LUND UNIVERSITY

The prognostic impact of COX-2 expression in breast cancer depends on oral contraceptive history, preoperative NSAID use, and tumor size

Simonsson, Maria; Björner, Sofie; Markkula, Andrea; Nodin, Björn; Jirström, Karin; Rose, Carsten; Borgquist, Signe; Ingvar, Christian; Jernström, Helena

The prognostic impact of COX-2 expression in breast cancer depends on oral contraceptive history, preoperative NSAID use, and tumor size

Maria Simonsson[1], Sofie Björner[1], Andrea Markkula[1], Björn Nodin[1], Karin Jirström[1], Carsten Rose[2], Signe Borgquist[1,3], Christian Ingvar[4], Helena Jernström[1]

[1]Lund University, Faculty of Medicine, Department of Clinical Sciences Lund, Oncology and Pathology, Lund, Sweden. [2]CREATE Health and Department of Immunotechnology, Lund University, Medicon Village, Lund, Sweden. [3]Skåne University Hospital, Oncology and Haematology, Sweden. [4]Lund University and Skåne University Hospital, Department of Clinical Sciences, Lund, Surgery, Lund, Sweden.

Corresponding Author:

Associate professor Helena Jernström, Lund University and Skåne University Hospital, Department of Clinical Sciences, Lund, Division of Oncology and Pathology, Lund University Cancer Center/Kamprad, Barngatan 2B, SE-221 85 Lund, Sweden. Phone: +46 46 17 76 19; Fax: +46 46 14 73 27; E-mail: helena.jernstrom@med.lu.se

Short title: COX-2 expression in breast cancer

Conflict of interest: The authors declare that they have no conflict of interest.

Word count: 4,116

Total number of figures and tables: 6

Key words: Breast cancer, cyclooxygenase-2, prognosis, oral contraceptives, non-steroidal anti-inflammatory drugs

Novelty and impact: The association between tumor-specific COX-2 expression and breast cancer prognosis remains unclear and clinical trials with adjuvant NSAIDs are ongoing. This team found COX-2 expression to be associated with less aggressive tumor characteristics and better short-term prognosis. The prognostic impact of COX-2 differed significantly according to tumor size, history of OC use, and preoperative NSAID use. These factors may need to be considered when evaluating outcome in clinical trials with NSAIDs for breast cancer patients.

Abstract

The association between tumor cyclooxygenase 2 (COX-2) expression and breast cancer prognosis has been inconsistent. The purpose of this study was to evaluate the prognostic significance of COX-2 tumor expression according to adjuvant treatment, and potential effect modifications of non-steroid anti-inflammatory drug (NSAID) use, and other tumor and lifestyle factors. A prospective cohort of 1,116 patients with primary breast cancer in Lund, Sweden, included 2002-2012 was followed until June 2014 (median 5 years). Tumor-specific COX-2 expression was evaluated on tissue microarrays using immunohistochemistry. Associations between COX-2 intensity (negative, weak-moderate, high) and patient and tumor characteristics as well as prognosis were analyzed. Tumor-specific COX-2 expression was available for 911 patients and was significantly associated with higher age at diagnosis and less aggressive tumor characteristics. Higher COX-2 expression was associated with lower risk for breast cancer events during the first five years of follow-up, $_{adj}$HR 0.60 (95%CI: 0.37-0.97), per category. The association between COX-2 expression and prognosis was significantly modified by oral contraceptive (OC) use ($P_{interaction}$=0.048), preoperative NSAID use ($P_{interaction}$=0.009), and tumor size ($P_{interaction}$=0.039). COX-2 negativity was associated with increased risk for events during the first five years in ever OC users, $_{adj}$HR 1.94 (1.01-3.72) and during the 11-year follow-up in preoperative NSAID users, $_{adj}$HR 4.51 (1.18-11.44) as well as in patients with large tumors, $_{adj}$HR 2.57 (1.28-5.15). In conclusion, this study, one of the largest evaluating COX-2 expression in breast cancer, indicates that the prognostic impact of COX-2 expression depends on host factors and tumor characteristics.

Word count: 245

Introduction

Breast cancer is a heterogeneous disease where most patients have a good prognosis. Improved tumor classification could identify subgroups of patients with a poorer prognosis who are in need of more personalized treatment [1]. In breast cancer, cyclooxygenase 2 (COX-2)-mediated prostaglandin aromatase gene activation increases both aromatase and estrogen levels that may lead to increased proliferation, primarily in patients with estrogen receptor positive (ER-positive) tumors and in postmenopausal patients [2]. COX-2 catalyzes the conversion of arachidonic acid to prostaglandins and have pro-inflammatory effects [2]. COX-2 expression in predominantly ER-negative tumors was shown to lead to Akt-pathway activation [2, 3]. Regarding prognosis, most [1, 3-19], but not all [20-27], studies have found an association between high COX-2 expression and worse prognosis. Whether COX-2 expression was associated with prognosis in the different studies also depended on tumor characteristics, type of breast cancer treatment, body constitution, and concomitant medications [1, 6]. Non-steroidal anti-inflammatory drugs (NSAIDs) including both selective COX-2 inhibitors and non-selective COX-1/2 inhibitors as well as acetylsalicylic acid (ASA) are widely available both on prescription and over-the-counter and may decrease COX-2 expression in breast cancer [28, 29].

Results from randomized trials of NSAID use in relation to prognosis in the preoperative setting have been inconsistent; some showed a decrease in tumor volume or anti-tumor transcriptional response [28, 30] others did not find any effect [31-33]. However, perioperative NSAID administration was shown to significantly improve short-term prognosis [34, 35]. There are several ongoing randomized clinical trials with perioperative or adjuvant NSAID in breast cancer (ClinicalTrials.gov identifiers; NCT00502684, NCT02429427, NCT01431053, NCT01806259, and NCT02141139), but to date no results have been published. It is currently unknown whether preoperative NSAID use impacts prognosis differently depending on the tumor COX-2 expression. The association between COX-2 and breast cancer prognosis may depend on several factors. A previous report from our group based on a subset of this cohort showed that the *COX2* rs5277 polymorphism, ER-status, and breast volume had a combined effect on the risk of early events and prognosis in different treatment groups [36].

Based on the above, we hypothesized that higher tumor COX-2 expression would be associated with worse prognosis in patients with ER-negative tumors and that the association might be modified by body constitution, preoperative NSAID use, or other tumor or patient characteristics. In contrast, increased COX-2 expression in patients with ER-positive tumor may lead to increased aromatase activation and estrogen levels. These effects could be counteracted by endocrine treatment, resulting in no impact by COX-2 on prognosis [1, 36]. The aim of this study was to evaluate the

prognostic and/or predictive significance of COX-2 tumor expression in relation to ER-status and treatment. A secondary aim was to analyze potential effect modifications of body constitution, NSAID or ASA use, and other tumor and lifestyle factors.

