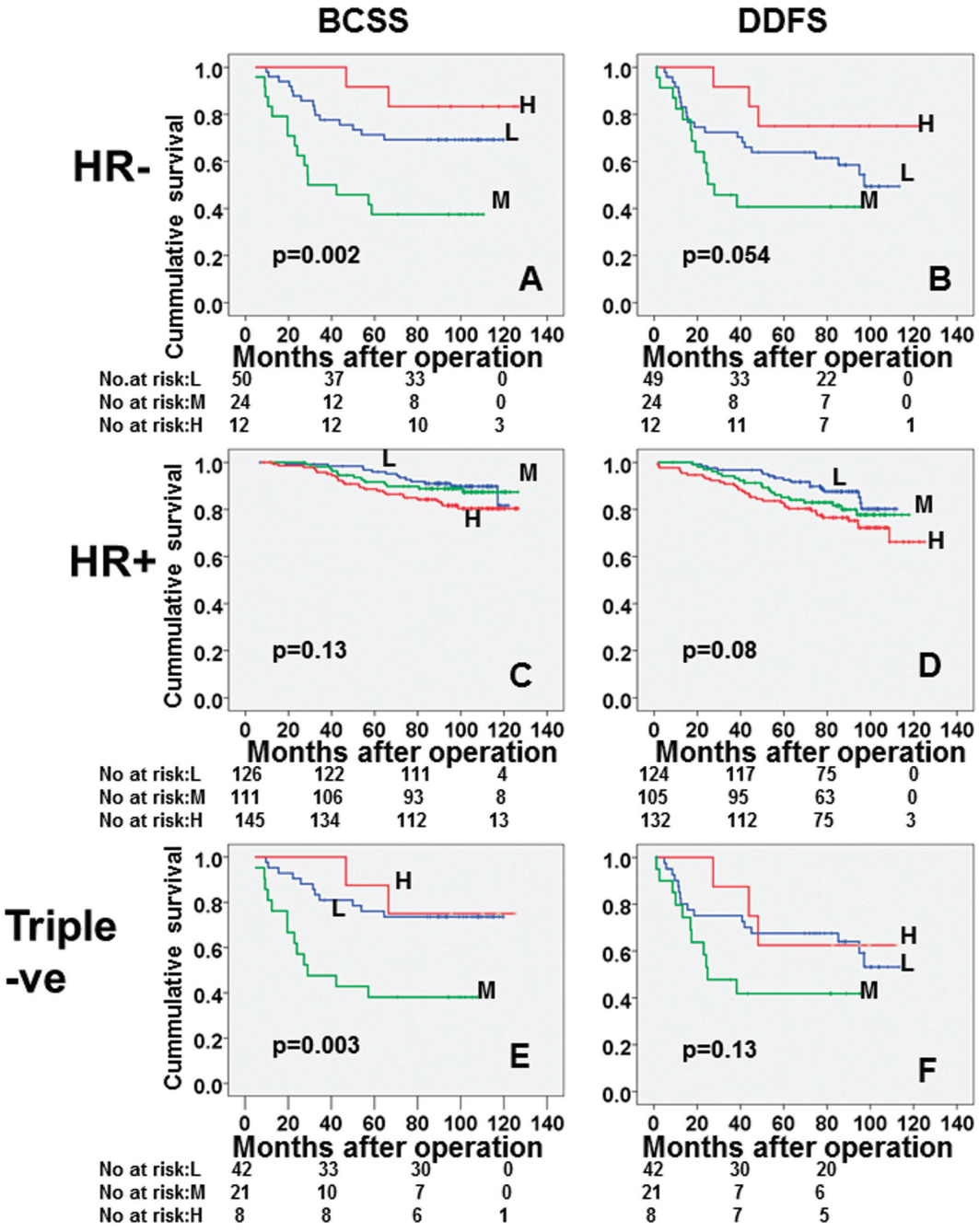

Fig. 3. Kaplan Meier survival plottings for COX-2 expression in hormone receptor (HR) negative and positive as well as in triple negative tumour groups of patients. **(A)** breast cancer specific survival (BCSS) and **(B)** distant disease free survival (DDFS) in HR-negative group. **(C)** BCSS and **(D)** DDFS in HR-positive group. **(E)** BCSS and **(F)** DDFS in triple negative group. In the survival plotting; L, M and H represent no/low, moderate and high expression of COX-2 respectively. HR+ denotes for hormone receptor positive (positive for oestrogen receptor (ER) and/or progesterone receptor (PgR)); HR-, hormone receptor negative (negative for both ER and PgR) and Triple-ve, triple negative (negative for ER, PgR and HER2).

