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**COX2 expression in prognosis and in prediction to endocrine therapy in early breast cancer patients**

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This study has never been presented elsewhere.

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1

2 **Key words**

3 Early breast cancer; COX2; prognostic and predictive factors; endocrine therapy

4

**ABSTRACT**

**Introduction** In breast cancer, the prognostic impact of COX2 expression varies widely between studies. We examined the prognostic value of COX2 expression in a large cohort of breast cancer patients treated with primary surgery between 1985 and 1994 and explained the variable results of COX2 expression found in the literature.

**Methods** A tissue microarray was constructed of available tumour material and ER, PgR, HER2, Ki67 and COX2 were examined by immunohistochemistry.

**Results** Median follow-up was 19 years. Fifty-five percent (n=369/677) of patients received no systemic treatment. COX2 was scored using a weighted histoscore. Analysis of COX2 expression in two groups based on the median (148; below versus above) showed an increased hazard ratio (HR) of 1.35 (95%CI 1.05-1.75, p=0.021) for disease free survival (DFS) and of 1.39 (95%CI 1.03-1.82, p=0.016) for overall survival (OS). However, COX2 did not remain independent in multivariate analysis. In patients with hormone receptor positive tumours, COX2 expression had a negative influence on outcome (low versus high: DFS: HR 1.37, 95%CI 1.07-1.76, p=0.013). This effect disappeared when endocrine therapy was administered (low versus high: DFS: HR 0.93, 95%CI 0.51-1.70, p=0.811) while it remained statistically significant when endocrine therapy was omitted (low versus high: DFS: HR 1.48, 95%CI 1.12-1.94, p=0.005).

**Conclusions** Our results show that COX2 plays a role in hormonal pathways. Our results can explain the results found in previous published studies.

## 1 INTRODUCTION

2  
3 Breast cancer is one of the most commonly diagnosed cancers and the leading cause of death from cancer in  
4 women in the western world [1]. Systemic treatment improves disease free survival (DFS) and overall survival  
5 (OS) in patients with early breast cancer [2]. The indication for systemic treatment is based on prognostic and  
6 predictive factors [3]. Prognostic factors estimate the patient's risk of relapse in the absence of systemic therapy,  
7 and include, amongst others, age at diagnosis, tumour grade, and lymph node status. Predictive factors estimate  
8 the responsiveness of a tumour to a specific treatment, for example expression of oestrogen receptor (ER) for  
9 endocrine therapy and overexpression/amplification of human epidermal growth factor receptor 2 (HER2) for  
10 trastuzumab. Prognostic and predictive factors are increasingly important as the relative risk of death from breast  
11 cancer is decreased by early diagnosis and improved treatment. Moreover, a substantial proportion of patients  
12 with early breast cancer may survive without adjuvant endocrine and/or chemotherapy. Novel markers are  
13 needed to further subcategorize patients for different systemic treatment regimens.

14  
15 Several epidemiologic and observational studies have examined the relationship between non-steroidal anti-  
16 inflammatory drugs (NSAIDs) and early breast cancer. In the prospective Women's Health Initiative  
17 Observational Study, regular use of NSAIDs was significantly correlated with a reduction in the incidence of  
18 breast cancer [4]. Furthermore, the findings of a meta-analysis of 14 studies suggest that use of NSAIDs may be  
19 associated with a small decrease in breast cancer risk [5]. The main target of NSAIDs is cyclooxygenase (COX),  
20 which exists in two isoforms: COX1 and COX2, which are regulated independently. COX1 is expressed in  
21 normal tissue whereas COX2 is expressed in various human malignancies, like colon and breast cancer. COX2  
22 catalyzes the conversion of arachidonic acid into prostaglandins and increased levels of prostaglandins are  
23 associated with carcinogenesis [6].

24  
25 A number of studies have examined the impact of COX2 expression in early breast cancer patients [7-22]. The  
26 results of these studies vary widely, which may be partly explained by the fact that most studies did not stratify  
27 patients according to systemic therapy. Moreover, in the majority of these studies, patients received some form  
28 of systemic therapy so reliable data on the prognostic impact of COX2 in the absence of systemic therapy is  
29 lacking. This might be important, especially for endocrine therapy, as COX2 catalyzes the conversion of  
30 arachidonic acids into prostaglandins which stimulates aromatase and thus formation of estrogens [23].

We examined the prognostic value of COX2 expression in a cohort of operable breast cancer patients in the Leiden University Medical Centre (LUMC) between 1985 and 1994. About one third of this group was treated with adjuvant endocrine or chemotherapy. We performed stratified analyses to systemic treatment and assessed the relationship between COX2 expression and established prognostic clinicopathological parameters. We used the guidelines for the reporting of tumour marker studies (REMARK) [24].

## **PATIENTS AND METHODS**

### **Patients**

The patient population was a consecutive series of all women with non-metastatic breast cancer who received a primary surgical resection (with or without radiotherapy) in the LUMC between 1985 and 1994. Patients with a prior history of cancer (other than basal cell carcinoma or *in situ* carcinoma) or bilateral tumours were excluded. Age at diagnosis, tumour grade, morphology, TNM stage, local and systemic therapy, locoregional/distant recurrence, second primaries and death were recorded. All tumours were histologically classified and graded by one pathologist (VS). Approval was obtained from the LUMC Medical Ethics Committee.

### **Preparation of tumour tissue microarrays**

Formalin fixed paraffin-embedded tumour blocks of the primary tumour were collected of the pathology department. All blocks were stored at room temperature. Sections were cut for haematoxylin and eosin staining and histopathologically representative tumour regions were indicated by a pathologist (MvdV) and used for preparation of tumour tissue microarray (TMA) blocks [25]. From each donor block, three 0.6 mm<sup>2</sup> tissue cores were punched from tumour areas and transferred into a recipient paraffin block using a custom-made precision instrument. Four µm thick sections of the TMAs were cut and processed for immunohistochemistry.

### **Immunohistochemistry**

Tissues were dewaxed in xylene and rehydrated in a graded alcohol series. Antigen retrieval was carried out for 5 minutes in a pressure cooker in 10 mmol/L sodium-citrate buffer (pH 6.0). Slides were immersed in 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity and in a blocking solution (DAKO protein free serum block) for 60 minutes to block nonspecific binding sites. Immunostaining was performed with COX2 (antihuman) monoclonal antibody (160112; Cayman Chemical Co., Ann Arbor, MI) diluted in antibody

diluent (DAKO) at a concentration of 1:100 for 30 minutes at room temperature. One tumour TMA was stained with and without antibody as a positive and negative control, respectively. Sections were treated with envision (DAKO) and visualization was performed using DAB (DAKO). Finally, tissues were counterstained with haematoxylin and dehydrated through graded alcohols and xylene. Immunostaining for ER, progesterone receptor (PgR), Ki67 and HER2 was carried out using established procedures with the following antibodies: NCL-L-ER-6F11 (Novocastra), monoclonal mouse antihuman progesterone receptor Clone PgR 636 (M3569, DAKO), monoclonal mouse antihuman ki67 antigen Clone MIB-1 (M7240, DAKO) and HercepTest™ for DAKO autostainer (K5207 DAKO) respectively [26].

