

# Impact of *COX2* genotype, ER status and body constitution on risk of early events in different treatment groups of breast cancer patients

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The *COX2* rs5277 (306G>C) polymorphism has been associated with inflammation-associated cancers. In breast cancer, tumor *COX-2* expression has been associated with increased estrogen levels in estrogen receptor (ER)-positive and activated Akt-pathway in ER-negative tumors. Our study investigated the impact of *COX2* genotypes on early breast cancer events and treatment response in relation to tumor ER status and body constitution. In Sweden, between 2002 and 2008, 634 primary breast cancer patients, aged 25–99 years, were included. Disease-free survival was assessed for 570 rs5277-genotyped patients. Body measurements and questionnaires were obtained preoperatively. Clinical data, patient- and tumor-characteristics were obtained from questionnaires, patients' charts, population registries and pathology reports. Minor allele(C) frequency was 16.1%. Genotype was not linked to *COX-2* tumor expression. Median follow-up was 5.1 years. G/G genotype was not associated with early events in patients with ER-positive tumors, adjusted HR 0.77 (0.46–1.29), but conferred an over 4-fold increased risk in patients with ER-negative tumors, adjusted HR 4.41 (1.21–16.02) ( $p_{\text{interaction}} = 0.015$ ). Chemotherapy-treated G/G-carriers with a breast volume  $\geq 850$  ml had an increased risk of early events irrespective of ER status, adjusted HR 8.99 (1.14–70.89). Endocrine-treated C-allele carriers with ER-positive tumors and a breast volume  $\geq 850$  ml had increased risk of early events, adjusted HR 2.30 (1.12–4.75). *COX2* genotype, body constitution and ER status had a combined effect on the risk of early events and treatment response. The high risk for early events in certain subgroups of patients suggests that *COX2* genotype in combination with body measurements may identify patients in need of more personalized treatment.

**Key words:** breast cancer, *COX2*, estrogen receptor, body constitution, treatment response

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Breast cancer is the most common cancer among women.<sup>1</sup> Despite an overall 5-year relative survival rate of almost 90% for breast cancer patients in Sweden,<sup>2</sup> it remains the primary cause of cancer death among women worldwide.<sup>1</sup> Resistance to available treatments is common and increases breast cancer mortality.<sup>3</sup> New prognostic and treatment-predictive markers that account for host factors may lead to more personalized breast cancer treatment and improved prognosis.

Accumulating evidence has suggested an association between inflammation and risk and prognosis of several cancers, including breast cancer.<sup>4,5</sup> Cyclooxygenase 2 (*COX-2*), the rate-limiting enzyme in the production of proinflammatory prostaglandins, induces inflammation as well as *cytochrome P450 19A1* (*CYP19A1*) aromatase gene transcription.<sup>6</sup> Tumor expression of *COX-2* promotes angiogenesis and treatment resistance, and may be one of the key steps in carcinogenesis.<sup>7,8</sup>

About 40% (range 17–89%) of invasive breast cancers express *COX-2*.<sup>9</sup> In breast cancer, *COX-2* expression has been associated with increased tumor grade and stage, human epidermal growth factor receptor-2 (HER-2) amplification, chemotherapy resistance<sup>10–12</sup> and a shorter disease-free survival.<sup>10,11</sup> *COX* inhibition has been associated with an

**What's new?**

In breast cancer, expression of the COX-2 gene means worse prognosis, and inhibiting COX-2 improves survival. But how does estrogen receptor (ER) figure into this? Could COX-2 expression affect tumors differently depending on ER status? Some evidence suggests that COX-2 spurs ER-negative tumors more than ER-positive ones. This team investigated the relationship between a patient's response to breast cancer treatment and COX2 genotype, ER status, and breast volume. They identified two subgroups of patients that responded poorly to treatment; knowing a patient's COX-2 genotype in concert with ER status and breast volume could help clinicians personalize treatment for higher risk individuals.

increased length of disease-free survival in breast cancer patients.<sup>13</sup>

COX-2 expression may have contrasting effects on tumors depending on the tumor's estrogen receptor (ER) status. COX-2-mediated prostaglandin *CYP19* activation increases aromatase and estrogen levels, which possibly stimulate the proliferation of ER-positive cancers.<sup>4,14</sup> COX-2 expression has been associated with more aggressive ER-negative tumors<sup>11,15</sup> and leads to the activation of the oncogenic Akt-pathway more often in ER-negative than in ER-positive tumors.<sup>15</sup> The relationship between COX-2 and ER status with respect to disease-free survival is unresolved.<sup>10,11,16</sup> However, one study reported that the worse prognosis for patients with COX-2-expressing ER-positive tumors may be neutralized by endocrine treatment.<sup>10</sup>