Materials and Methods

Patient material

This study is based on 1,116 patients who were included in a prospective cohort of primary breast cancer patients (BC-Blood Study) at the Skåne University Hospital in Lund, Sweden that has been previously described in depth [37, 38]. The patients in the present study were included between October 2002 and June 2012, were between 24 and 99 years of age, and did not have any history of cancer during the past 10 years. Patients were followed from inclusion to the first breast cancer event, distant metastasis, or death by any cause, respectively. Patients without events were censored at the last follow-up or death prior to July 1st 2014. Breast cancer events included ipsilateral, contralateral, axillary lymph node, and distant metastases. The first breast cancer event of any type was considered the primary endpoint i.e. disease-free survival. Secondary endpoints were distant metastasis i.e. distant-metastasis-free survival, and overall survival. Information on breast cancer events or death was obtained from the Regional Tumor Registry, the Population Registry, patient charts, or pathology reports. As previously described, the follow-up rates of the patients were high [37]. During the time period this cohort was compiled, a total of 2,170 female patients were operated for breast cancer in Lund. This number also included patients with a non-primary breast cancer as well as patients who had been diagnosed with other cancers during the past 10 years. The median age of the 2,170 patients was 61 years. ER-status was available for 1,928 patients and 85.4% had ER-positive tumors. Progesterone receptor (PgR)-status was available for 1,914 patients and 70.1% had PgR-positive tumors. For the present study, patients with preoperative treatment and *in situ* tumors were excluded. For survival analyses, the final study cohort consisted of 911 patients with successfully stained TMA, see flowchart (Fig. 1) for inclusion an exclusion of patients. The REMARK (REporting recommendations for tumor MARKer prognostic study) guidelines were followed for this study [39]. Breast cancer treatment was prescribed according to the standard of care at Skåne University Hospital at the time the patients were included. Prior to surgery, patients were asked to fill out questionnaires regarding reproductive history, use of exogenous hormones or other medications, and other lifestyle factors including smoking, and alcohol and coffee consumption. The patients' anthropometric factors including weight, height, waist and hip circumference, and breast volume [40], were measured by a research nurse at the preoperative visit. Tumor characteristics including

histological grade, axillary lymph node status, tumor size, proliferation marker Ki67, ER, PgR, and HER2 status were obtained from medical records and pathology reports. The tumors were analyzed at the Department of Pathology at Skåne University Hospital. ER and PgR status were determined as previously described [41]. HER2 assessment was routinely analyzed as of November 2005 in patients younger than 70 years as described before [36]. HER2 status was therefore included in subgroup analyses of patients included in the study between November 2005 and December 2012. Ki67 was only available as of March 2009.

Information regarding the type of adjuvant treatment was collected from patient charts and questionnaires. Breast cancer treatments were registered up to the last follow-up or death and prior to any breast cancer event. NSAID was defined as all medications classified as NSAIDs in the World Health Organizations ATC code system [42] and therefore acetylsalicylic acid (ASA) was not included in the NSAIDs but recorded as a separate variable. Written informed consents were collected from all patients and the study was approved by the ethics committee at Lund University (Dnr75-02, Dnr37-08, Dnr658-09, Dnr58-12, Dnr379-12, Dnr227-13, Dnr277-15, and Dnr458-15).

Tissue microarray and immunohistochemistry

Tissue microarrays (TMAs), containing duplicate 1.00 mm cores, were constructed from selected areas with invasive breast cancer from blocks of formalin-fixed paraffin-embedded tissue. 4-µm thick TMA sections were deparaffinized and pretreated using an automatic PT-link system followed by staining using COX-2 antibody (ab15191, diluted 1:750 for 30 min at pH7, Abcam, Cambridge, UK) and EnVision FLEX high-pH kit, in an Autostainer Plus, according to the manufacturer's instructions (DAKO, Glostrup, Denmark). Heart muscle and non-cancerous tumor adjacent breast epithelium were used as negative controls. Lung cancer and colon cancer tissue were used as positive controls. Cytoplasmic tumor-specific COX-2 expression was evaluated for staining intensity (0=negative, 1=weak, 2=moderate, 3=strong) as well as for positive fraction (0-100%), and a joint score for the invasive cancerous cells in the two cores were obtained. The immunohistochemical staining was evaluated by two independent observers (SBj and MS) that were blinded to patient information. In cases that showed discrepancy (11.1%), a re-evaluation was made and consensus was reached. In most cases (90.3%) for which the staining was positive, COX-2 was expressed in the majority of the cells (75-100%). Therefore, the fraction of COX-2-positive cells was excluded from further analyses. The concordance for staining intensity between the individual cores of correlated pairs was 91.9% for the senior evaluator (SBj) and 89.8% for the second evaluator (MS). All cores were evaluated jointly to obtain a pooled score based on the intensity represented in the majority of positively stained invasive cancerous cells. The pooled score was classified as positive if at least one core was classified

as positive. For six patients with bilateral tumors where the COX-2 intensity differed, the highest intensity was used. A sensitivity analysis was also conducted where the lowest intensity was used instead. Patients with missing COX-2 status were not included in the analyses.

Statistical methods

The IBM SPSS Statistics, version 22.0 (IBM Corp. Armonk, NY, USA) were used for the statistical analyses. Height (m), weight (kg), waist-to-hip ratio (WHR), age at first full-term pregnancy (years) and age at menarche (years) were all used as continuous variables. The following variables were dichotomous: body mass index (BMI; ≥ 25 kg/m^2), breast volume (≥ 850 mL as per previously defined cut-off [40]), current smoker prior to surgery, alcohol abstainer prior to surgery, nulliparity, any chemotherapy, radiotherapy, tamoxifen treatment, and AI-treatment. Trastuzumab treatment was entered as a covariate in subgroup analyses of patients included as of November 2005. Tumor features were described as any axillary lymph node involvement or number of involved axillary lymph nodes (0, 1-3, 4+), histological grade (I-III), and invasive tumor size (≤ 20 mm vs ≥ 21 mm or skin or muscular involvement).

LogRank tests were used for univariable analyses and Cox regression was used to calculate Hazard Ratios (HRs) in multivariable analyses. Adjustments were made for age (≥ 50 years), invasive tumor size (≥ 21 mm or skin or muscular involvement independent of size), any axillary lymph node involvement, ER-status, and histological grade (I-III). Formal interaction analyses between COX-2 expression (negative/weak to moderate/strong) and patient characteristics and tumor characteristics were performed. A prior power calculation assuming 900 patients, an accrual interval of 10 years and additional follow-up after the accrual interval of 2 years, and a frequency of 10% COX-2 negative tumors showed that the study was able to detect true HRs of 0.69 or 1.51 with 80% power and α of 0.05 [43]. All *P*-values were two-sided and a *P*-value<0.05 was considered significant. Nominal *P*-values are presented without adjustment for multiple testing.

Results

Patients were followed for up to 11 years, median follow-up time was 5.0 years for patients still at risk. During this time, 88 patients had died due to any cause, and 110 patients experienced a breast cancer recurrent event, of which 69 had distant metastases. COX-2 staining was successful for 911 tumors (92.6%). The distribution of COX-2 intensity is presented in Table 1. There were no significant differences between the included and the missing cases regarding tumor characteristics.

COX-2 in relation to patient and tumor characteristics

As presented in Table 1, higher intensity of tumor-specific COX-2 expression was significantly associated with increasing age at diagnosis (P_{trend}=0.001) and with lower frequency of oral contraceptive (OC) use prior to age 20 years (P_{trend}=0.013). COX-2 negativity was associated with higher frequency of preoperative consumption of ASA (χ^2 P=0.033), compared to weak, moderate, or strong expression. As described in Table 2, higher COX-2 intensity was significantly associated with a lower histological grade and Ki67, higher frequency of ER-positive tumors, PgR-positive tumors, and lower frequency of HER-2 positive tumors and triple negative (ER-PgR-HER2-) tumors. Further, higher COX-2 intensity was also associated with higher frequency of adjuvant tamoxifen and aromatase inhibitor treatment, and lower frequency of chemotherapy. COX-2 negativity was significantly associated with lower frequency of smaller invasive tumors. In summary, COX-2 expression was significantly associated with less aggressive tumor characteristics.