### **Evaluation of immunostaining**

It has been shown that TMAs with 2 cores from each tumour is a valuable and accurate method for analysis of protein expression in large archival cohorts and correlates highly with whole-section staining [27,28]. COX2 immunohistochemical staining was scored independently and in a blind manner using a weighted histoscore. The proportion of cells with cytoplasmic staining was multiplied by the intensity of staining to provide a score of 0 to 300. Score = (0 x percentage of cytoplasm not stained) + (1 x percentage weakly stained) + (2 x percentage moderately stained) + (3 x percentage strongly stained) [29]. The first investigator (JvN) scored all cores and the second investigator (CF) scored 33% of cores to ensure consistency, both in a blind manner. The interclass correlation coefficient of cores scored was excellent: 0.928 [30]. The intra-observer variability analysed using the cohen's kappa coefficient was 0.87. All tumours with discordant scores were re-evaluated by both investigators. The mean score of all cores scored by the first investigator was used for analysis.

ER and PgR immunohistochemical staining was scored using a weighted histoscore generated by an automated image analysis machine, the Ariol SL-50 Image Analysis System. The suitability of this system has been reported previously [31,32]. Tumour epithelium was marked for analysis, and marked areas quality assured by a pathologist (DF). The system was trained on the basis of the cellular characteristics of epithelial, stromal, and infiltrating non-tumourous cells to produce a trained classifier applicable to all nuclear stains. A positive hormone receptor status was defined as a histoscore of at least 10. Ki67 expression (percentage of positive cells) was also evaluated using the Ariol System. There are different cut points when using Ki67, we used 5. HER2 staining intensity was graded (by DF) in accordance with the HercepTest protocol system as 0, 1+, 2+ or 3+.

Samples scored as 0, 1+ or 2+ were considered negative for HER2 overexpression, 3+ was considered to be positive.

#### **Study design**

The design comprises a retrospective cohort study (1985-1994). No stratification or matching was used. The end of follow-up period is 1 January 2009 or death or date of lost of follow-up. Objectives of this retrospective analyses are (independent) correlation of COX2 with survival, relation with systemic therapy and relation with established prognostic clinicopathological parameters

#### **Statistical Analysis**

All data was analysed using the statistical package SPSS for Windows 15.0 (SPSS Inc, Chicago, IL, USA). Descriptive data are given as mean (+/- standard deviation, SD) or median (range). DFS was calculated from the date of surgery up to the first date of locoregional or distant recurrence, contralateral breast cancer or death by any cause, whichever came first. OS was defined from the date of surgery up to the date of death from any cause.

To examine if COX2 expression was correlated with DFS and OS, univariate Cox analyses were performed. COX2 expression was normally distributed within the population, for statistical analysis samples were divided into two equal groups (high and low) based on the median histoscore, this cut off was selected to maximise statistical power. Multivariate analyses were performed using the Cox proportional hazards model entering COX2 expression with other significant variables (defined as those with  $p < 0.1$ ) on univariate analysis. The relationship between COX2 expression and established prognostic factors was investigated using the chi-square test without Bonferroni correction. All testing was two-tailed with 0.05 as level of significance [33]. Missing data were not used for analyses.

### **RESULTS**

#### **Patients**

A total of 667 patients with non-metastatic breast cancer were treated with a primary surgical resection in the LUMC during the study period. TMAs were made from available paraffin tumour blocks from 574/667 (86%) patients. Of these, 369/574 (64%) patients received no systemic treatment, the remaining 205 patients received a



mixture of endocrine and/or chemotherapy (Figure 1). The median follow-up for patients alive was 19 years (range 0-23). Clinicopathological and local treatment characteristics are shown in Table 1.

#### **Distribution of prognostic factors**

ER staining was successful in 94% of tumours (cores were missing, folded or contained no invasive tumour in 6%). Median tumour ER histoscore was 45.77 (range 0-254). PgR staining was successful in 93% of tumours; median tumour PgR histoscore was 25.59 (range 0-300). The interquartile ranges for ER and PgR were 132 and 126 respectively. HER2 expression was successful in 74% of tumours. Overexpression of HER2 was seen in 10%, 90% of tumours had no HER2 overexpression. Ki67 staining was successful in 93%; median tumour Ki67 expression was 2.20 (0-63) and the interquartile range was 6.19.

#### **COX2 expression in tumour tissue**

In tumour cells, COX2 staining was observed in the cytoplasm with a granular staining pattern (Figure 2). COX2 staining was successful in 88% of tumours (cores were missing, folded or contained no invasive tumour in 12%). COX2 expression was available for 316/369 (86%) cases with no systemic treatment and for 188/205 (92%) cases receiving systemic treatment (Figure 1), no significant differences were observed between cases with data available for COX2 expression and those for whom data was not available with respect to conventional prognostic markers (data not shown). Median tumour COX2 histoscore was 148.33 (range 3-278), meaning high COX2 had a histoscore >148 and low COX2 a histoscore ≤148.

#### **Prognostic value of COX2 expression**

To analyse the prognostic value of COX2 we analysed data from the patients who were treated with local therapy only (Table 2, Figure 3). In univariate analysis, high COX2 expression resulted in a worse DFS (HR 1.35, 95%CI 1.05-1.75, p=0.021) and more deaths (HR 1.39, 95%CI 1.06-1.82, p=0.016) compared to low COX2 expression. In multivariate analysis, COX2 expression did not provide prognostic information independently of other prognostic variables for DFS (HR 1.07 (95%CI 0.81–1.41), p=0.633) or OS (HR 1.03 (95%CI 0.77– 1.37), p=0.849). For DFS, only age, nodal status, tumour grade and surgical therapy were statistically significant factors in multivariate analysis. Age, nodal status and surgical therapy were independent prognostic variables for OS.

## COX2 expression in the hormonal pathway

To investigate the influence of COX2 expression on survival in relation to hormone receptor and endocrine therapy we first investigated COX2 expression in patients with hormone receptor negative tumours (Figure 4). In these patients, COX2 expression had no influence on overall survival (log-rank  $p=0.593$ ). In univariate analyses of patients with hormone receptor positive tumours, however, low COX2 expression was associated with better survival compared to a high COX2 expression (DFS: HR 1.37 95%CI 1.07-1.76,  $p=0.013$ ; OS: HR 1.44, 95%CI 1.11-1.86,  $p=0.006$ ). Subsequently, within patients with hormone receptor positive tumours, we analysed the effect of endocrine therapy administration in relation to COX2 expression (Figure 5). COX2 expression levels did not differentiate survival in patients treated with endocrine therapy in univariate analyses (low versus high: DFS: HR 0.93, 95%CI 0.51-1.70,  $p=0.811$ ; OS: HR 0.91, 95%CI 0.50-1.68,  $p=0.767$ ). In contrast, within patients who did not receive endocrine therapy, COX2 expression had an influence on outcome (low versus high: DFS: HR 1.48, 95%CI 1.12-1.94,  $p=0.005$ ; OS: HR 1.58, 95%CI 1.18-2.10,  $p=0.002$ ). In univariate analyses. In multivariate analyses, only age, nodal status and surgical therapy were independent prognostic factors. Nevertheless, these results suggest that the clinical impact of COX2 expression in breast cancer patients is closely related to the ER status of the tumour.