Body constitutions such as breast volume have been associated with inflammation and COX-2 expression. Overall obesity in women has been associated with breast tissue inflammation in breast cancer patients and increased COX-2 levels.<sup>14,17</sup> A large breast volume has been associated with a higher ratio of adipose tissue in breasts<sup>18,19</sup>; adipose tissue releases several proinflammatory factors, promoting local inflammation.<sup>20</sup>

COX2 polymorphisms have been associated with cancer risk, prognosis and breast tumor characteristics.<sup>21–23</sup> The synonymous COX2 rs5277 polymorphism (306G>C, V102V) has been directly associated with several cancers associated with inflammation, suggesting that it may play a role in COX-2-mediated inflammation.<sup>24–26</sup> Although studies have reported conflicting associations between rs5277 and cancer-related inflammation, two have found that G/G carriers have an increased risk of inflammation-associated cancers.<sup>23,27</sup> Female radiologic technologists with the G/G genotype were more likely to develop breast cancer after ionizing radiation exposure.<sup>27</sup> In addition, as reviewed by Pereira *et al.*, pooled data indicate that G/G carriers have an increased risk of colorectal adenomas.<sup>23</sup> Nevertheless, one meta-analysis found a borderline significant increased risk of breast cancer for C/C-allele carriers.<sup>26</sup> It is unknown whether the rs5277 genotype is associated with patient or tumor characteristics among breast cancer patients or could provide prognostic information.

To identify more inflammation-prone breast cancer subtypes, it is of interest to investigate the combined effects of inflammation-associated host factors such as body constitution, breast cancer treatment, ER status and COX2 genotype

on breast cancer prognosis. We hypothesized that rs5277 genotypes can affect the risk of early events and treatment response and that the impact is further modified by ER status and body constitution. Hence, the aim of our study was to investigate the impact of rs5277 on early events and treatment response in relation to tumor ER status and to determine whether any effect is modified by body constitution. Tumor COX-2 expression in relation to the COX2 genotype was also evaluated.

**Material and Methods****Study population**

As of October 2002, women diagnosed with a first breast cancer at the Skåne University Hospital in Lund, Sweden were invited preoperatively to participate in the ongoing prospective cohort BC-blood study. The Skåne University Hospital in Lund serves almost 300,000 inhabitants. Since patients are not referred to other hospitals for surgery, the study is considered population-based. During the time the cohort was compiled, 1,090 patients received breast cancer surgery at the hospital. Approximately 58% of these patients were included in the study. Patients were mostly missed due to lack of available research nurses. The included patients were similar to the nonincluded patients regarding age and hormone receptor status. Patients with a prior history of breast cancer or another cancer diagnosis within the past 10 years were excluded. The vast majority of the patients who were diagnosed in Lund were ethnic Swedes; however, ethnicity information was not obtained during our study.

This article is based on data collected from 634 patients initiating treatment between October 2002 and October 2008. The median follow-up time for the 634 patients was 5.1 years (IQR 3.0–7.1 years). Treatment was administered according to the standard of care in Skåne University Hospital. The patients were asked to fill-out questionnaires preoperatively, 3–6 months after surgery and then 1, 2, 3, 5, 7 and 9 years postoperatively. The follow-up rates in the present cohort are high.<sup>28</sup> Written informed consent was obtained from all patients, and the study was approved by the ethics committee of the Lund University (Dnr 75-02, 37-08 and 658-09).

At the preoperative visit, around 30 ml of EDTA plasma and 14 ml of serum was collected. The research nurses also measured body weight, height and waist and hip circumferences during the preoperative visit. The volume of each breast

was measured with plastic cups, as previously described.<sup>29</sup> The waist circumference was measured at the umbilicus; the hip circumference was measured at the widest part between the hip and trochanter major. The questionnaire included questions regarding date of surgery, reproductive history, exogenous hormone use, smoking history (yes/no/occasional smoker) and alcohol consumption. Regular smokers and occasional smokers during the preoperative visit were classified as "preoperative smokers." A body mass index (BMI) cutoff value of 25 kg/m<sup>2</sup> was used according to WHO's classifications of "overweight."<sup>30</sup> Central obesity was considered to be present if the waist-to-hip ratio (WHR) was above 0.85.<sup>30</sup> Alcohol consumption frequency was divided into five categories (never, not more than once a month, two to three times every month, two to three times every week, four or more times a week) based on the alcohol use disorders identification test (AUDIT).<sup>31</sup> A breast volume cutoff of 850 ml was chosen based on an earlier publication.<sup>32</sup> Mammography-detected tumors in patients aged 45–74 at the time of diagnosis were considered to be screening detected. This was the age category invited to mammography screening in Sweden during the study inclusion period. Antidepressant and COX-inhibitor use were coded as dummy variables based on the information obtained from the preoperative questionnaire.<sup>33</sup>

Information regarding the type of adjuvant treatment, sentinel node biopsy results, axillary node dissection and type of surgery was collected from patient charts. Treatment information was also collected from questionnaires and was recorded up to the time of the last follow-up appointment or death, prior to any event. Data on tumor size, histological type and grade and number of involved axillary lymph nodes were obtained from each patient's pathology report. ER and progesterone receptor (PgR) status were determined as previously described.<sup>34,35</sup> COX-2 expression was assessed in tumor microarrays (TMAs).