COX-2 expression in relation to prognosis

Patients with weak or moderate COX-2 tumor intensity were combined into one group in the survival analyses to avoid violation of the assumption of proportional hazards. This resulted in three categories of COX-2 intensity: negative, weak to moderate, and strong. Higher COX-2 expression was associated with lower risk for any breast cancer event in the univariable analysis (LogRank P_{trend}=0.020), but not in the multivariable analysis, adjusted HR ($_{adj}$HR) 0.73 (95% CI: 0.49-1.09) per category, adjusted for age and tumor characteristics. However, higher COX-2 expression was independently associated with lower risk for events during the first five years of follow-up (LogRank P_{trend}=0.0003), $_{adj}$HR 0.60 (95% CI: 0.37-0.97) per category, Fig. 2A, Table 3. COX-2 expression was not independently associated with distant metastasis-free survival or overall survival, Fig. 2B-C.

COX-2 and OC use in relation to prognosis

Formal interaction analyses were performed to investigate whether there were any effect modifications by patient or tumor characteristics on the association between COX-2 expression and prognosis. There was a significant interaction between COX-2 and ever use of OCs ($_{adj}$HR 0.39; $P_{interaction}$=0.048). Higher COX-2 expression was associated with a lower risk for events among patients who had ever used OCs (n=642; LogRank P_{trend}=0.005), $_{adj}$HR 0.64 (95% CI; 0.41-0.97) per category. Conversely, there were no events among never OC users with COX-2 negative tumors, but this was not significant (n=269; LogRank P_{trend}=0.71), Fig 3A, while ever OC users with COX-2 negative tumors had the highest risk for events, Fig 3B. Four categories were computed according to any COX-2

expression and ever OC use to illustrate these differences. Ever OC users with COX-2 negative tumors had a significantly worse prognosis in the univariable (LogRank P=0.011) but not in the multivariable model, $_{adj}$HR 1.51 (95% CI: 0.85-2.68). However, during the first five years, these patients had a nearly 2-fold risk for breast cancer events compared to the other groups (LogRank P<0.0001), $_{adj}$HR 1.94 (95% CI: 1.01-3.72), Fig. 3C. There was no effect modification of OC use prior to age 20 years on the association between COX-2 expression and risk for events.

COX-2 and NSIADs in relation to prognosis

Preoperative NSAID use was not associated with risk of breast cancer events among all patients (LogRank P=0.95) or in different adjuvant treatment groups (all LogRank P_s>0.3). However, there was a significant interaction between preoperative NSAID use and higher COX-2 expression on risk for events (adjusted HR 0.24; $P_{interaction}$=0.009), where higher COX-2 expression was associated with a better prognosis among patients who had used NSAID preoperatively (n=131; LogRank P_{trend}=0.003), $_{adj}$HR 0.19 (95% CI; 0.06-0.66) per category, Fig. 3D, but not among the other patients (n=777; LogRank P_{trend}=0.19), Fig. 3E. Four groups were constructed based on any COX-2 expression and preoperative NSAID use and patients with preoperative NSAID use and COX-2 negative tumors had a significantly higher risk for events, $_{adj}$HR 4.51 (95% CI: 1.18-11.44), compared to all other patients, Fig. 3F. Preoperative ASA use was not associated with risk for breast cancer events in the total study population or when stratified according to adjuvant treatments (all Ps>0.3). There were no significant effect modifications of any of the other patient characteristics presented in Table 1 on the association between COX-2 and risk for events (all adjusted $P_{interactions}$>0.11).

COX-2 and tumor size in relation to prognosis

Regarding tumor characteristics, there was a significant interaction between COX-2 and tumor size ($_{adj}$HR 0.44; $P_{interaction}$=0.039), where COX-2 was prognostic among patients with larger tumors, i.e. ≥21 mm or skin or muscular involvement, (LogRank P_{trend}=0.003), adjusted HR 0.58 (95% CI: 0.33-1.03), but not among patients with smaller tumors (LogRank P_{trend}=0.89), Fig. 3G-H. Four groups were constructed based on any COX-2 intensity and tumor size. Patients with large COX-2 negative tumors had a worse prognosis compared to the other patients (LogRank P_{trend}<0.0001), $_{adj}$HR 2.57 (95% CI; 1.28-5.15), Fig. 3I. Due to small numbers, no interaction analyses were conducted between COX-2 and Ki67. There were no effect modifications of any of the other tumor characteristics presented in Table 2 and the association between COX-2 expression and risk for events (all $P_{interactions}$>0.25).

COX-2 in relation to prognosis in different treatment groups

Stratified analyses were performed according to adjuvant treatment with chemotherapy, radiotherapy, tamoxifen, AIs, or trastuzumab. Higher COX-2 expression was significantly associated with lower risk for events among chemotherapy-treated patients in the univariable analysis (LogRank P_{trend}=0.012), but not in the multivariable analysis, $_{adj}$HR 0.61 (95% CI: 0.32-1.18) per category and the hazard was similar for the first five years. Further, COX-2 expression was not associated with prognosis among patients who had received radiotherapy, tamoxifen, AIs, or trastuzumab for the entire follow-up (all LogRank P_s>0.19). However, during the first five years, higher COX-2 expression was borderline associated with lower risk for events among tamoxifen-treated patients, $_{adj}$HR 0.46 (95% CI: 0.19-1.11) per category, but not in the other treatment groups.

Sensitivity analyses

Sensitivity analyses were conducted where the lowest intensity from the patients with bilateral tumors was used instead of the highest intensity. This did not materially change the results in any of the analyses. Further adjustments for OC or NSAID use did not materially change the results.

Discussion

The present study investigated the prognostic value of tumor-specific COX-2 expression in relation to prognosis. The main finding of the current study was that higher COX-2 expression was associated with less aggressive tumor features and a favorable prognosis. COX-2 negativity was independently associated with a worse prognosis the first five years, which is in contrast to the majority of previous studies. Moreover, the association between COX-2 expression and risk for events depended on history of OC use, preoperative NSAID use, and tumor size, where significant effect modifications were observed.

Tumor COX-2 expression has previously been associated with a shorter disease-free survival in many [1, 3-19] but not all [20-27] studies. However, most of the previous studies had 200 patients or less [5, 8-11, 13, 14, 18, 23, 24, 26]. Further, multivariable analyses to elucidate the independent prognostic value of COX-2 expression, were only performed in a subset of these studies [1, 3-8, 13, 16-19]. The association between COX-2 expression and prognosis remained significant after multivariable adjustments only in two

smaller studies with around 200 patients [6, 19], but not in the two largest studies with 1,576 and 2,001 patients, respectively [4, 16]. In other studies, COX-2 was only independently associated with worse prognosis in subgroups of patients who were either node-negative [8], had high Ki67 [6], ER-negative [3, 18], or ER-positive tumors [7]. There are many possible explanations for the diverse results between previous studies including study populations with different body constitutions, reproductive patterns, lifestyle factors, medication use, ethnic backgrounds, and study end-points. Further, the methods for immunohistochemical analysis of COX-2 have been diverse, with various antibodies, lack of standardization of immunohistochemical staining, and analysis of COX-2 tumor expression [44]. Classifications of COX-2 positivity and negativity differed significantly between published studies, which makes comparisons between studies difficult. In the present study, a polyclonal rabbit antibody was used. COX-2 expression was based on intensity since the vast majority of the COX-2 positive tumors expressed COX-2 in over 75% of the cells. A clean negative group without any staining was rare among published studies [4, 14, 16, 20, 26]. In the present study, the COX-2 negative group had the worst prognosis. This group may have been missed in studies where COX-2 negative tumors were combined with tumors with weak or moderate staining. To our knowledge, a dose-dependent association between COX-2 expression and prognosis has not been previously investigated.