## COX2 expression and clinicopathological parametres

Increased COX2 expression was observed with increasing grade and stage ( $p<0.0001$ ), and tumours from older patients exhibited increased COX2 expression ( $p<0.004$ ; Table 3). Both in ER negative and PgR negative tumours exhibited higher COX2 expression levels than hormone receptor positive tumours (Table 3). There was no significant correlation between Ki67 or HER2 expression and COX2 expression.

## DISCUSSION

In breast cancer, a number of studies have explored the relationship between COX2 expression and clinical outcome (Table 4) [7-22]. However, to the best of our knowledge, this is the first study to investigate and assess the true impact of COX2 expression on prognosis in breast cancer (i.e. outcome in the absence of systemic therapy). In the current study, elevated levels of COX2 expression were associated with an increased risk of relapse and death in the absence of endocrine or chemotherapy in univariate analysis. However, in multivariate analysis, COX2 expression did not provide additional prognostic information over conventional markers like age and lymph node status. Therefore, our data do not support a role for COX2 expression as a prognostic marker

1 independent of conventional clinicopathological criteria. This contradicts much of the published literature (see  
2 below). Although the number of patients in the untreated group in the current study is relatively small (369), the  
3 hazard ratio for COX2 expression in the multivariate analysis (1.07 for DFS and 1.03 for OS) approaches one.  
4 This suggests that even in a significantly larger patient group, COX2 expression would not provide statistically  
5 significant additional prognostic information.

6  
7 COX2 plays an evident role in the hormonal pathway. We hypothesised that COX2 expression is associated with  
8 a worse outcome in patients with hormone receptor positive tumours compared to patients with hormone  
9 receptor negative tumours due to the relationship between COX2 and aromatase expression. In addition, we  
10 suggested that administration of endocrine therapy would abolish this prognostic effect of COX2 expression  
11 within patients with hormone receptor positive tumours. In patients with hormone negative disease, COX2 had  
12 no influence on survival. In addition, both Kaplan Meier survival analyses and Cox regression analyses showed  
13 that COX2 expression had no additional effect in patients with hormone receptor positive tumours who received  
14 endocrine therapy. However, in the subgroup of patients in which endocrine therapy was not administrated,  
15 COX2 expression was of prognostic significance. These findings suggest that COX2 plays a role in the hormonal  
16 pathway in breast cancer. Additionally, these findings suggest that endocrine therapy could be omitted in patients  
17 who express of low levels of COX2 with hormone sensitive tumours at a low risk according to current risk  
18 stratification. However to prove this, a randomised controlled trial is needed.

19  
20 We reviewed 16 previous studies examining the immunohistochemical expression of COX2 expression in breast  
21 tumours (Table 4) [7-22]. Cross-comparison between studies and our data was not easy since many studies  
22 included very small numbers of tumours, or had short median follow-up (2-9 years) and there was a lack of  
23 standardization of COX2 analysis. Most studies used the same antibody (Cayman Chemical Co., Ann Arbor,  
24 MI), but different concentrations and incubation times. Also, different methods for evaluation of COX2  
25 expression were used, for example, Spizzo *et al* examined COX2 expression using the product of the percentage  
26 of stained cells and the intensity, whilst Ristimaki *et al* compared just the intensity of stained cells [16,20]. We  
27 used the weighted histoscore because this combines the intensity as well as the percentage of cells stained.  
28 Finally, in published studies COX2 “positivity” was seen in a range of 5-85% of cases. However, arbitrary  
29 distinctions between COX2 positive and COX2 negative cases were used. COX2 expression was defined as any  
30 positive staining by Schmitz *et al*, in contrast to Park *et al* where at least 80% of cells needed to be stained before

1 the tumour was classified positive [15,17]. This diversity in methods used for staining, interpretation and  
2 analysis of COX2 expression in previous studies complicates comparisons between studies.

3  
4 In spite of the diversity of methods discussed above, we suggest our results can provide a unifying explanation  
5 of the results found in the other studies. Denkert *et al* showed a correlation with COX2 expression and DFS in  
6 multivariate analysis [9]. However, 63% of tumours in Denkert's study were ER positive (PgR was not shown)  
7 and only 23% of patients received endocrine therapy. Therefore, at least 40% of this population was hormone  
8 receptor positive and did not receive endocrine therapy. Similar results were seen in the study of Zerkowski *et al*  
9 where at least 70% of tumours were hormone receptor positive and only 57% of patients received endocrine  
10 therapy. The three largest studies, included a majority of patients treated prior to 1998 [10,16,22]. We regard this  
11 as important because results reported by the Early Breast Cancer Trialist' Collaborative Group (following meta-  
12 analysis of randomised trials of endocrine therapy in early breast cancer patients) showed a correlation between  
13 tumour ER expression and response to endocrine therapy[34]. Prior to this report, endocrine therapy was given  
14 on the basis of menopausal status; following the publication of the meta-analysis, endocrine therapy was given  
15 based on hormonal status. We believe that the existence of cohorts of ER positive patients who did not received  
16 endocrine therapy can explain the results of the earlier studies This may also explain the similarity between our  
17 findings and those of Haffty *et al* who also showed that COX2 expression was correlated with survival in  
18 hormone receptor positive tumours, but not in hormone receptor negative tumours [10]. This hypothesis is  
19 readily testable by future studies exploring the impact of COX2 expression in ER positive patients stratified by  
20 treatment with endocrine therapy.

21  
22 In conclusion, in patients who did not receive systemic treatment, increased COX2 expression was not  
23 independently prognostic for an increased risk of relapse and death from breast cancer after correction for  
24 current clinicopathological markers. COX2 expression was prognostic in patients with hormone receptor positive  
25 breast cancer who did not receive endocrine therapy. Studies measuring the impact of COX2 expression in the  
26 context of systemic therapies should be reinterpreted in light of this finding and a randomised controlled trial is  
27 needed to prove our results.