#### TMA and immunohistochemistry

TMAs were constructed by sampling cores from representative tumor regions of formalin-fixed paraffin-embedded tissue blocks, using a semiautomated tissue array device (Beecher Instruments, Sun Prairie, WI). Duplicate cores (1.0 mm) from primary tumors were mounted into recipient blocks. For COX-2 staining, 4- $\mu$ m TMA sections were deparaffinized and pretreated using an automatic PT-link system (DAKO, Glostrup, Denmark), followed by staining using COX-2 antibody (ab15191, diluted 1:250, Abcam, Cambridge, UK) and EnVision FLEX high-pH kit, in an Autostainer Plus, according to the manufacturer's instructions (DAKO, Glostrup, Denmark).

COX-2 expression was evaluated by two independent observers. The fraction was estimated in absolute percent and staining intensity was estimated in a scale of 0.0–3.0 for each tissue core. Discrepant annotations were discussed until consensus was reached. A pooled value of the patient's tissue cores was used in the statistical analyses.

For all breast cancer patients treated in Skåne University Hospital in Lund, HER2 was routinely analyzed as of November 2005. HER2 protein was detected using Herceptest (DAKO K5206, Copenhagen, Denmark). Gene amplification was assessed using a *HER2* FISH pharmDx™ Kit (DAKO K5331), according to the manufacturer's instructions. Among the 570 patients included in the survival analyses, data on the HER-2 status were missing for 320 patients; 280 of these were included in the study before November 2005. Tumors were considered triple negative if ER, PgR and HER-2 status were all negative; 320 patients lacked triple negative status information. Of the patients included after November 2005, 40 were missing data on triple negative status. Thirty-nine patients lacked HER-2 status only and one patient lacked HER-2, ER and PgR status.

The tumors were analyzed at the Department of Pathology at Skåne University Hospital in Lund. Information concerning breast cancer events including local or regional recurrence, new breast cancer, or distant metastases was obtained from patient charts, pathology reports and the Regional Tumor Registry. The date of death was obtained from the Swedish Population Registry.

#### Genotyping

Genomic deoxyribonucleic acid (DNA) was extracted from the leukocyte portion of whole blood using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI). The genotyping was performed at the Region Skåne Competence Centre (RSKC Malmö), Malmö University Hospital, Malmö, Sweden. SNP rs5277 was analyzed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a Sequenom MassARRAY® platform (Sequenom, San Diego, CA) and iPLEX reagents according to the manufacturers' protocol. Sequenom MassARRAY® software (Sequenom) was used for multiplex SNP analysis design. Over 10% of the samples were run in duplicate with a concordance of 100%. Patients lacking SNP rs5277 information were excluded from the SNP analyses ( $n = 6$ ). The rs5277 minor allele C was defined according to the Database of Single Nucleotide Polymorphisms (dbSNP).

#### Data analyses

The statistical analyses were performed using IBM SPSS Statistics 19.0 (Chicago, IL). Each patient's BMI was calculated by dividing weight in kilograms by the square of their height in meters (kg/m<sup>2</sup>). The WHR was calculated as waist circumference divided by hip circumference. The COX2 genotype was recorded as G/G, G/C or C/C in the analyses of COX2 genotype in relation to tumor and patient characteristics. The COX2 genotype was analyzed in relation to patient characteristics (age at diagnosis, weight, height, BMI, WHR, age at menarche and total breast volume) with the nonparametric Kruskal–Wallis or Jonckheere–Terpstras tests because these variables were continuous and not normally distributed. Chi-square and linear-by-linear analyses were used to investigate