In the present study, higher COX-2 expression was significantly associated with favorable tumor characteristics. Previous studies have also found significant associations between COX-2 expression and tumor characteristics, but the results have been inconsistent, ranging from favorable characteristics [25, 45] to unfavorable characteristics [4, 7]. The effect estimate between COX-2 and prognosis was significantly modified by tumor size, where COX-2 expression was associated with prognosis only among patients with larger tumor sizes in the present study. Furthermore, all but one event among the patients with COX-2 negative tumors occurred within five years, and this event occurred right after five years. This may explain findings in the largest previous study [16], where patients were included at least 12 months after the diagnosis. Therefore, some patients with COX-2 negative tumors and a poor prognosis would have been missed in contrast to patients with COX-2 negative tumors and a good prognosis. This may have led to survival bias. A strength of the current study is that it is a prospective cohort, which minimizes the risk for bias and patients with early recurrences were not missed.

Higher COX-2 expression was associated with higher frequency of ER-positive tumors in the present study. Activated aromatase leads to higher estrogen levels and increased proliferation in ER-positive

10

tumors [2]. The aromatase gene is a downstream target of COX-2 [2]. According to a paper by van Nes *et al.* that included both an original study and a review [1], the majority of patients in two large studies [4, 7] were included before tamoxifen was prescribed according to hormonal receptor status of the tumor. Since the former studies also included tamoxifen-treated patients with ER-negative tumors, data from newer studies are needed to elucidate the importance of COX-2 in today's setting. The current study included patients as of 2002 and selection of treatment regimens are based on hormone receptor status. The result of the original study including 667 patients by van Nes *et al.* showed no significant association between COX-2 expression (over the median level) in endocrine-treated patients with ER-positive tumors and prognosis. This is partly in line with the current study, where there was a borderline association between higher COX-2 expression and lower risk of early events within the first five years, but not with later events, among tamoxifen-treated patients. However, the median follow-up time of the current study was relatively short and patients with ER-positive tumors tend to relapse late [46].

In the present study, COX-2 expression was significantly associated with lower Ki67. Martin *et al.* reported a trend towards lower Ki67 after neoadjuvant treatment with the selective COX-2 inhibitor celecoxib [32]. However, a recent neoadjuvant randomized controlled phase II trial of celecoxib versus exemestane did not show any antiproliferative effect of celecoxib [33]. In the present study, there was no statistically significant difference in Ki67 among patients with and without reported preoperative NSAID use (data not shown). However, patients who reported preoperative NSAID use and whose tumors were COX-2 negative had the worst prognosis and there was a highly significant interaction between NSAID use and COX-2 expression on risk for breast cancer events. This is to our knowledge the first study to report preoperative NSAID use to modify the effect estimate on the association between COX-2 expression and breast cancer prognosis. Thus, tumor-specific COX-2 expression in randomized controlled trials of preoperative or adjuvant NSAID treatment merits further investigation.

OC use prior to age 20 years was significantly associated with lower COX-2 expression in the present study and low COX-2 expression was associated with worse short-term prognosis. In line with this, OC use prior to age 20 years was previously reported to be associated with a worse prognosis among patients younger than 50 years in this cohort [47]. Early OC use may carry long-term impact on the tumor environment. However, any history of OC use prior to diagnosis, and not early OC use modified the association between COX-2 expression and prognosis. Ever OC use was also associated

with improved prognosis among AI-treated patients 50 years or older in the previous study [47]. However, there was no association between COX-2 expression and prognosis in AI-treated patients in the present study. OC use could be a confounding factor in the association between COX-2 and prognosis in breast cancer in studies not taking OCs into account. In Sweden, the majority of the female population have used OCs [48]. OC use has increased worldwide since the 1970s [49] and patterns of OC use differ between countries. This could potentially impact the results between COX-2 expression and prognosis in breast cancer patients from different countries. In the current study, no data on ethnic background was collected but the majority of the patients in the Skåne University Hospital in Lund were Swedes. Skåne University Hospital in Lund has a catchment area of 300.000 inhabitants and the patients are not referred to other hospitals for surgery. This study is therefore considered population-based.

In a previous study of a subgroup of the current cohort, breast size interacted with *COX2* rs5277 genotype on prognosis [36]. However, in the present study, there were no effect modifications of anthropometric factors on the association between COX-2 expression and prognosis, which was not explored in the previous study. In the present study, new slides of the TMAs were re-stained with a more diluted antibody compared with the previous study in order to obtain a wider distribution of staining intensities. Similar to the previous study, there was no association between *COX2* rs5277 genotype and COX-2 expression (data not shown).

Conclusions

This study is one of the largest cohorts evaluating tumor-specific COX-2 expression in breast cancer. Higher COX-2 expression was associated with lower risk for early breast cancer events and less aggressive tumor characteristics. The prognostic impact of COX-2 expression differed significantly according to tumor size, history of OC use, and preoperative NSAID use. The findings warrant validation in an independent cohort or randomized trial. If validated, tumor size, history of OC use, and tumor-specific COX-2 expression may need to be considered when evaluating outcome in clinical trials with NSAIDs for breast cancer patients.

Acknowledgements

References

1. van Nes JG, de Kruijf EM, Faratian D, van de Velde CJ, Putter H, Falconer C, Smit VT, Kay C, van de Vijver MJ, Kuppen PJ, Bartlett JM. COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients. *Breast Cancer Res Treat* 2011;**125**: 671-85.

2. Hugo HJ, Saunders C, Ramsay RG, Thompson EW. New Insights on COX-2 in Chronic Inflammation Driving Breast Cancer Growth and Metastasis. *J Mammary Gland Biol Neoplasia* 2015;**20**: 109-19.

3. Glynn SA, Prueitt RL, Ridnour LA, Boersma BJ, Dorsey TM, Wink DA, Goodman JE, Yfantis HG, Lee DH, Ambs S. COX-2 activation is associated with Akt phosphorylation and poor survival in ER-negative, HER2-positive breast cancer. *BMC Cancer* 2010;**10**: 626.

4. Ristimäki A, Sivula A, Lundin J, Lundin M, Salminen T, Haglund C, Joensuu H, Isola J. Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. *Cancer Res* 2002;**62**: 632-5.

5. Park K, Han S, Shin E, Kim HJ, Kim JY. Cox-2 expression on tissue microarray of breast cancer. *Eur J Surg Oncol* 2006;**32**: 1093-6.

6. Park BW, Park S, Park HS, Koo JS, Yang WI, Lee JS, Hwang H, Kim SI, Lee KS. Cyclooxygenase-2 expression in proliferative Ki-67-positive breast cancers is associated with poor outcomes. *Breast Cancer Res Treat* 2012;**133**: 741-51.

7. Haffty BG, Yang Q, Moran MS, Tan AR, Reiss M. 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* 2008;**71**: 1006-13.

8. Sicking I, Rommens K, Battista MJ, Bohm D, Gebhard S, Lebrecht A, Cotarelo C, Hoffmann G, Hengstler JG, Schmidt M. Prognostic influence of cyclooxygenase-2 protein and mRNA expression in node-negative breast cancer patients. *BMC Cancer* 2014;**14**: 952.

9. Zhang XH, Huang DP, Guo GL, Chen GR, Zhang HX, Wan L, Chen SY. Coexpression of VEGF-C and COX-2 and its association with lymphangiogenesis in human breast cancer. *BMC Cancer* 2008;**8**: 4.

10. O'Connor JK, Avent J, Lee RJ, Fischbach J, Gaffney DK. Cyclooxygenase-2 expression correlates with diminished survival in invasive breast cancer treated with mastectomy and radiotherapy. *Int J Radiat Oncol Biol Phys* 2004;**58**: 1034-40.

11. Costa C, Soares R, Reis-Filho JS, Leitao D, Amendoeira I, Schmitt FC. Cyclo-oxygenase 2 expression is associated with angiogenesis and lymph node metastasis in human breast cancer. *J Clin Pathol* 2002;**55**: 429-34.