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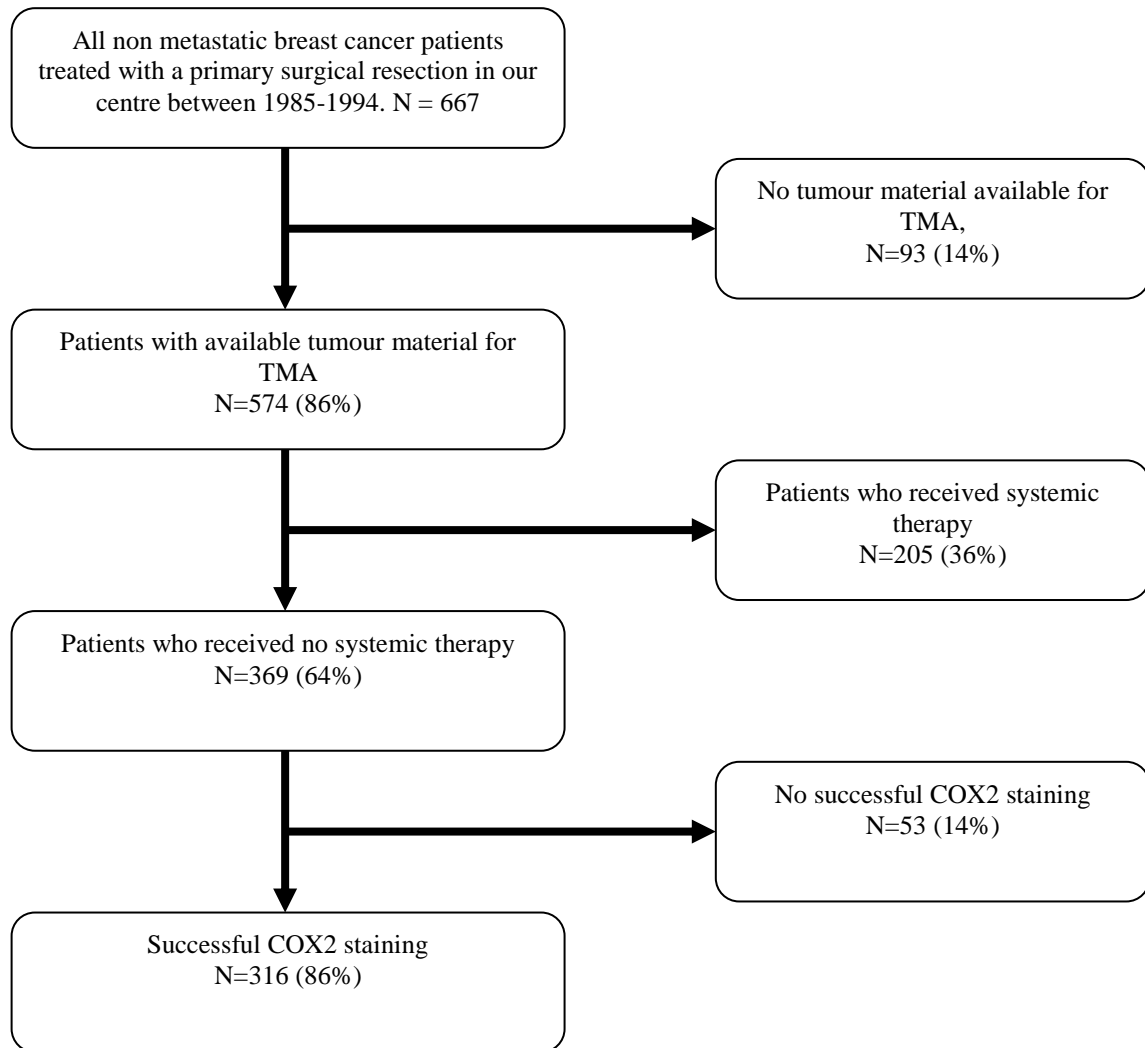
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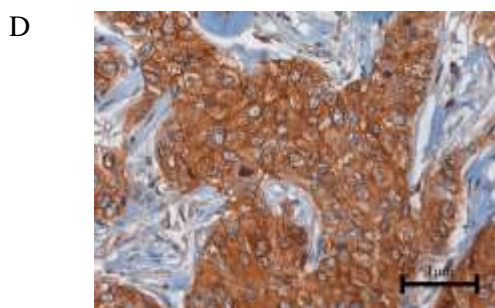
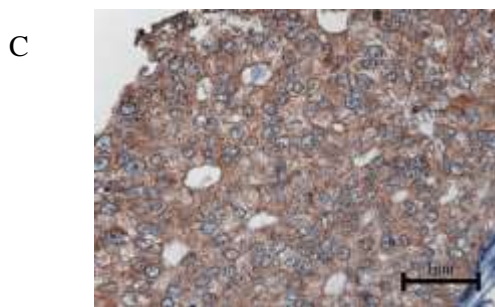
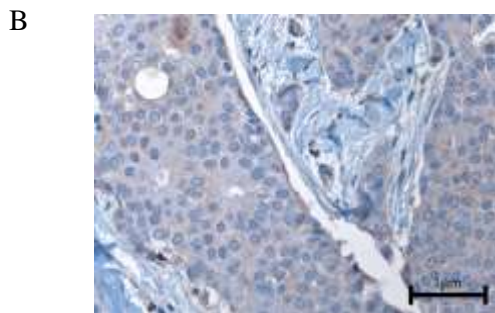
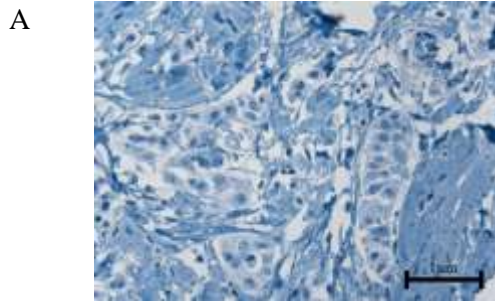
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## FIGURES AND TABLES

**Figure 1** Patient selection illustrated in a diagram

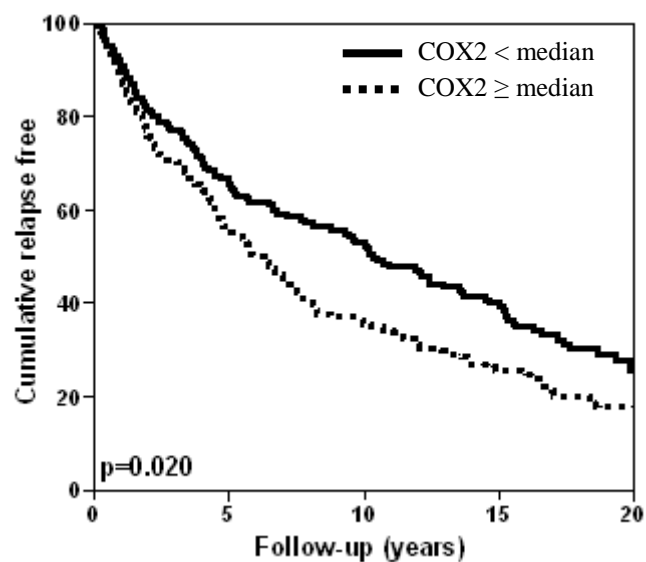


**Figure 2** COX2 staining: (A) not stained; (B) largely weakly stained; (C) largely moderately stained and (D) largely strong stained.