the relationship between the *COX2* genotype and the categorical variables breast volume  $\geq 850$  ml (yes/no), parous (yes/no), preoperative use of antidepressants (yes/no), any hormone replacement therapy use (HRT) (yes/no), preoperative smoking (yes/no), alcohol consumption frequency, tumor size (*in situ*,  $\leq 20$ , 21–50,  $\geq 51$  mm, skin or muscle involvement or 21 mm or larger), histological grade (I–III or III), axillary node involvement (0, 1–3, 4+ or axillary node involvement (yes/no)), ER status (yes/no), PgR status (yes/no), HER2 status (amplified/not amplified) and triple negative tumor (yes/no). Due to the small number of homozygous minor allele carriers, the G/C and C/C genotypes were combined to a single “Any C” genotype for the *COX-2* tumor expression and the survival analyses. *COX-2* expression was analyzed in relation to Any C or G/G genotype and ER status using Mann–Whitney *U*-test. Receiver operating characteristics curve (ROC) was used when determining optimal cutoff for high *COX-2* expression. Derived groups were analyzed in strata of ER-positive and ER-negative tumors and a cutoff level of 2.325 was set for ER-negative tumors.

For analyses of breast cancer-free survival, patients were followed from inclusion to the first breast cancer event. Patients without events were followed until the last follow-up or death prior to January 1, 2013. Of 634 patients, 570 were included in the survival analyses.

For the univariable survival analysis, the Log-Rank test was used to analyze the risk of early cancer events in relation to rs5277 and ER status and their relation to host factors and chemotherapy.

For the multivariable analysis, Cox regression was used to calculate hazard ratios (HRs) in relation to rs5277, adjusting for age (linear), invasive tumor size ( $\geq 21$  mm or muscular or skin involvement), axillary lymph node involvement (yes/no) and histological grade III (yes/no). Since few patients had an invasive tumor size  $\geq 51$  mm or muscular or skin involvement, these patients were combined with the patients with invasive tumor sizes between 21 and 50 mm in the multivariable analyses. In the multivariable analyses, categorical variables combining *COX2* genotype and patient characteristics were used. They consisted of the four different combinations of the G/G genotype *vs.* the Any C genotype compared to the bivariate variable breast volume  $\geq 850$  ml (yes/no). Adjustments were also made using breast volume and WHR as continuous variables. Breast volume was not normally distributed and was therefore transformed using the natural logarithm (ln). Prior power calculations assuming 600 patients with an accrual interval of 6 years and additional follow-up time of 2 years and 30% of patients with a variant allele demonstrated that the study was able to detect true HRs between 0.722 and 1.440 with 80% power and an  $\alpha$  of 5%.<sup>36</sup> Furthermore, simulations with 80% failure rates were also performed and showed that the study was powered to detect an increased HR of 1.9 with a genotype frequency of 30%.

A *p*-value  $< 0.05$  was considered significant. All *p*-values were two-tailed. Since this was an exploratory study, nomi-

nal *p*-values are presented without adjustments for multiple testing. The report is based on the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) criteria.<sup>37</sup>

## Results

The SNP rs5277 minor allele (C) frequency was 16.1%. The patients' ages at breast cancer diagnosis ranged from 25 to 99 years, with a median age of 59.8 years.

### Patient and tumor characteristics

Patient and tumor characteristics for all 634 patients and for the 570 patients included in the survival analyses are shown in Table 1. Only a few significant associations between rs5277 genotype and patient characteristics were observed. There was an association between weight and the number of G alleles. Patients with a G/C genotype had a lower median BMI than patients with the other genotypes.

Patients with a C/C genotype had a more advanced nodal involvement. There was a borderline significant association between an increasing number of C-alleles and ER-negative tumors, but G/G carriers were not more likely to be ER-positive than C-allele carriers.

### Early events in relation to *COX2* genotype

Patients who had received preoperative treatment ( $n = 42$ ) (including one patient who had no information regarding interstitial laser thermotherapy and one patient who presented with and received treatment for another cancer between the initial surgery and reoperation), who were diagnosed with carcinoma *in situ* ( $n = 14$ ), and/or had metastases detected earlier than 3 months after study inclusion ( $n = 2$ ), were excluded from the survival analyses. A flowchart of patients included and excluded in the analyses is presented in Figure 1. After exclusion, 86 of the included patients were diagnosed with some type of breast cancer event (ipsi/contralateral, regional or distant metastasis) during the 9-year follow-up time period; 54 of these patients had distant metastases. The median follow-up time was 5.1 years (IQR 3.0–7.1 years) for the 570 genotyped patients with invasive tumors and no distant metastasis detected on the postoperative metastasis screen.

For all patients, rs5277 was not associated with early events (Fig. 2a; Log Rank 1 df;  $p = 0.99$ ). Similar results were observed when patients who had received preoperative treatment were included. In a multivariable model including the 570 patients who had not received preoperative treatment, the G/G genotype did not predict early events (HR 1.01; 95% CI 0.63–1.61;  $p = 0.97$ ), adjusting for tumor size, axillary nodal involvement, age and histological grade III. Adding the use of endocrine treatment, chemotherapy and radiation treatment or WHR and breast volume (either as dichotomous or continuous variables) to the model did not affect the results.