The biological variability of breast tumours cannot be overlooked. The prognostic importance of COX-2 over expression has been reported for patients with ER-negative as well as HER2-positive tumours, but without any significant impact on survival in the ER-positive group (Glynn et al., 2010). In contrast, in the present series, we did not observe any such impact of COX-2 expression on survival of patients with HER2-positive cancer (data not shown). Glynn and colleagues reported a relationship between COX-2 over expression and activation of the Akt cell signalling pathway in ER-negative and HER2-positive breast cancer (Glynn et al., 2010). However, we did not find any association between COX-2 and HER2 in the present series, which is similar to the observations made by Witton et al (2004). In this Oslo I Breast Cancer cohort, the percentage of HER2-positive tumours was low compared to the earlier report (Glynn et al., 2010).

In our present study, triple negative breast carcinomas were found to express significantly less COX-2 compared to other tumours (non-triple negative tumours), and high grade tumours constituted the bulk of the triple negative tumours that expressed no/low COX-2. These findings are in contrast to the observations by Kim and co-workers, who reported higher COX-2 expression in luminal B and triple negative tumour types (Kim et al., 2012). However, another report did not show any association (Darb-Esfahani et al., 2009).

Expression of aromatase, a critical enzyme in the conversion of androgens into oestrogens, is positively correlated with COX-2 expression in breast carcinomas (Oliveira et al., 2006). Aromatase expression has also been positively associated with oestrogen receptor expression in breast cancer (Lykkesfeldt et al., 2009). Moreover, high COX-2 expression in breast cancer is associated with tumour PGE2 expression (Badawi et al., 2003; Timoshenko et al, 2003), which may impact breast cancer risk (Badawi et al., 2003). COX-2 induced synthesis of PGE2 leading to aromatase induction, and subsequent increase in the conversion of androgen into oestradiol may explain the relationship between COX-2 expression and HR-positive breast tumours. This