**Figure 3** Kaplan Meyer for (A) disease free survival and (B) overall survival. COX2 expression is divided into two groups based on the median histoscore of 148.

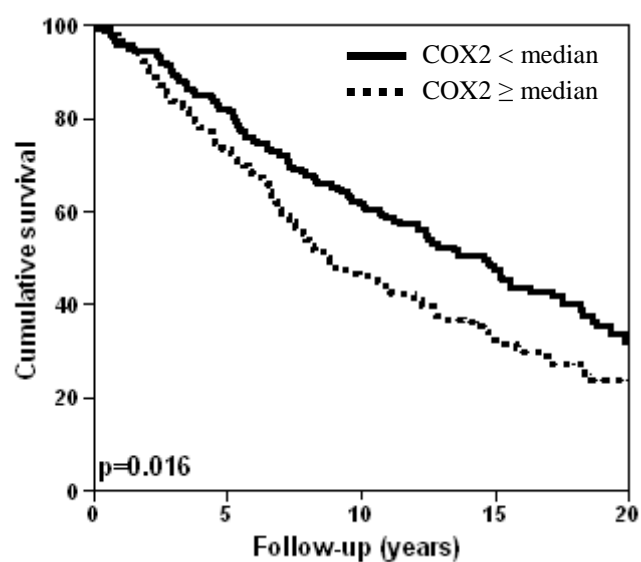
(A)



*Patients at risk*

COX2<median	162	107	84	53	14
COX2≥median	154	85	54	33	9

(B)

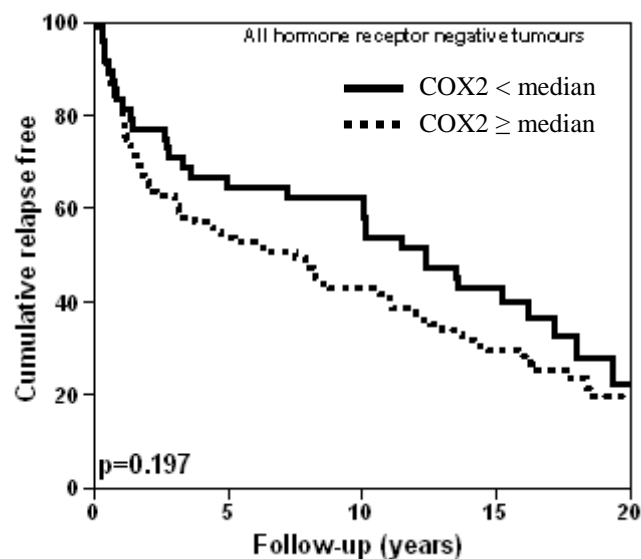


*Patients at risk*

COX2<median	162	133	99	63	19
COX2≥median	154	113	70	41	12

**Figure 4** COX2 expression and survival in all patients with hormone receptor negative tumours. Kaplan Meyer A and B show the disease free survival and overall survival respectively. COX2 expression is divided into two groups based on median histoscore of 148.

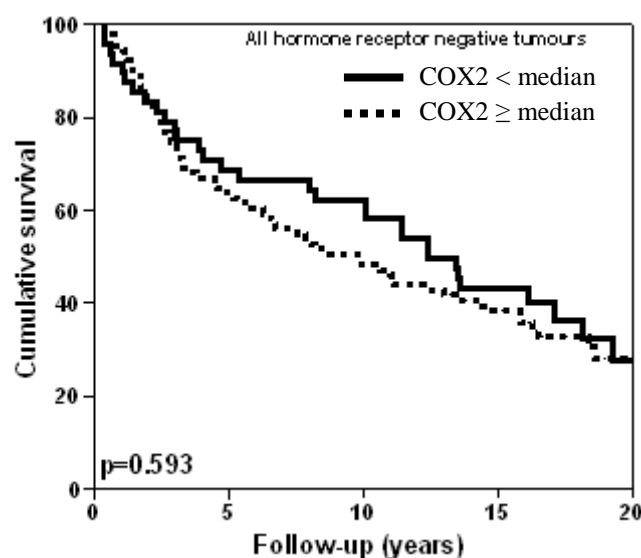
(A)



*Patients at risk*

COX2<median	48	31	29	14	4
COX2≥median	91	49	39	25	5

(B)

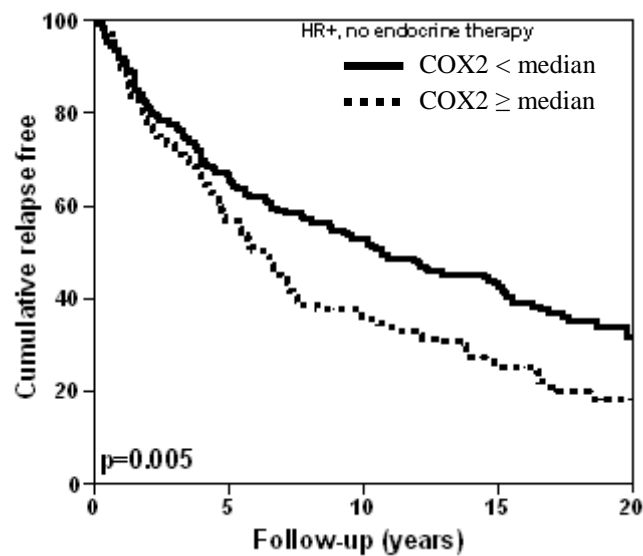


*Patients at risk*

COX2<median	48	39	29	15	6
COX2≥median	91	58	44	31	7

**Figure 5** COX2 expression and survival in all patients with hormone receptor positive tumours. Kaplan Meyer A and B show the disease free survival and overall survival for patients who did not receive endocrine therapy and C and D for whom who did. HR = hormone receptor.

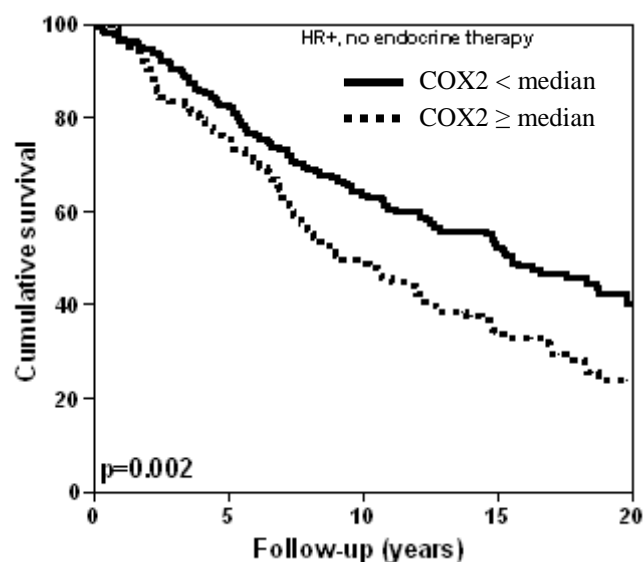
(A)