### Early events in relation to *COX2* genotype and ER status

There was a significant interaction between ER status and *COX2* genotype on the risk of early events ( $p_{\text{interaction}} = 0.015$ ).

Table 1. Patient and tumor characteristics for all 634 patients, and according to rs5277 genotype for the 570 patients included in the survival analyses (in gray)

	All patients			Patients included in the survival analyses			Stratified according to rs5277					p
	Median (IQR) or n (%)			Median (IQR) or n (%)			GG		GC		CC	
	634	Missing	570	Missing	400 (70.2)	157 (27.5)	13 (2.3)					
Age at diagnosis, years	59.6 (51.1–66.1)	–	59.8 (51.8–66.4)	–	60.2 (52.7–66.4)	58.7 (50.1–66.3)	57.0 (51.3–63.0)	0.18 <sup>2</sup>				
Weight, kg	68.0 (61.0–76.2)	2	68.0 (60.9–76.3)	2	69.0 (62.0–78.0)	66.0 (58.5–75.0)	64.9 (59.0–77.0)	0.008 <sup>2</sup>				
Height, m	1.66 (1.62–1.70)	1	1.65 (1.62–1.70)	1	1.66 (1.62–1.70)	1.66 (1.62–1.70)	1.67 (1.58–1.68)	>0.3 <sup>3</sup>				
BMI, kg/m <sup>2</sup>	24.6 (22.3–27.8)	3	24.6 (22.3–27.7)	3	24.9 (22.6–28.3)	23.8 (21.4–26.9)	24.6 (21.3–27.2)	0.023 <sup>3</sup>				
Breast volume	1000 (625–1450)	88	1000 (600–1450)	76	1000 (600–1525)	813 (600–1325)	900 (650–1000)	0.21 <sup>3</sup>				
Breast volume ≥850 ml	312 (57.1)	88	283 (57.3)	76	209 (60.6)	68 (49.3)	6 (54.5)	0.075 <sup>4</sup>				
Waist–hip ratio	0.84 (0.78–0.89)	4	0.84 (0.78–0.88)	2	0.84 (0.78–0.89)	0.82 (0.78–0.89)	0.83 (0.78–0.87)	>0.3 <sup>3</sup>				
Age at menarche, years	13 (12–14)	5	13 (12–14)	3	13 (12–14)	13 (13–14)	14 (12–15)	>0.3 <sup>2</sup>				
Parous	537 (84.7)	–	488 (85.6)	–	346 (86.5)	130 (82.8)	12 (92.3)	>0.3 <sup>4</sup>				
Alcohol use	1	1	–	1	–	–	–	–				
Never	70 (11.1)	–	63 (11.1)	–	43 (10.8)	17 (10.8)	3 (23.1)	>0.3 <sup>4</sup>				
Not more than once a month	170 (26.9)	–	151 (26.5)	–	103 (25.8)	46 (29.3)	2 (15.4)	–				
2–4 times per month	238 (37.6)	–	216 (38.0)	–	158 (39.6)	53 (33.8)	5 (38.5)	–				
2–3 times per week	124 (19.6)	–	110 (19.3)	–	75 (18.8)	32 (20.4)	3 (23.1)	–				
4 or more times per week	31 (4.9)	–	29 (5.1)	–	20 (5.0)	9 (5.7)	0	–				
Preoperative smoker	135 (21.3)	–	121 (21.2)	–	86 (21.5)	31 (19.7)	4 (30.8)	>0.3 <sup>4</sup>				
Smoker at any visit	146 (23.0)	–	129 (22.6)	–	91 (22.8)	34 (21.7)	4 (30.8)	>0.3 <sup>4</sup>				
Preoperative use of COX inhibitors	92 (14.6)	5	83 (14.6)	3	60 (15.1)	20 (12.8)	3 (23.1)	>0.3 <sup>4</sup>				
Preoperative use of antidepressants	68 (10.7)	–	56 (9.8)	–	38 (9.5)	15 (9.6)	3 (23.1)	0.27 <sup>4</sup>				
Ever use of HRT, %	287 (45.3)	1	265 (46.6)	–	191 (47.9)	68 (43.3)	6 (46.2)	>0.3 <sup>5</sup>				
Screening detected tumor <sup>6</sup>	310 (58.6)	–	292 (60.6)	–	209 (61.8)	78 (59.1)	5 (41.7)	>0.3 <sup>5</sup>				
Tumor size (pT)	–	4	–	–	–	–	–	–				
≥21 mm (≥2)	174 (27.6)	–	151 (26.5)	–	109 (27.3)	38 (24.2)	4 (30.8)	>0.3 <sup>4</sup>				
In situ	14 (2.2)	–	–	–	–	–	–	>0.3 <sup>4</sup>				
≤20 mm (1)	442 (70.2)	–	419 (73.5)	–	291 (72.8)	119 (75.8)	9 (69.2)	–				
21–50 mm (2)	159 (25.2)	–	141 (24.7)	–	102 (25.5)	35 (23.3)	4 (30.8)	–				
≥51 mm (3)	10 (1.6)	–	9 (1.6)	–	7 (1.8)	2 (1.3)	0	–				
skin or muscle involvement (4)	5 (0.8)	–	1 (0.2)	–	0	1 (0.6)	0	–				