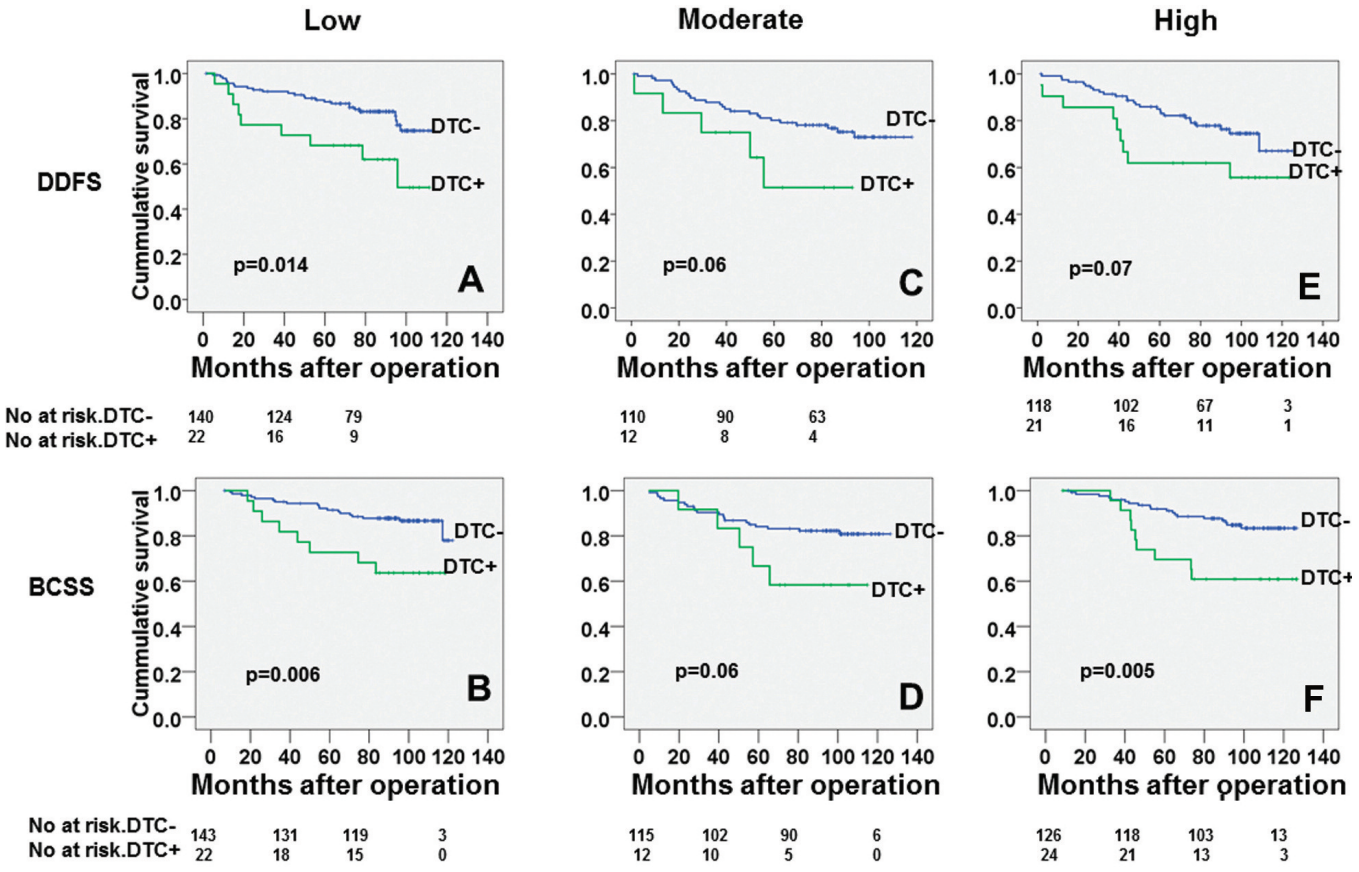

Fig. 4. Kaplan Meier survival plottings for COX-2 expression with disseminated tumour cells (DTC) status. **(A)** distant disease free survival (DDFS) and **(B)** breast cancer specific survival (BCSS) in no/low COX-2 expression group of patients. **(C)** DDFS and **(D)** BCSS in moderate COX-2 expression group. **(E)** DDFS and **(F)** BCSS in high COX-2 expression group. In the survival plotting; DTC represents disseminated tumour cells in bone marrow, Low is for no/low expression and Moderate for moderate expression and High for high expression of COX-2 respectively.

hypothesis is supported by reports of poor survival in patients with HR-positive breast cancer expressing high COX-2 (Ristimaki et al., 2002; Haffty et al., 2008; van Nes et al., 2011).

Other traditional prognostic parameters such as nodal status, histological grade, and tumour size did not show any association with COX-2 expression in the present series, which is similar to some earlier reports (Davies et al., 2003; Kelly et al., 2003; Witton et al., 2004; Leo et al., 2006; Barisik et al., 2010) but is in contrast with other reports (Ristimaki et al., 2002; Denkert et al., 2003; Wulfing et al., 2003; Cho et al., 2006; Kim et al., 2012).

The present study found that a higher percentage of tumours with high COX-2 expression did not show evidence of necrosis and had only minimal/mild inflammatory cell infiltrate. Mononuclear inflammatory cell infiltrate in gastric adenoma was observed in mice following treatment with the COX-2 inhibitor celecoxib, suggesting an intense inflammatory reaction following inhibition of COX-2 activity (Saukkonen et al., 2003). Similarly, low mononuclear inflammatory cell infiltration was observed in cervical carcinomas with high COX-2 expression (Ferrandina et al., 2002). A similar phenomenon may explain a low inflammatory cellular infiltrate in tumours expressing high COX-2 in the present breast cancer series.