*Patients at risk*

COX2<median	168	112	88	63	16
COX2≥median	127	72	45	26	8

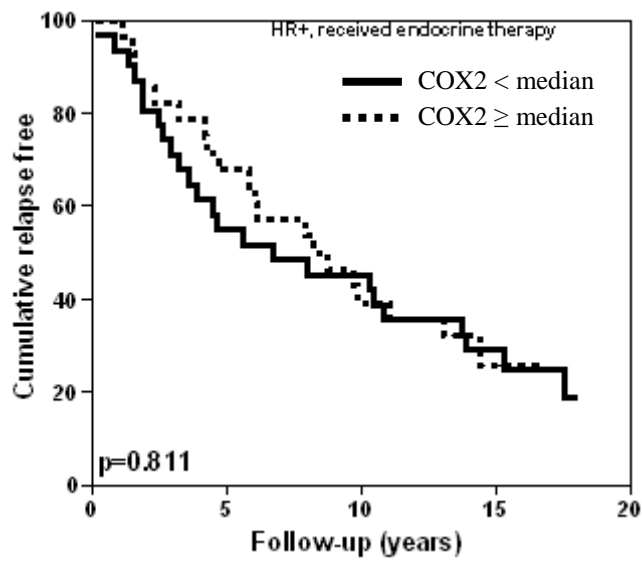
(B)



*Patients at risk*

COX2<median	168	139	106	76	21
COX2≥median	127	96	61	35	10

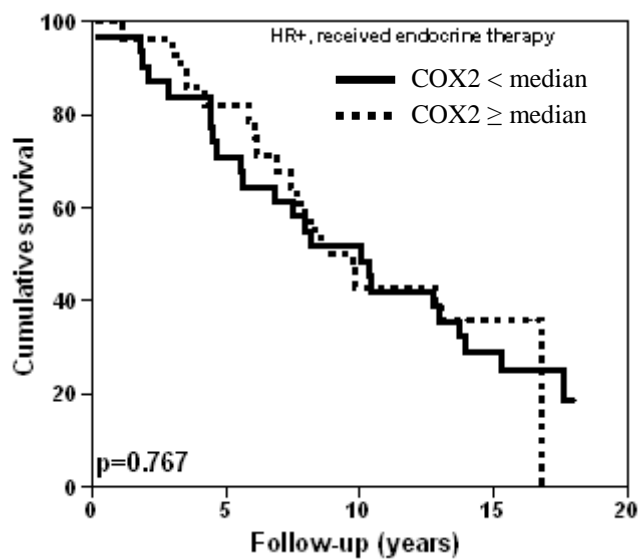
1 (C)

2 *Patients at risk*

COX2<median	31	17	14	7	0
COX2≥median	28	19	11	3	0

3

4 (D)

5 *Patients at risk*

COX2<median	31	22	16	7	0
COX2≥median	28	23	12	5	0

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**Table 1.** Clinicopathological and survival data of all patients with non metastatic breast cancer treated with a primary surgical resection in our centre between 1985 and 1994.

	All patients		Patients not on TMA*		Patients on TMA		On TMA, no systemic Therapy	
	N	%	N	%	N	%	N	%
<b>Age (years)</b>								
Median (range)	57	23-96	52	26-85	57	23-96	59	29-96
Mean (SD)	57	14	52	13	58	14	60	14
<40	66	10	18	19	48	8	22	6
40-50	169	25	24	26	145	25	85	23
51-60	157	24	25	27	132	23	84	23
>60	275	41	26	28	249	43	178	48
<b>Extent of disease</b>								
Early breast cancer	547	82	82	88	465	81	304	82
Locally advanced	120	18	11	12	109	19	65	18
<b>Tumour stage</b>								
pT1	269	40	58	62	211	37	157	43
pT2	295	44	23	25	272	47	165	45
pT3/4	78	12	6	7	72	13	38	10
pTmissing	25	4	6	7	19	3	9	2
<b>Grade</b>								
I	87	13	7	8	80	14	50	14
II	298	45	16	17	282	49	194	53
III	209	31	6	7	203	35	117	32
Missing	73	11	64	69	9	2	8	2
<b>Histological type</b>								
Ductal	541	81	28	30	513	89	329	89
Other	54	8	1	1	53	9	33	9
Missing	72	11	64	69	8	1	7	2
<b>Nodal stage</b>								
pN0	365	55	59	63	307	54	265	72
pN+	302	45	34	37	267	47	104	28
<b>Oestrogen receptor</b>								
Negative	unknown		unknown		203	35	126	34
Positive	unknown		unknown		337	59	221	60
Missing	unknown		unknown		34	6	22	6
<b>Progesterone receptor</b>								
Negative	unknown		unknown		223	39	138	37
Positive	unknown		unknown		313	55	204	55
Missing	unknown		unknown		38	7	27	7
<b>HER2</b>								
0+/1+/2+	unknown		unknown		387	66	248	67
3+	unknown		unknown		44	8	23	6
Missing	unknown		unknown		152	27	98	27
<b>Ki67 expression</b>								
<5	unknown		unknown		360	63	231	63
>5	unknown		unknown		176	31	112	30
Missing	unknown		unknown		38	7	26	7
<b>COX2</b>								
Low: <148	unknown		unknown		254	44	162	44
High: >148	unknown		unknown		250	44	154	42
Missing	unknown		unknown		70	12	53	14
<b>Local treatment</b>								
MST without RT	250	38	27	29	223	39	149	40
MST with RT	122	18	14	15	108	19	58	16
BCS without RT	5	1	0	0	5	1	4	1
BCS with RT	290	44	52	56	238	42	158	43
<b>Systemic treatment</b>								
Chemotherapy alone	134	20	22	24	112	20	0	0
Endocrine therapy alone	83	12	8	9	75	13	0	0
Both	20	3	2	2	18	3	0	0
None	430	65	61	66	369	64	369	100
<b>Survival data</b>								
Local recurrence	73	11	19	20	54	9	38	10
Regional recurrence	22	3	4	4	18	3	9	2
Distant recurrence	274	41	31	33	243	42	148	40
Contralateral breast cancer	54	8	10	11	44	8	33	9
Death	413	62	43	46	370	65	240	65
<b>Total</b>	<b>667</b>	<b>100</b>	<b>93</b>	<b>100</b>	<b>574</b>	<b>100</b>	<b>369</b>	<b>100</b>

1  
2 **Abbreviations** BCS: breast conserving surgery; MST: mastectomy; RT: radiotherapy; TMA: tissue micro array.  
3 Locally advanced breast cancer are all T3 and T4 cancer or any tumour stage with N2 or N3 disease. Numbers  
4 and percentage are calculated on available data, missing data are not shown. \*If tumour of a patient is not on the  
5 TMA means that there was no (not enough) available tumour.  
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**Table 2.** Univariate and multivariate analysis of (A) disease free survival and (B) overall survival.