**Table 1.** Patient and tumor characteristics for all 634 patients, and according to rs5277 genotype for the 570 patients included in the survival analyses (in gray) (Continued)

	All patients			Patients included in the survival analyses			Stratified according to rs5277			p
	Median (IQR) or n (%)			Missing			Rs5277 <sup>1</sup>			
	634	Missing	570	Missing	400 (70.2)	157 (27.5)	13 (2.3)			
Axillary node involvement		2								
Any lymph node involvement	251 (39.7)		217 (38.1)		155 (38.9)	55 (35.0)	7 (53.8)		>0.3 <sup>4</sup>	
0	380 (60.1)		351 (61.8)		243 (61.1)	102 (65.0)	6 (46.2)		0.008 <sup>4</sup>	
1–3	183 (29.0)		163 (28.7)		118 (29.6)	43 (27.4)	2 (15.4)			
4+	69 (10.9)		54 (9.5)		37 (9.3)	12 (7.6)	5 (38.5)			
Histological grade		6		1						
Grade III	133 (21.2)		111 (19.5)		73 (18.3)	35 (22.3)	3 (25.0)		0.24 <sup>5</sup>	
I	164 (26.1)		156 (27.4)		115 (28.7)	38 (24.2)	3 (25.0)		0.17 <sup>5</sup>	
II	331 (52.7)		302 (53.1)		212 (53.0)	84 (53.5)	6 (50.0)			
III	133 (21.2)		111 (19.5)		73 (18.3)	35 (22.3)	3 (25.0)			
Hormone receptor status										
ER+	528 (85.9)	19	495 (86.6)	2	354 (88.7)	132 (84.1)	9 (75.0)		0.057 <sup>5</sup>	
PgR+	424 (68.9)	19	397 (69.9)	2	285 (71.4)	106 (67.5)	6 (50.0)		0.13 <sup>5</sup>	
HER-2 status <sup>7</sup>		48		40						
Positive	35 (13.4)		32 (12.8)		20 (11.6)	10 (14.3)	2 (28.6)		0.25 <sup>5</sup>	
Negative	226 (86.6)		218 (87.2)		153 (88.4)	60 (85.7)	5 (71.4)			
Triple negative tumor <sup>7</sup>		48		40						
Yes	24 (9.2)		22 (8.8)		12 (6.9)	9 (12.9)	1 (14.3)		0.13 <sup>5</sup>	
No	237 (90.8)		228 (91.2)		161 (93.1)	61 (87.1)	6 (85.7)			

<sup>1</sup>6 patients lacked genotype information.

<sup>2</sup>Jonckheere–Terpstra.

<sup>3</sup>Kruskal–Wallis.

<sup>4</sup>Chi-square.

<sup>5</sup>Linear by linear.

<sup>6</sup>Including women 45–74 years at diagnosis due to previous Swedish screening protocols.

<sup>7</sup>Including women included in the study as of November 2005. HER-2 status routinely analyzed in patients <70 years with invasive tumors as of November 2005.