12. Surowiak P, Materna V, Matkowski R, Szczuraszek K, Kornafel J, Wojnar A, Pudelko M, Dietel M, Denkert C, Zabel M, Lage H. Relationship between the expression of cyclooxygenase 2 and MDR1/P-glycoprotein in invasive breast cancers and their prognostic significance. *Breast Cancer Res* 2005;**7**: R862-70.

13. Zhou L, Li K, Luo Y, Tian L, Wang M, Li C, Huang Q. Novel prognostic markers for patients with triple-negative breast cancer. *Hum Pathol* 2013;**44**: 2180-7.

14. Schmitz KJ, Callies R, Wohlschlaeger J, Kimmig R, Otterbach F, Bohr J, Lee HS, Takeda A, Schmid KW, Baba HA. Overexpression of cyclo-oxygenase-2 is an independent predictor of unfavourable outcome in node-negative breast cancer, but is not associated with protein kinase B (Akt) and mitogen-activated protein kinase (ERK1/2, p38) activation or with Her-2/neu signalling pathways. *J Clin Pathol* 2006;**59**: 685-91.

15. Dillon MF, Stafford AT, Kelly G, Redmond AM, McIlroy M, Crotty TB, McDermott E, Hill AD, Young LS. Cyclooxygenase-2 predicts adverse effects of tamoxifen: a possible mechanism of role for nuclear HER2 in breast cancer patients. *Endocr Relat Cancer* 2008;**15**: 745-53.

16. Holmes MD, Chen WY, Schnitt SJ, Collins L, Colditz GA, Hankinson SE, Tamimi RM. COX-2 expression predicts worse breast cancer prognosis and does not modify the association with aspirin. *Breast Cancer Res Treat* 2011;**130**: 657-62.

17. Spizzo G, Gastl G, Wolf D, Gunsilius E, Steurer M, Fong D, Amberger A, Margreiter R, Obrist P. Correlation of COX-2 and Ep-CAM overexpression in human invasive breast cancer and its impact on survival. *Br J Cancer* 2003;**88**: 574-8.

18. Witton CJ, Hawe SJ, Cooke TG, Bartlett JM. Cyclooxygenase 2 (COX2) expression is associated with poor outcome in ER-negative, but not ER-positive, breast cancer. *Histopathology* 2004;**45**: 47-54.

19. Denkert C, Winzer KJ, Muller BM, Weichert W, Pest S, Kobel M, Kristiansen G, Reles A, Siegert A, Guski H, Hauptmann S. Elevated expression of cyclooxygenase-2 is a negative prognostic factor for disease free survival and overall survival in patients with breast carcinoma. *Cancer* 2003;**97**: 2978-87.

20. Gunnarsson C, Jansson A, Holmlund B, Ferraud L, Nordenskjöld B, Rutqvist LE, Skoog L, Stål O. Expression of COX-2 and steroid converting enzymes in breast cancer. *Oncol Rep* 2006;**16**: 219-24.

21. Ahn HJ, Jung SJ, Kim TH, Oh MK, Yoon HK. Differences in Clinical Outcomes between Luminal A and B Type Breast Cancers according to the St. Gallen Consensus 2013. *J Breast Cancer* 2015;**18**: 149-59.

22. Kargi A, Uysal M, Bozcuk H, Coskun HS, Savas B, Ozdogan M. The importance of COX-2 expression as prognostic factor in early breast cancer. *J BUON* 2013;**18**: 579-84.

23. Mohammad MA, Zeeneldin AA, Abd Elmageed ZY, Khalil EH, Mahdy SM, Sharada HM, Sharawy SK, Abdel-Wahab AH. Clinical relevance of cyclooxygenase-2 and matrix metalloproteinases (MMP-2 and MT1-MMP) in human breast cancer tissue. *Mol Cell Biochem* 2012;**366**: 269-75.

24. Nassar A, Radhakrishnan A, Cabrero IA, Cotsonis G, Cohen C. COX-2 expression in invasive breast cancer: correlation with prognostic parameters and outcome. *Appl Immunohistochem Mol Morphol* 2007;**15**: 255-9.

25. Dhakal HP, Naume B, Synnestvedt M, Borgen E, Kaaresen R, Schlichting E, Wiedswang G, Bassarova A, Holm R, Giercksky KE, Nesland JM. Expression of cyclooxygenase-2 in invasive breast carcinomas and its prognostic impact. *Histol Histopathol* 2012;**27**: 1315-25.

26. Kelly LM, Hill AD, Kennedy S, Connolly EM, Ramanath R, Teh S, Dijkstra B, Purcell R, McDermott EW, O'Higgins N. Lack of prognostic effect of Cox-2 expression in primary breast cancer on short-term follow-up. *Eur J Surg Oncol* 2003;**29**: 707-10.

27. Wülfing P, Diallo R, Müller C, Wülfing C, Poremba C, Heinecke A, Rody A, Greb RR, Böcker W, Kiesel L. Analysis of cyclooxygenase-2 expression in human breast cancer: high throughput tissue microarray analysis. *J Cancer Res Clin Oncol* 2003;**129**: 375-82.

28. Lustberg MB, Povoski SP, Zhao W, Ziegler RM, Sugimoto Y, Ruppert AS, Lehman AM, Shiels DR, Mrozek E, Ramaswamy B, Layman RM, Brueggemeier RW, et al. Phase II trial of neoadjuvant exemestane in combination with celecoxib in postmenopausal women who have breast cancer. *Clin Breast Cancer* 2011;**11**: 221-7.

29. Barnes NL, Warnberg F, Farnie G, White D, Jiang W, Anderson E, Bundred NJ. Cyclooxygenase-2 inhibition: effects on tumour growth, cell cycling and lymphangiogenesis in a xenograft model of breast cancer. *Br J Cancer* 2007;**96**: 575-82.

30. Brandao RD, Veeck J, Van de Vijver KK, Lindsey P, de Vries B, van Elssen CH, Blok MJ, Keymeulen K, Ayoubi T, Smeets HJ, Tjan-Heijnen VC, Hupperets PS. A randomised controlled phase II trial of pre-operative celecoxib treatment reveals anti-tumour transcriptional response in primary breast cancer. *Breast Cancer Res* 2013;**15**: R29.

31. Bundred NJ, Cramer A, Morris J, Renshaw L, Cheung KL, Flint P, Johnson R, Young O, Landberg G, Grassby S, Turner L, Baildam A, et al. Cyclooxygenase-2 inhibition does not improve the reduction in ductal carcinoma in situ proliferation with aromatase inhibitor therapy: results of the ERISAC randomized placebo-controlled trial. *Clin Cancer Res* 2010;**16**: 1605-12.

32. Martin LA, Davies GL, Weigel MT, Betambeau N, Hills MJ, Salter J, Walsh G, A'Hern R, Dowsett M. Pre-surgical study of the biological effects of the selective cyclo-oxygenase-2 inhibitor celecoxib in patients with primary breast cancer. *Breast Cancer Res Treat* 2010;**123**: 829-36.

33. Aristarco V, Serrano D, Gandini S, Johansson H, Macis D, Guerrieri-Gonzaga A, Lazzeroni M, Feroce I, Pruneri G, Pagani G, Toesca A, Caldarella P, et al. A Randomized, Placebo-Controlled, Phase II, Presurgical Biomarker Trial of Celecoxib Versus Exemestane in Postmenopausal Breast Cancer Patients. *Cancer Prev Res (Phila)* 2016;**9**: 349-56.

34. Retsky M, Demicheli R, Hrushesky WJ, Forget P, De Kock M, Gukas I, Rogers RA, Baum M, Sukhatme V, Vaidya JS. Reduction of breast cancer relapses with perioperative non-steroidal anti-inflammatory drugs: new findings and a review. *Curr Med Chem* 2013;**20**: 4163-76.