Upregulation of COX-2 expression is linked to induce angiogenesis (Kuwano et al., 2004). Chang and colleagues (Chang et al., 2004) reported tumour-associated angiogenesis with increased microvessel density in invasive tumours from COX-2 transgenic mice that correlated with tumour development and progression. Moreover, a positive association of COX-2 expression with tumour vascularity in human breast cancer has been reported (Costa et al., 2002; Davies et al., 2003). However, the present analyses did not find a statistically significant association of COX-2 with tumour vascularity, which is similar to a previous report (Thorat et al., 2009). COX-2 expression has been linked with inflammatory cytokine-induced angiogenesis (Kuwano et al., 2004). Through PGE2, COX-2 has been linked to induce angiogenesis through the release of angiogenesis regulatory factors such as VEGF, VEGFR, Ang 1 and Ang2 (Chang et al., 2004; Kuwano et al., 2004). The contradictory findings on the relationship between angiogenesis and COX-2 expression in the present series may be explained by the induction of various molecules that promote angiogenesis independent of COX-2 expression (Kuwano et al., 2004).

DTC in bone marrow is an established prognostic factor in invasive breast cancer that has been reported to be associated with different characteristics of primary tumours and to have prognostic significance (Wiedswang et al., 2003; Braun et al., 2005). In the present study, we did not find a significant association between COX-2 expression and DTC in bone marrow in contrary to a report by Lucci et al (2009). Different findings on the association between DTC and COX-2 may be due to the evaluation of different study populations with different primary tumour characteristics, in addition to methodological differences (Lucci et al., 2009). The present study identified a higher percentage of node-negative tumours, smaller tumours, a lower frequency of HER2 positivity and vascular invasion, and higher HR positivity than was reported by Lucci and colleagues (Lucci et al., 2009). Moreover, the DTC detection rate was lower in our series compared to the earlier study (Lucci et al., 2009).

In conclusion, the present analyses demonstrated that COX-2 expression in invasive breast carcinomas is positively associated with hormone receptor expression, while triple negative breast carcinomas showed low COX-2 expression. Further study is necessary to better understand the role of COX-2 in hormone-dependent breast tumours.

Acknowledgements. We are grateful to Ellen Hellesylt, Mette Førsund and Leni Tøndevold Moripen for the high quality immunohistochemistry, Berit Sandstad and Junbai Wang for statistical support and Oslo Breast Cancer Micrometastasis Project for permitting the use of data and The Norwegian Radium Hospital Foundation and The Norwegian Cancer Society for financial support.

References

Badawi A.F. and Badr M.Z. (2003). Expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor-gamma and levels of prostaglandin E2 and 15-deoxy-delta12,14-prostaglandin J2 in human breast cancer and metastasis. Int. J. Cancer 103, 84-90.

Barisik N.O., Keser S.H., Gul A.E., Sensu S., Kandemir N.O., Kucuk H.F., Gumus M. and Karadayi N. (2010). The value of COX-2 expression in the prognostic parameters of invasive ductal carcinoma of the breast. Med. Oncol. 28, 703-708.

Bennett A., Charlier E.M., McDonald A.M., Simpson J.S., Stamford I.F., and Zebro T. (1977). Prostaglandins and breast cancer. Lancet 2, 624-626.

Boland G.P., Butt I.S., Prasad R., Knox W.F. and Bundred N.J. (2004). COX-2 expression is associated with an aggressive phenotype in ductal carcinoma in situ. Br. J. Cancer 90, 423-429.

Braun S., Vogl F.D., Naume B., Janni W., Osborne M.P., Coombes R.C., Schlimok G., Diel I.J., Gerber B., Gebauer G., Pierga J.Y., Marth C., Oruzio D., Wiedswang G., Solomayer E.F., Kundt G., Strobl B., Fehm T., Wong G.Y., Bliss J., Vincent-Salomon A. and Pantel K. (2005). A pooled analysis of bone marrow micrometastasis in breast cancer. N. Engl. J. Med. 353, 793-802.

Chang S.H., Liu C.H., Conway R., Han D.K., Nithipatikom K., Trifan O.C., Lane T.F. and Hla T. (2004). Role of prostaglandin E2-dependent angiogenic switch in cyclooxygenase 2-induced breast cancer progression. Proc. Natl. Acad. Sci. USA 101, 591-596.