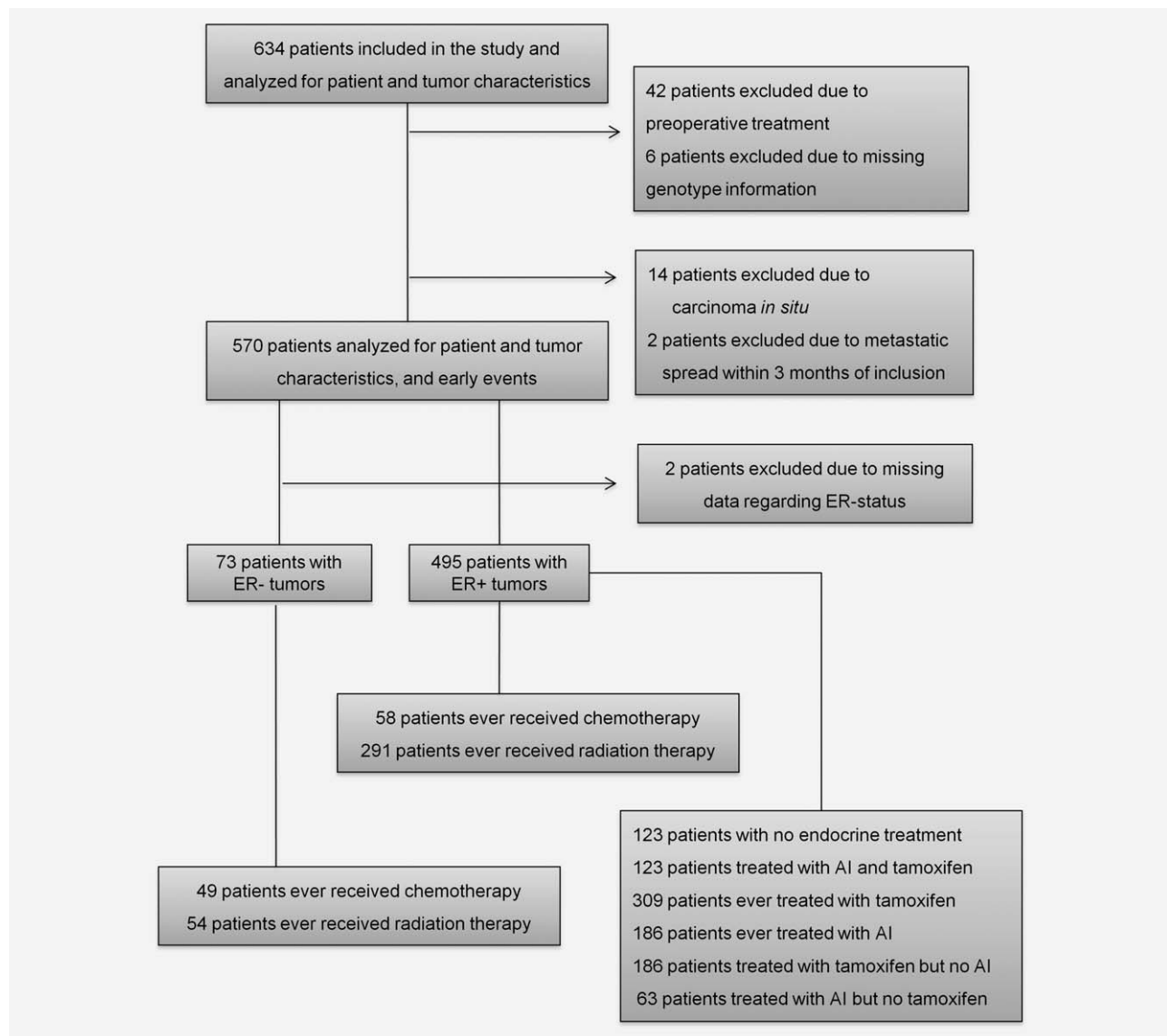


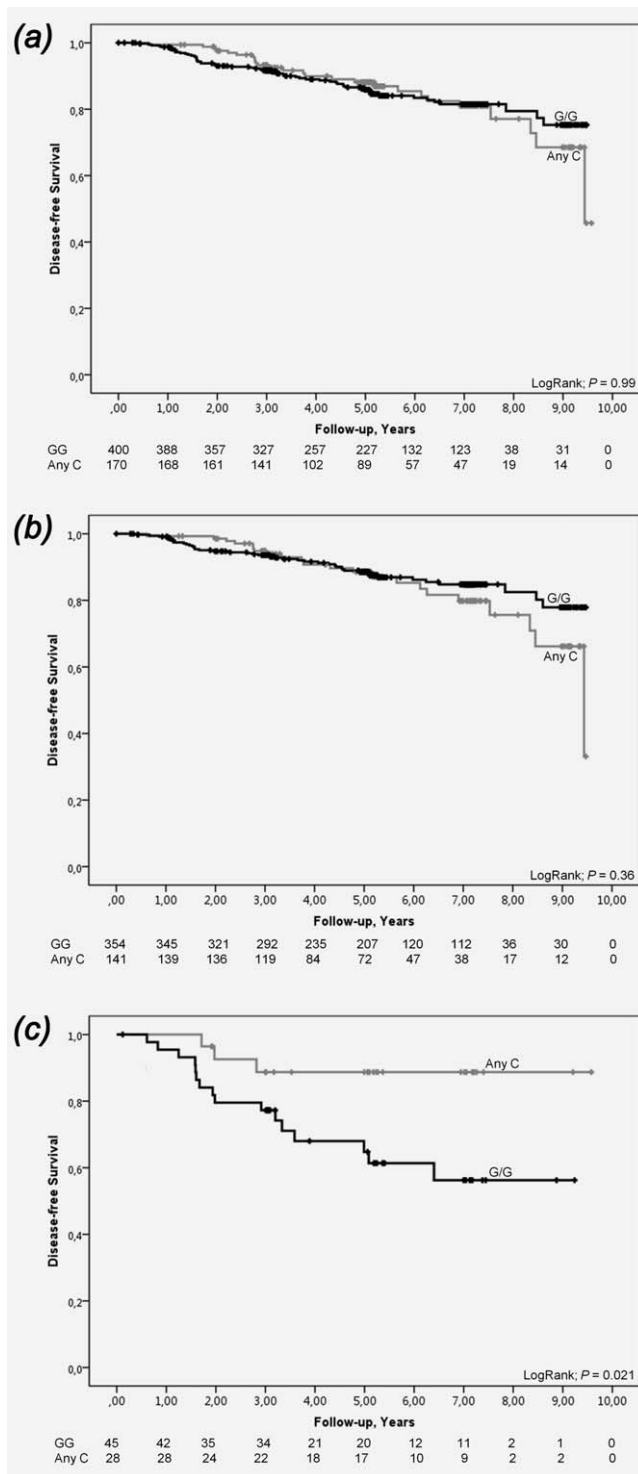
Figure 1. Flow chart of the selection process of patients.