35. Forget P, Vandenhende J, Berliere M, Machiels JP, Nussbaum B, Legrand C, De Kock M. Do intraoperative analgesics influence breast cancer recurrence after mastectomy? A retrospective analysis. *Anesth Analg* 2010;**110**: 1630-5.

36. Markkula A, Simonsson M, Rosendahl AH, Gaber A, Ingvar C, Rose C, Jernström H. Impact of COX2 genotype, ER status and body constitution on risk of early events in different treatment groups of breast cancer patients. *Int J Cancer* 2014;**135**: 1898-910.

37. Simonsson M, Söderlind V, Henningson M, Hjertberg M, Rose C, Ingvar C, Jernström H. Coffee prevents early events in tamoxifen-treated breast cancer patients and modulates hormone receptor status. *Cancer Causes Control* 2013;**24**: 929-40.

38. Simonsson M, Veerla S, Markkula A, Rose C, Ingvar C, Jernström H. CYP1A2 - a novel genetic marker for early aromatase inhibitor response in the treatment of breast cancer patients. *BMC Cancer* 2016;**16**: 256.

39. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. REporting recommendations for tumour MARKer prognostic studies (REMARK). *Eur J Cancer* 2005;**41**: 1690-6.

40. Markkula A, Bromée A, Henningson M, Hietala M, Ringberg A, Ingvar C, Rose C, Jernström H. Given breast cancer, does breast size matter? Data from a prospective breast cancer cohort. *Cancer Causes Control* 2012;**23**: 1307-16.

41. Simonsson M, Markkula A, Bendahl PO, Rose C, Ingvar C, Jernström H. Pre- and postoperative alcohol consumption in breast cancer patients: impact on early events. *SpringerPlus* 2014;**3**: 261.

42. WHO Collaborating Centre for Drug Statistics Methodology O, Norway. Guidelines for ATC classification and DDD assignment 2015 Oslo, 2015.

43. Dupont WD, Plummer WD, Jr. Power and sample size calculations. A review and computer program. *Control Clin Trials* 1990;**11**: 116-28.

44. Urban J, Kuzbicki L, Szatkowski G, Stanek-Widera A, Lange D, Chwirot BW. Stromal, rather than epithelial cyclooxygenase-2 (COX-2) expression is associated with overall survival of breast cancer patients. *BMC Cancer* 2014;**14**: 732.

45. Nakopoulou L, Mylona E, Papadaki I, Kapranou A, Giannopoulou I, Markaki S, Keramopoulos A. Overexpression of cyclooxygenase-2 is associated with a favorable prognostic phenotype in breast carcinoma. *Pathobiology* 2005;**72**: 241-9.

46. Osborne CK, Yochmowitz MG, Knight WA, 3rd, McGuire WL. The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 1980;**46**: 2884-8.

47. Huzell L, Persson M, Simonsson M, Markkula A, Ingvar C, Rose C, Jernström H. History of oral contraceptive use in breast cancer patients: impact on prognosis and endocrine treatment response. *Breast Cancer Res Treat* 2015;**149**: 505-15.

48. Kopp Kallner H, Thunell L, Brynhildsen J, Lindeberg M, Gemzell Danielsson K. Use of Contraception and Attitudes towards Contraceptive Use in Swedish Women--A Nationwide Survey. *PLoS One* 2015;**10**: e0125990.

49. United Nations department of Economic and Social Affairs Population Division. World Fertility Report 2009 (United Nations publication). http://www.un.org/esa/population/publications/WFR2009_Web/Main.html, 2011.

Figure legends

Figure 1

Flowchart of included and excluded patients and COX-2 staining intensity (20X, scale bar represent 20 μm).

Figure 2

COX-2 expression in relation to:

A. Disease-free survival

COX-2 expression was significantly associated with risk for early breast cancer events.

B. Distant metastasis-free survival

COX-2 expression was not associated with distant-metastasis free-survival.

C. Overall survival

COX-2 expression was not associated with overall survival.

Number of patients at each time-point during follow-up and number of events (NoE) are indicated below each graph. The number of patients decreased with each follow-up since this is an ongoing cohort.

Figure 3

COX-2 expression in relation to disease-free survival, according to ever OC use, A-C, preoperative NSAID use, D-F, and tumor size, G-I.

Number of patients at each time-point during follow-up and number of events (NoE) are indicated below each graph. The number of patients decreased with each follow-up since this is an ongoing cohort.

Figure 1

```
                                              ┌─────────────────────────┐                        ------------►  Excluded
                                              │ Patients receiving surgery│
                                              │   for breast cancer       │                        ───────────►  Included
                                              │  Oct 2002-June 2012       │
                                              │      n = 2,170            │
                                              └─────────────────────────┘
                                                          │
                                                          ├ ─ ─ ─ ─ ─ ─►  ┌─────────────────────────┐
                                                          │               │ Patients not included   │
                                                          │               │   in the cohort         │
                                                          │               │     n = 1,054           │
                                                          │               └─────────────────────────┘
                                              ┌─────────────────────────┐
                                              │ Patients with primary    │
                                              │ breast cancer included   │
                                              │  Oct 2002-June 2012      │
                                              │      n = 1,116           │
                                              └─────────────────────────┘
                                                          │
                                                          ├ ─ ─ ─ ─ ─ ─►  ┌─────────────────────────┐
                                                          │               │ Preoperative            │
                                                          │               │   treatment             │
                                                          │               │     n = 51              │
                                                          │               └─────────────────────────┘
                                                          │
                                                          ├ ─ ─ ─ ─ ─ ─►  ┌─────────────────────────┐
                                                          │               │ In situ carcinoma       │
                                                          │               │     n = 39              │
                                                          │               └─────────────────────────┘
                                                          │
                                                          ├ ─ ─ ─ ─ ─ ─►  ┌─────────────────────────┐
                                                          │               │ Distant metastasis      │
                                                          │               │ ≤ 0.3 years from        │
                                                          │               │   baseline              │
                                                          │               │     n = 8               │
                                                          │               └─────────────────────────┘
```