Cho M.H., Yoon J.H., Jaegal Y.J., Choi Y.D., Lee J.S., Lee J.H., Nam J.H., Choi C., Lee M.C., Park C.S., Woo J.S. and Min K.W. (2006). Expression of cyclooxygenase-2 in breast carcinogenesis and its relation to HER-2/neu and p53 protein expression in invasive ductal carcinoma. Breast 15, 390-398.

Costa C., Soares R., Reis-Filho J.S., Leitao D., Amendoeira I. and

Schmitt F.C. (2002). Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. J. Clin. Pathol. 55, 429-434.

Darb-Esfahani S., Loibl S., Muller B.M., Roller M., Denkert C., Komor M., Schluns K., Blohmer J.U., Budczies J., Gerber B., Noske A., du B.A., Weichert W., Jackisch C., Dietel M., Richter K., Kaufmann M. and von M.G. (2009). Identification of biology-based breast cancer types with distinct predictive and prognostic features: role of steroid hormone and HER2 receptor expression in patients treated with neoadjuvant anthracycline/taxane-based chemotherapy. Breast Cancer Res. 11, R69-

Davies G., Salter J., Hills M., Martin L.A., Sacks N. and Dowsett M. (2003). Correlation between cyclooxygenase-2 expression and angiogenesis in human breast cancer. Clin. Cancer Res. 9, 2651-2656.

Denkert C., Winzer K.J., Muller B.M., Weichert W., Pest S., Kobel M., Kristiansen G., Reles A., Siegert A., Guski H. and Hauptmann S. (2003). Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma. Cancer 97, 2978-2987.

Dhakal H.P., Naume B., Synnestvedt M., Borgen E., Kaaresen R., Schlichting E., Wiedswang G., Bassarova A., Giercksky K.E. and Nesland J.M. (2008). Vascularization in primary breast carcinomas: its prognostic significance and relationship with tumor cell dissemination. Clin. Cancer Res. 14, 2341-2350.

Dias Pereira P., Lopes C.C., Matos A.J., Santos M., Gärtner F., Medeiros R. and Lopes C. (2009). COX-2 expression in canine normal and neoplastic mammary gland. J. Comp. Pathol. 140, 247-253.

Ellis I.O., Schnitt S.J., Sastre-Garau X., Bussolati G., Tavassoli F.A., Eusebi V., Peterse J.L., Mukai K., Tabar L., Jacquemier J., Cornelisse C.J., Sasco A.J., Kaaks R., Pisani P., Goldgar D.E., Devilee P., Cleton-Jansen M.J., Børresen-Dale A.L., van't Veer L. and Sapino A. (2003). Invasive Breast Carcinoma. In: World Health Organization Classification of Tumours Pathology and Genetics. Tumours of the Breast and Female Genital Organs. Tavassoli F.A. and Devilee P. (eds). IARC Press. Lyon, France. pp 13-59.

Elston C.W. and Ellis I.O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19, 403-410.

Ferrandina G., Lauriola L., Zannoni G.F., Distefano M.G., Legge F., Salutari V., Gessi M., Maggiano N., Scambia G. and Ranelletti F.O. (2002). Expression of cyclooxygenase-2 (COX-2) in tumour and stroma compartments in cervical cancer: clinical implications. Br. J. Cancer 87, 1145-52.

Galamb O., Spisák S., Sipos F., Tóth K., Solymosi N., Wichmann B., Krenács T., Valcz G., Tulassay Z and Molnár B. (2010). Reversal of gene expression changes in the colorectal normal-adenoma pathway by NS398 selective COX2 inhibitor. Br. J. Cancer 16, 765-773.

Glynn S.A., Prueitt R.L., Ridnour L.A., Boersma B.J., Dorsey T.M., Wink D.A., Goodman J.E., Yfantis H.G., Lee D.H. and Ambs S. (2010). COX-2 activation is associated with Akt phosphorylation and poor survival in ER-negative, HER2-positive breast cancer. BMC Cancer 10, 626-

Gruver A.M., Portier B.P. and Tubbs R.R. (2011). Molecular pathology of breast cancer: the journey from traditional practice toward embracing the complexity of a molecular classification. Arch. Pathol. Lab. Med. 135, 544-557.