(A)

			UNI	UNI	UNI		MULTI	MULTI	MULTI
Characteristic		n	HR	95% CI	p-value		HR	95% CI	p-value
Age	<60	191	1.000		<0.001		1.000		0.002
	≥60	178	1.917	1.500 - 2.450			1.571	1.176 - 2.099	
Tumour stage	pT1	157	1.000		< 0.001		1.000		0.124
	pT2,3,4	203	1.903	1.479 – 2.449			1.228	0.933 – 1.780	
Nodal stage	pN0	265	1.000		< 0.001		1.000		< 0.001
	pN+	104	2.900	2.252 – 3.734			2.245	1.670 - 3.019	
Grade	I/II	244	1.000		0.001		1.000		0.040
	III	117	1.503	1.169 – 1.932			1.343	1.014 - 1.778	
Morphology	Ductal	329	1.000		0.373				
	Other	33	1.198	0.805 – 1.785					
ER	Negative	126	1.000		0.992				
	Positive	221	0.992	0.776 – 1.292					
PgR	Negative	138	1.000		0.300				
	Positive	204	0.877	0.683 – 1.125					
Ki67	<5	231	1.000		0.953				
	>5	112	0.992	0.763 – 1.290					
HER2	Negative	248	1.000		0.988				
	Positive	23	0.996	0.598 – 1.661					
COX2	<148	162	1.000		0.021		1.000		0.633
	>148	154	1.352	1.047 – 1.746			1.069	0.813 - 1.405	
Surgery	MST	207	1.000		< 0.001		1.000		0.014
	BCS	162	0.505	0.393 – 0.647			0.600	0.399 - 0.902	
Radiotherapy	No	152	1.000		0.010		1.000		0.080
	Yes	217	0.728	0.573 – 0.927			1.392	0.961 - 2.016	

**Abbreviations** BCS: breast conserving surgery; ER: oestrogen receptor; HER2: human epidermal growth factor receptor 2; MST: mastectomy; PgR: progesterone receptor.



1 (B)

			UNI	UNI	UNI		MULTI	MULTI	MULTI
Characteristic		n	HR	95% CI	p-value		HR	95% CI	p-value
Age	<60	191	1.000		<0.001		1.000		<0.001
	>=60	178	2.814	2.156-3.672			2.119	1.566-2.869	
Tumour stage	pT1	157	1.000		<0.001		1.000		0.175
	pT2,3,4	203	2.130	1.618-2.803			1.267	0.900-1.782	
Nodal stage	pN0	265	1.000		<0.001		1.000		<0.001
	pN+	104	2.809	2.159-3.655			2.268	1.676-3.069	
Grade	I/II	244	1.000		0.005		1.000		0.053
	III	117	1.457	1.117-1.899			1.337	0.997-1.793	
Morphology	Ductal	329	1.000		0.310				
	Other	33	1.245	0.816-1.900					
ER	Negative	126	1.000		0.548				
	Positive	221	0.921	0.705-1.204					
PgR	Negative	138	1.000		0.128				
	Positive	204	0.817	0.629-1.060					
Ki67	<5	231	1.000		0.700				
	>5	112	0.947	0.717-1.250					
HER2	Negative	248	1.000		0.769				
	Positive	23	1.080	0.647-1.804					
COX2	<148	162	1.000		0.016		1.000		0.849
	>148	154	1.390	1.062-1.818			1.028	0.770-1.373	
Surgery	MST	207	1.000		<0.001		1.000		0.016
	BCS	162	0.386	0.293-0.508			0.592	0.387-0.905	
Radiotherapy	No	152	1.000		<0.001		1.000		0.267
	Yes	217	0.579	0.449-0.747			1.240	0.848-1.814	

2

3 **Abbreviations** BCS: breast conserving surgery; ER: oestrogen receptor; HER2: human epidermal growth factor

4 receptor 2; MST: mastectomy; PgR: progesterone receptor.

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**Table 3** Correlation between COX2 expression in groups and well established prognostic factors using chi-square test. COX2 expression was divided into two groups based on the median histoscore: Histoscore <148: low COX2 expression; histoscore  $\geq$  148: high COX2 expression.

COX2			COX2		
Low			High		p-value
Age	n	%	n	%	0.004
<40	25	60	17	41	
40-50	78	63	46	37	
50-60	54	45	65	55	
>60	98	45	122	56	
Tumour stage					<0.001
pT1	115	65	63	35	
pT2,3,4	130	42	180	58	
Nodal stage					0.269
pN0	139	53	124	47	
pN+	116	48	126	52	
Grade					<0.001
I	48	75	16	25	
II	132	53	118	47	
III	71	39	112	61	
Morphology					0.317
Ductal	224	50	226	50	
Other	27	57	20	43	
Oestrogen receptor					0.015
Negative	82	43	108	57	
Positive	166	54	139	46	
Progesterone receptor					<0.001
Negative	82	39	127	61	
Positive	167	58	120	42	
Ki67					0.321
<5	162	48	173	52	
>5	85	53	75	47	
HER2					0.059
Negative	120	50	121	50	
Positive	7	30	16	70	

**Abbreviation** HER2: human epidermal growth factor receptor 2.

1 **Table 4.** Immunohistochemical studies of COX2 expression in invasive non metastatic breast cancer.