Patients receiving surgery for breast cancer Oct 2002-June 2012 *n* = 2,170

Patients not included in the cohort *n* = 1,054

Patients with primary breast cancer included Oct 2002-June 2012 *n* = 1,116

Preoperative treatment *n* = 51

In situ carcinoma *n* = 39

Distant metastasis ≤ 0.3 years from baseline *n* = 8

Missing on TMA or no evaluable core for COX-2 expression n = 107

No tumor on TMA *n* = 34

No evaluable core for COX-2 expression *n* = 73

Available COX-2 score and included in analyses *n* = 911

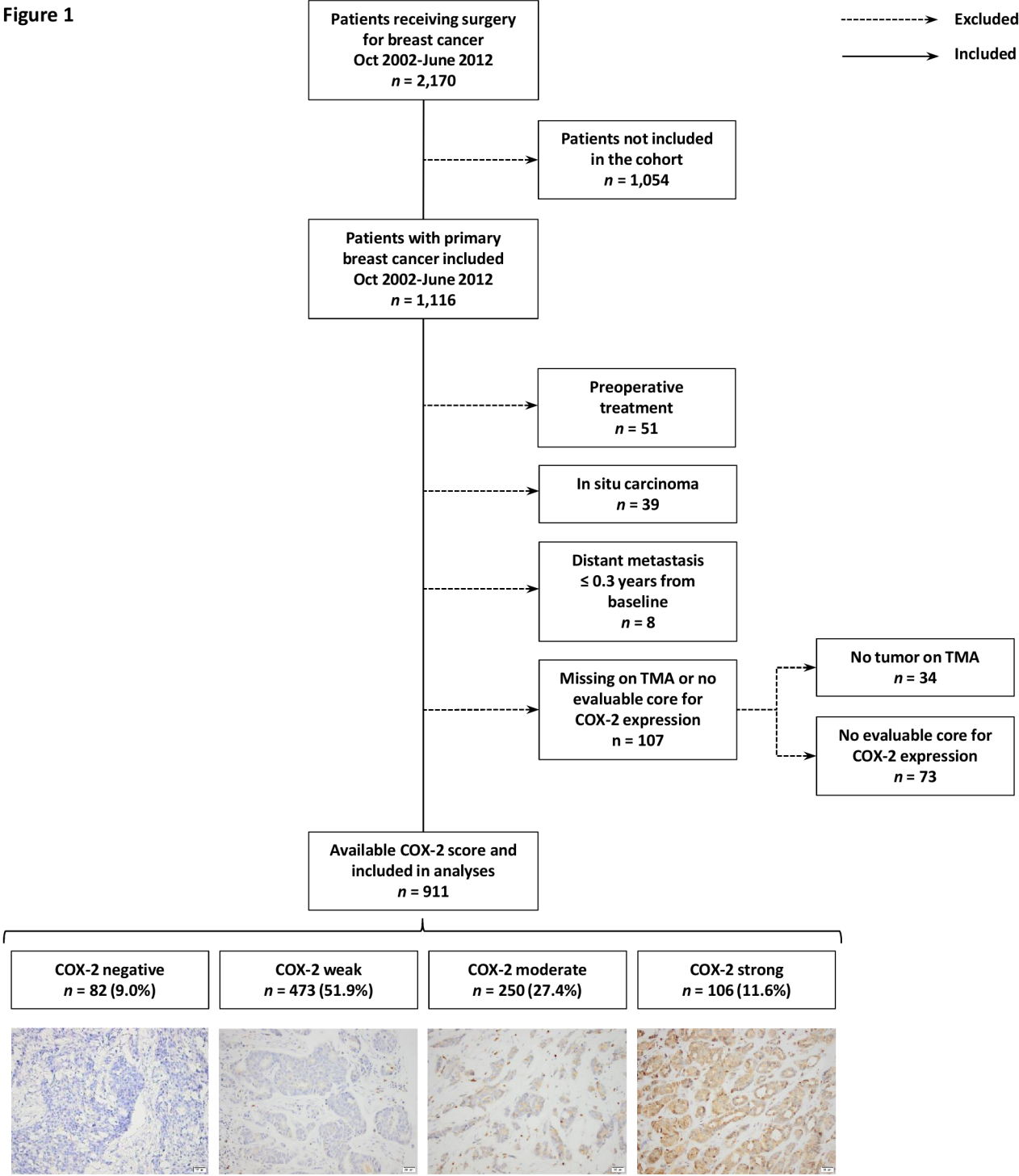| COX-2 negative *n* = 82 (9.0%)	COX-2 weak *n* = 473 (51.9%)	COX-2 moderate *n* = 250 (27.4%)	COX-2 strong *n* = 106 (11.6%)

Table 1. Patient characteristics at inclusion and treatments in relation to COX-2 expression during the first postoperative year.

	All	Missing	COX-2 staining intensity				Non-evaluable COX-2 status
			Negative	Weak	Moderate	Strong	
	n=911		n=82 (9.0%)	n=473 (51.9%)	n=250 (27.4%)	n=106 (11.6%)	n=73 (7.4%)
	Median (IQR) or %		Median (IQR) or %	Median (IQR) or %	Median (IQR) or %	Median (IQR) or %	Median (IQR) or %
Age at inclusion, years	61.2 (52.6-68.3)	0	60.3 (48.0-67.3)	60.1 (51.5-67.8)	62.8 (56.3-69.3)	63.7 (55.4-68.8)	56.0 (48.2-66.1)
Age of 50 years or older at inclusion	81.3	0	72.0	78.6	88.4	84.0	69.9
Age at menarche, years	13.0 (12.0-14.0)	6	13.0 (12.0-14.0)	13.0 (12.0-14.0)	13.0 (12.0-14.0)	13.0 (12.0-14.0)	13.0 (13.0-14.0)
Height, meters	1.65 (1.62-1.70)	25	1.65 (1.61-1.69)	1.66 (1.62-1.70)	1.65 (1.62-1.69)	1.65 (1.62-1.70)	1.65 (1.62-1.70)
Weight, kgs	70.0 (62.0-78.5)	25	70.0 (62.4-79.0)	70.0 (62.0-78.5)	69.0 (61.0-78.0)	70.5 (62.0-80.0)	66.0 (61.3-73.0)
BMI ≥25 kg/m^2	51.7	27	57.7	50.1	50.0	58.1	38.4
Waist to hip ratio	0.86 (0.81-0.90)	36	0.88 (0.83-0.91)	0.85 (0.80-0.90)	0.86 (0.81-0.91)	0.88 (0.84-0.91)	0.85 (0.79-0.90)
Total breast volume, mL [e]	1000 (650-1550)	142	1000 (700-1600)	1000 (650-1550)	1000 (600-1563)	1000 (625-1600)	900 (625-1100)
Breast volume ≥ 850 mL [e]	57.5	142	67.6	57.0	55.1	57.0	55.7
Parous	87.9	1	90.2	88.2	87.6	85.8	89.0
Current smoker	20.1	2	24.4	18.8	19.8	23.6	26.0
Alcohol abstainer	10.7	6	15.9	10.6	9.3	10.5	12.5
Coffee consumption, 2+ cups/day	81.0	4	81.7	82.6	79.0	78.3	83.6
Ever oral contraceptive use	70.5	1	79.0	70.4	68.8	68.9	72.6
Any oral contraceptives use prior to age 20 years	32.2	5	44.3	33.1	28.9	27.4	41.1
Ever treatment for menopausal symptoms	44.7	3	46.3	44.1	46.8	41.5	38.4
Preoperative NSAIDs use during past week at inclusion	14.4	3	17.1	15.9	9.6	17.0	17.8
Preoperative ASA use during past week at inclusion	7.2	4	13.4	5.1	8.5	8.5	5.5

[a] Jonckheere-Terpstra, [b] Kruskal-Wallis, [c] Linear-by-Linear Association, [d] Chi-square, [e] breast volume was not analyzed for women with previous breast surgeries n=102.

Breast volume was missing for an additional 40 patients. IQR = Interquartile range, NSAIDs = Non steroidal anti-inflammatory drugs, ASA = Acetylsalicylic acid

Table 2. Tumor characteristics at inclusion in relation to COX-2 expression during the first postoperative year.