Haffty B.G., Yang Q., Moran M.S., Tan A.R. and Reiss M. (2008). Estrogen-dependent prognostic significance of cyclooxygenase-2 expression in early-stage invasive breast cancers treated with breast-conserving surgery and radiation. Int. J. Radiat. Oncol. Biol. Phys. 71, 1006-1013.

Half E., Tang X.M., Gwyn K., Sahin A., Wathen K. and Sinicrope F.A. (2002). Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma in situ. Cancer Res. 62, 1676-1681.

Howe L.R. (2007). Inflammation and breast cancer. Cyclooxygenase/prostaglandin signaling and breast cancer. Breast Cancer Res. 9, 210.

Hwang D., Scollard D., Byrne J. and Levine E. (1998). Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J. Natl. Cancer Inst. 90, 455-460.

Kelly L.M., Hill A.D., Kennedy S., Connolly E.M., Ramanath R., Teh S., Dijkstra B., Purcell R., McDermott E.W. and O'Higgins N. (2003). Lack of prognostic effect of Cox-2 expression in primary breast cancer on short-term follow-up. Eur. J. Surg. Oncol. 29, 707-710.

Kim H.S., Moon H.G., Han W., Yom C.K., Kim W.H., Kim J.H. and Noh D.Y. (2012). COX2 overexpression is a prognostic marker for Stage III breast cancer. Breast Cancer Res. Treat. (in press).

Kuwano T., Nakao S., Yamamoto H., Tsuneyoshi M., Yamamoto T., Kuwano M. and Ono M. (2004). Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. FASEB J. 18, 300-310.

Leo C., Faber S., Hentschel B., Hockel M. and Horn L.C. (2006). The status of cyclooxygenase-2 expression in ductal carcinoma in situ lesions and invasive breast cancer correlates to cyclooxygenase-2 expression in normal breast tissue. Ann. Diagn. Pathol. 10, 327-332.

Lucci A., Krishnamurthy S., Singh B., Bedrosian I., Meric-Bernstam F., Reuben J., Broglio K., Mosalpuria K., Lodhi A., Vincent L. and Cristofanilli M. (2009). Cyclooxygenase-2 expression in primary breast cancers predicts dissemination of cancer cells to the bone marrow. Breast Cancer Res.Treat. 117, 61-68.

Lykkesfeldt A.E., Henriksen K.L., Rasmussen B.B., Sasano H., Evans D.B., Moller S., Ejlertsen B. and Mouridsen H.T. (2009). In situ aromatase expression in primary tumor is associated with estrogen receptor expression but is not predictive of response to endocrine therapy in advanced breast cancer. BMC Cancer 9, 185.

Mann J.R., Backlund M.G. and DuBois R.N. (2005). Mechanisms of disease: Inflammatory mediators and cancer prevention. Nat. Clin. Pract. Oncol. 2, 202-210.

Nakopoulou L., Mylona E., Papadaki I., Kapranou A., Giannopoulou I., Markaki S. and Keramopoulos A. (2005). Overexpression of cyclooxygenase-2 is associated with a favorable prognostic phenotype in breast carcinoma. Pathobiology 72, 241-249.

Nassar A., Radhakrishnan A., Cabrero I.A., Cotsonis G. and Cohen C. (2007). COX-2 expression in invasive breast cancer: correlation with prognostic parameters and outcome. Appl. Immunohistochem. Mol. Morphol. 15, 255-259.

Naume B., Borgen E., Kvalheim G., Karesen R., Qvist H., Sauer T., Kumar T. and Nesland J.M. (2001). Detection of isolated tumor cells in bone marrow in early-stage breast carcinoma patients: comparison with preoperative clinical parameters and primary tumor characteristics. Clin. Cancer Res. 7, 4122-4129.

Oliveira V.M., Piato S. and Silva M.A. (2006). Correlation of cyclooxygenase-2 and aromatase immunohistochemical expression in invasive ductal carcinoma, ductal carcinoma in situ, and adjacent

normal epithelium. Breast Cancer Res.Treat. 95, 235-241.

Perrone G., Santini D., Vincenzi B., Zagami M., La Cesa A., Bianchi A., Altomare V., Primavera A., Battista C., Vetrani A., Tonini G. and Rabitti C. (2005). COX-2 expression in DCIS: correlation with VEGF, HER-2/neu, prognostic molecular markers and clinicopathological features. Histopathology 46, 561-568.