Study	Total	Median Follow-up	Kind of patients	Inclusion in	ER+	PgR+	Systemic therapy?	Tissue	COX2 Ab	Score of COX2	Definition COX2 positive	COX2 positive % (overexpr)	Pathological correlates	Clinical correlates
Boland <i>et al.</i> 2004 [7]	65	NR	Archival material of a single centre.	NR	60%	NR	NR	Whole section	Santa Cruz Biotechn, San Diego, CA; 1:100 AND Cayman Chemical Co, Ann Arbor, MI	0: no staining 1: weak 2: moderate 3:strong	2 or 3	41 (63%)	ER- Ki67>10% HER2+	NR
Costa <i>et al.</i> 2002 [8]	46	Min 2 years	Single centre, all patients treated with mastectomy	NR	61%	NR	NR	Whole section	Santa Cruz Biotechn, San Diego, CA; 1:10	Brown cytoplasmic staining.	Brown	8 (17%)	pN+ Sialyl-Tn expression Microvessel count Apoptotic index	Uni: DFS shorter (only 26 pts used). Not in multivariate
Denkert <i>et al.</i> 2003 [9]	221	87 months (2-132)	Archival material, of a single centre, 1991-1996, residents of Berlin	1991-1996	63%	NR	HT: 23%	Whole section	Cayman Chemical Co, Ann Arbor, MI; 1:1000	Product of percentage (5 groups:0-4) and intensity (0-3). Negative: 0-6 points, positive 7-12 points	7-12 points	80 (36%)	pN+ Larger tumour size Poor differentiation Vascular invasion ER- Morphology	Multivariate: DFS (HR 1.90, 95%CI 1.00-3.59, p= 0.049) and OS (RR 1.14, 95%CI 0.67-1.93, p= 0.64)
Haffty <i>et al.</i> 2008 [10]	504	7 years	Single centre, 1975-2003, all breast conserving therapy	1975-2003	53%	50%	CT: 32% HT: 38%	TMA	Cayman Chemical Co, Ann Arbor, MI; 1:50	0: no staining 1: weak 2: moderate 3:strong	2 or 3, if >10% staining	250 (58%)	Younger age Larger tumour size	Multivariate ER+ pts: RFS (RR 2.95, 95%CI 1.20-7.86, p=0.03), DR (RR 1.82, 95%CI 0.97-3.44, p=0.06) and OS (RR 2.22, 95%CI 1.15-4.26, p=0.02). ER-pts: no correlation with survival
Half <i>et al.</i> 2002 [11]	57	19 months	Archival material of a single centre.	1995-2002	63%	46%	NR	Whole section	Cayman Chemical Co, Ann Arbor, MI; 1:500	Product of percentage (5 groups: 0-4) and intensity (0-3): low (0-4), medium (5-8), high (9-12)	High	18 (43%)	None found	NR
Hwang <i>et al.</i> 1998 [12]	44	NR	NR	NR	NR	NR	NR	Whole section	Homemade anti-COX2, 1:50	NR	NR	2 (5%)	NR	NR
Kelly <i>et al.</i> 2003 [13]	106	23 months	Achieval material	NR	NR	NR	NR	Whole section	Cayman Chemical Co, Ann Arbor, MI; 1:500	Product of percentage (5 groups:0-4) and intensity (0-3)	none (0) low (1-4) medium (5-8) high (9-12)	90 (85%) (detected)	None found	None found
O'Connor <i>et al.</i> 2004 [14]	23	48 months	Single centre, all patients treated with mastectomy and radiotherapy	1994-2004	65%	NR	CT: 96% HT: NR	Whole section	Alexis Biochemicals, Montreal, Canada; 1:500	Computer-assisted scoring. Percentage of cells expressing COX2 and the level of COX2 staining intensity	>80% intensity >75% expression		Younger age (for percentage, not intensity)	Univariate: DFS and OS
Park <i>et al.</i> 2006 [15]	178	56 months (13-18)	Single center, all patients are N+ and treated with CT between 1994-1996	1994-1996	58%	NR	CT, FAC: 100% HT: ?	TMA	Cayman Chemical Co, Ann Arbor, MI; 1:200	Absence, weak, moderate or strong. Absence for immunoreactivity or weak staining intensity: discharged	>80% of observed cells are positive	39% (70)	Poor differentiation	Multivariate: DFS (RR 1.91, 95%CI 1.24-2.76, p=0.04) and OS (RR 1.73, 95%CI 1.16-2.14, p=0.03)
Ristimäki <i>et al.</i> 2002 [16]	1576	Median 6.8 years (5.1-7.8)	All patients diagnosed with breast cancer between 1991-1992 of 50% of the Finnish population.	1991-1992	55%	51%	HT: 57% of patients >50 CT: 37% of patients <50	TMA	Cayman Chemical Co, Ann Arbor, MI; 2.5 µg/ml	0: no staining 1: weak 2: moderate 10-90% 3:strong >90%	Score 2 or 3	589 (37%)	Large tumour size ER-, PgR-, HER2+ High ki67 and/or high p53 expression pN+ Tumour grade and type	Reduced DDFS, not in multivariate-

## COX2 expression &amp; breast cancer

Schmitz <i>et al.</i> 2006 [17]	113	7 years	Single centre, 1989-1996, all pN0	1989-1996	62%	NR	Yes, unknown percentage	Whole section	DCS, Hamburg, Germany; 1:250	Negative (no immunoreactivity) or positive	Positive staining.	43% (49)	Poor differentiation	Multivariate: disease specific OS (RR 3.51, 95%CI 1.10-11.21, p=0.03)
Singh-Ranger <i>et al.</i> 2004 [18]	30		Single centre	NR	NR	NR		Whole section	Rabbit IgG antibody to COX2, Oxford Biosciences; 1/NR	Product of percentage (5 groups:0-4) and intensity (0-3)	0: none 1-4: low 5-8: medium 9-12: high	11 (37%)	None	NR
Soslow <i>et al.</i> 2000 [19]	17	NR	Randomly, one centre	NR	NR	NR	NR	Whole section	COX-2 polyclonal (PG-27, Lot No. 0599; Oxford Biomedical research, Oxford, MI); 1/500	Product of percentage (5 groups:0-4) and intensity (0-3)	0: none 1-4: low 5-8: medium 9-12: high	7 (42%)	NR	NR
Spizzo <i>et al.</i> 2003 [20]	212	11 years	Single centre, 1980-1992	1980-1992	64%	56%	NR	Whole section	Cayman Chemical Co, Ann Arbor, MI; 1:100	Product of percentage (5 groups:0-4) and intensity (0-3)	Score >4	103 (49%)	Age Morfology Ep-CAM	Poor DFS and OS, not in multivariate
Witton <i>et al.</i> 2004 [21]	179	6 years (0-15)	Randomly selected, single centre, 1984 - 1993.	1984-1993	59%	NR	All systemic therapy	Whole section	Cayman Chemical Co, Ann Arbor, MI; 0.625 µg/ml	Weighted histoscore: exact percentage * intensity (0-3)	Histoscore > 129.25 based on 10 cases with adjacent normal breast epithelium.	38 (21%)	None significant found	Multivariate: In all pts: reduced DFS (p=0.02) next to ER, pN, tumour size. In ER- reduced disease related survival and time to relapse (both p=0.05) In ER+: no association
Zerkowski <i>et al.</i> 2007 [22]	669	9 years (0-53)	Patients treated in 1953-1983 from a single centre.	1953-1983	70%	55%	Full treatment information unavailable. Most local therapy +/- HT. CT in 10%	TMA	Cayman Chemical Co, Ann Arbor, MI; 1:250 ON incl polyclona AB for AQUA	Using automatic analysis by AQUA	By AQUA. Fluorescent: 19.3	(44%)	Low ER/PgR High grade	Multivariate high COX2 worse OS (RR 1.66, 95%CI 1.12-2.16, p=0.01).
Van Nes (current study)	369 (677)	19 years	Cohort, single institute	1985-1994	64%	60%	0%	TMA	Cayman Chemical Co, Ann Arbor, MI; 1:100	Weighted histoscore: exact percentage * intensity (0-3)	Above median histoscore	162 (51%)	Age Larger tumour size/stage Poor differentiation ER-, PgR-, HER2+	Reduced DFS and OS, not in multivariate

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Abbreviations: CT: chemotherapy; DDFS: distant disease free relapse; DFS: disease free survival; ER: oestrogen receptor; HT: hormone therapy; NR: not reported; PgR: progesterone receptor; OS: overall survival; RR: relative risk; TMA: tissue micro array