		COX-2 staining intensity					Number and (%)
		Number and (%) or median and (IQR)					Non-evaluable COX-2 status
	All	Negative	Weak	Moderate	Strong	*P*-value	
	n = 911	n = 82 (9.0)	n = 473 (51.9)	n = 250 (27.4)	n = 106 (11.6)		n = 73 (7.4)
Invasive tumor size						0.064[a]	
1 - 20 mm	652 (71.6)	47 (57.3)	342 (72.3)	189 (75.6)	74 (69.8)		57 (78.1))
21 - 50 mm	244 (26.8)	33 (40.2)	125 (26.4)	56 (22.4)	30 (28.3)		14 (19.2)
> 50 mm	13 (1.4)	1 (1.2)	5 (1.1)	5 (2.0)	2 (1.9)		2 (2.7)
Skin or muscular involvement	2 (0.2)	1 (1.2)	1 (0.2)	0 (0.0)	0 (0.0)		0 (0.0)
Missing	0	0	0	0	0		0
≥ 21 mm or Skin or muscular involvement	259 (28.4)	35 (42.7)	131 (27.7)	61 (24.4)	32 (30.2)	**0.015[a]**	16 (21.9)
Axillary nodal involvement						0.97[a]	
0	552 (60.7)	50 (61.0)	281 (59.5)	158 (63.5)	63 (59.4)		51 (69.9)
1-3	277 (30.4)	25 (30.5)	149 (31.6)	69 (27.7)	34 (32.1)		17 (23.3)
4+	80 (8.8)	7 (8.5)	42 (8.9)	22 (8.8)	9 (8.5)		5 (6.8)
Missing	2	0	1	1	0		0
Any axillary lymph node involvement	357 (39.3)	32 (39.0)	191 (40.5)	91 (36.5)	43 (40.6)	0.77[a]	22 (30.1)
Histological grade						**<0.0001[b]**	
I	217 (23.8)	9 (11.0)	102 (21.6)	84 (33.6)	22 (20.8)		25 (34.7)
II	461 (50.6)	23 (28.0)	239 (50.5)	130 (52.0)	69 (65.1)		31 (43.1)
III	233 (25.6)	50 (61.0)	132 (27.9)	36 (14.4)	15 (14.2)		16 (22.2)
Missing	1	0	0	0	0		0
Hormone receptor status							
ER-positive	800 (87.9)	43 (53.1)	419 (88.6)	238 (95.2)	100 (94.3)	**<0.0001[b]**	66 (90.4)
PgR-positive	650 (71.4)	34 (42.0)	338 (71.5)	196 (78.4)	82 (77.4)	**<0.0001[b]**	50 (68.5)
Missing	2	1	1	0	0		0
HER2 amplification[c]	70 (11.5)	10 (19.6)	37 (12.3)	17 (9.8)	6 (7.1)	**0.028[b]**	9 (16.1)
Missing	303	31	173	77	22		17
Triple negative tumors	51 (8.4)	20 (39.2)	26 (8.7)	3 (1.7)	2 (2.4)	**<0.0001[b]**	5 (8.9)
Missing	303	31	173	77	22		17
Ki67[e]							
Continuos	16 (10-28)	33 (17-55)	16 (10-28)	14 (8-22)	13 (9-19)	**<0.0001[d]**	15 (9-28)
≥20%	134 (36.0)	25 (71.4)	73 (38.0)	25 (27.2)	11 (20.8)	**<0.0001[b]**	10 (37.0)
Missing	539	47	281	158	53		46
Treatments by last follow-up prior to any event[f]							
Chemotherapy	232 (25.5)	42 (51.2)	128 (27.1)	42 (16.8)	20 (18.9)	**<0.0001[b]**	18 (2.7)
Radiotherapy	580 (63.7)	57 (69.5)	295 (62.4)	158 (63.2)	70 (66.0)	0.61[a]	42 (57.5)
Tamoxifen[g]	483 (60.4)	31 (72.1)	249 (59.4)	139 (58.4)	64 (64.0)	0.31[a]	32 (48.5)
Aromatase inhibitors[g]	313 (39.1)	15 (34.9)	169 (40.3)	87 (36.6)	42 (42.0)	0.66[a]	23 (34.8)
Trastuzumab[h]	55 (78.6)	8 (80.0)	32 (86.5)	9 (52.9)	6 (100)	**0.022[a]**	6 (66.7)
Type of event							
Any breast cancer event	110 (12.1)	15 (18.3)	58 (12.3)	31 (12.4)	6 (5.7)		6 (8.2)
Distant metastasis	69 (7.6)	9 (11.0)	38 (8.0)	18 (7.2)	4 (3.8)		4 (5.5)
Death	88 (9.7)	13 (15.9)	46 (9.7)	23 (9.2)	6 (5.7)		6 (8.2)

[a] Chi-square, [b] Linear-by-Linear Association [c] HER2 was not routinely analzyed until November 2005. Patients included before November 2005 were therefore considered missing, n=260. HER2 was missing for an additional 43 patients. [d] Jonckheere-Terpstra. [e] For patients included as of March 2009 [f] Most patients received more than one type of treatment [g] In patients with ER+ tumors [h] Trastuzumab is presented for patients included as of November 2005 with HER2 positive tumors. ER = estrogen receptor, PgR = progesterone receptor, HER2= Human epidermal growth factor receptor.
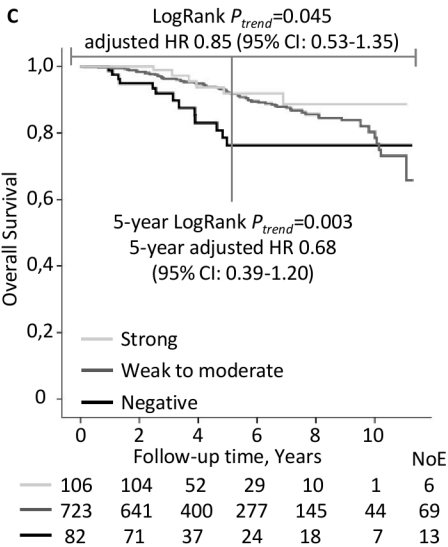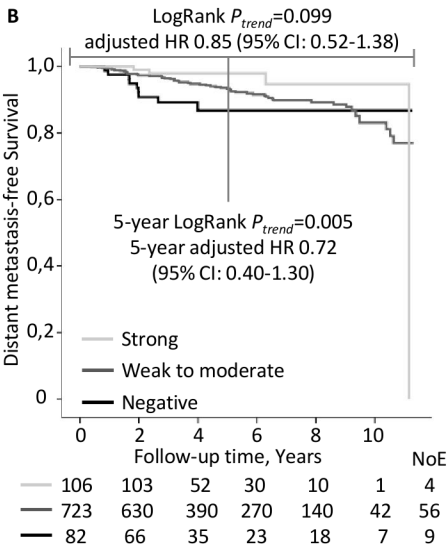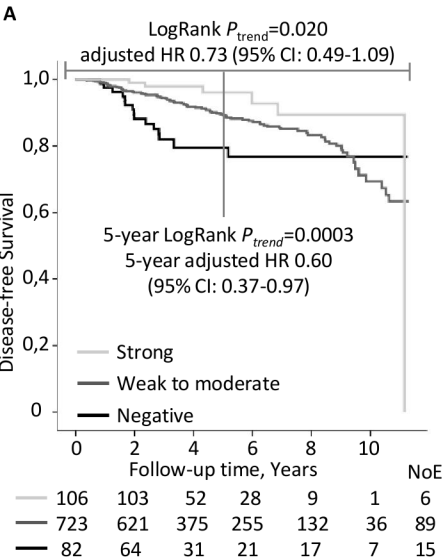
Figure 2

Table 3. Multivariable models

	Entire follow-up period			First five years of follow-up		
	HR	**95% CI**	*P*-value	**HR**	**95% CI**	*P*-value
COX-2 (negative/weak to moderate/high)	0.73	0.49-1.09	0.12	0.60	0.37-0.97	0.037
Age at diagnosis (≥50 years)	0.66	0.44-0.99	0.044	0.56	0.35-0.90	0.017
ER-status (positive)	0.47	0.28-0.79	0.004	0.44	0.24-0.80	0.44
Histological grade (I-III)	0.99	0.72-1.37	0.97	1.19	0.81-1.76	0.38
Invasive tumor size (≥21 mm or skin or muscular involvement)	2.02	1.36-3.00	0.001	1.91	1.19-3.08	0.008
Any axillary lymph node involvement (≥1)	1.57	1.07-2.32	0.023	1.93	1.20-3.10	0.007

CI = Confidence interval, COX-2 = Cyclooxygenase 2, HR = Hazard ratio