Perrone G., Zagami M., Santini D., Vincenzi B., Gullotta G., Morini S., Battista C., Guerriero G., Altomare V., Tonini G. and Rabitti C. (2007). COX-2 expression in lobular in situ neoplasia of the breast: correlation with histopathological grading system according to the Tavassoli classification. Histopathology 51, 33-39.

Ranger G.S., Jewell A., Thomas V. and Mokbel K. (2004). Elevated expression of cyclooxygenase-2 in breast cancer and ductal carcinoma in situ has no correlation with established prognostic markers. J. Surg. Oncol. 88, 100-103.

Ristimaki A., Sivula A., Lundin J., Lundin M., Salminen T., Haglund C., Joensuu H. and Isola J. (2002). Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res. 62, 632-635.

Rozic J.G., Chakraborty C. and Lala P.K. (2001). Cyclooxygenase inhibitors retard murine mammary tumor progression by reducing tumor cell migration, invasiveness and angiogenesis. Int. J. Cancer 93, 497-506.

Saukkonen K., Tomasetto C., Narko K., Rio M.C. and Ristimaki A. (2003). Cyclooxygenase-2 expression and effect of celecoxib in gastric adenomas of trefoil factor 1-deficient mice. Cancer Res. 63, 3032-3036.

Singh-Ranger G., Kirkpatrick K.L., Clark G.M. and Mokbel K. (2003). Cyclo-oxygenase-2 (COX-2) mRNA expression correlates with progesterone receptor positivity in human breast cancer. Curr. Med. Res. Opin. 19, 131-134.

Soslow R.A., Dannenberg A.J., Rush D., Woerner B.M., Khan K.N., Masferrer J. and Koki A.T. (2000).COX-2 is expressed in human pulmonary, colonic, and mammary tumors. Cancer 89, 2637-2645.

Spizzo G., Gastl G., Wolf D., Gunsilius E., Steurer M., Fong D., Amberger A., Margreiter R. and Obrist P. (2003). Correlation of COX-2 and Ep-CAM overexpression in human invasive breast cancer and its impact on survival. Br. J. Cancer 88, 574-578.

Thorat M.A., Mehrotra S., Morimiya A. and Badve S. (2009). COX-2 expression does not correlate with microvessel density in breast cancer. Pathobiology 76, 39-44.

Timoshenko A.V., Xu G., Chakrabarti S., Lala P.K. and Chakraborty C. (2003). Role of prostaglandin E2 receptors in migration of murine and human breast cancer cells. Exp. Cell Res. 289, 265-274.

van Nes J.G., de Kruijf E.M., Faratian D., van d., V, Putter H., Falconer C., Smit V.T., Kay C., van d., V, Kuppen P.J. and Bartlett J.M. (2011). COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. Breast Cancer Res. Treat. 125, 671-685.

Wiedswang G., Borgen E., Karesen R., Kvalheim G., Nesland J.M., Qvist H., Schlichting E., Sauer T., Janbu J., Harbitz T. and Naume B. (2003). Detection of isolated tumor cells in bone marrow is an independent prognostic factor in breast cancer. J. Clin. Oncol. 21, 3469-3478.

Witton C.J., Hawe S.J., Cooke T.G. and Bartlett J.M. (2004). Cyclooxygenase 2 (COX2) expression is associated with poor outcome in ER-negative, but not ER-positive, breast cancer. Histopathology 45, 47-54.

Wulfing P., Diallo R., Muller C., Wulfing C., Poremba C., Heinecke A., Rody A., Greb R.R., Bocker W. and Kiesel L. (2003). Analysis of cyclooxygenase-2 expression in human breast cancer: high throughput tissue microarray analysis. J. Cancer Res. Clin. Oncol. 129, 375-382.

Zerkowski M.P., Camp R.L., Burtness B.A., Rimm D.L. and Chung G.G. (2007). Quantitative analysis of breast cancer tissue microarrays shows high cox-2 expression is associated with poor outcome. Cancer Invest. 25, 19-26.

Accepted May 4, 2012