Therefore, the following analyses were stratified according to the ER status of the tumor.

For the 495 patients with ER-positive tumors, the COX2 genotype was not associated with risk of early events (Fig. 2b; Log Rank 1 df;  $p = 0.36$ ). In a multivariable model of patients with ER-positive tumors, C-allele carriers had a non-significantly increased risk of early events (adjusted HR 1.30; 95% CI 0.78–2.18;  $p = 0.32$ ) adjusted for age and tumor characteristics. Further adjustment for treatment did not materially change the results. C-allele carriers had a borderline significant increased risk of early events (adjusted HR 1.73; 95% CI 0.98–3.03;  $p = 0.057$ ) after adjustment for WHR >0.85, breast volume  $\geq 850$ , age and tumor characteristics. The results were materially the same in a model using WHR and breast volume as continuous variables. The risk of early events was especially high for C-allele carriers

with a larger body size represented by a breast volume  $\geq 850$  ml.

For the 73 patients with ER-negative tumors, the G/G genotype was associated with a significantly increased risk of early events (Fig. 2c; Log Rank 1 df;  $p = 0.021$ ). In a multivariable model, G/G carriers had an increased risk of early events (adjusted HR 4.41; 95% CI 1.21–16.02;  $p = 0.024$ ). Adding the use of chemotherapy and radiation treatment to the model did not materially change the results. G/G carriers with ER-negative tumors had an increased risk of early events (adjusted HR 4.60; 95% CI 1.25–16.88;  $p = 0.021$ ) in a model adjusted for WHR >0.85, breast volume  $\geq 850$ , age and tumor characteristics. The results were materially the same in a model using WHR and breast volume as continuous variables. The risk for early events was especially high for patients with a G/G



**Figure 2.** Kaplan–Meier estimates of breast cancer-free survival in relation to *COX2* rs5277 genotype and ER status. There was a significant interaction between ER status and *COX2* rs5277 genotype on risk of early events ( $p_{\text{interaction}} = 0.015$ ). As this is an ongoing cohort, there are fewer patients with longer follow-up times. (a) Among all patients with invasive tumors (Log Rank;  $p = 0.99$ ). (Adjusted HR 1.01; 95% CI 0.63–1.61;  $p = 0.97$ ). (b) Among patients with invasive ER-positive tumors (Log Rank;  $p = 0.36$ ). Adjusted HR adjusted HR 1.30; 95% CI 0.78–2.18;  $p = 0.32$ ). (c) Among patients with invasive ER-negative tumors (Log Rank;  $p = 0.021$ ). Adjusted HR 4.41 (95% CI 1.21–16.02;  $p = 0.024$ ).

genotype and a larger body size represented by a WHR >0.85 or a breast volume  $\geq 850$  ml. The results regarding both ER-positive and ER-negative tumors were somewhat more pronounced in patients 50 years or older than in younger patients.

**COX-2 expression in relation to COX2 genotype, ER status and risk for early events**

Tumor COX-2 expression was available for 465 of the patients included in the survival analyses. The vast majority of tumor cells expressed COX-2, median COX-2 expression was 2.6 (IQR 2.5–2.7). The median COX-2 expression did not differ according to the patients’ *COX2* genotype ( $p = 0.86$ ), but was significantly lower in ER-negative compared to ER-positive tumors ( $p < 0.0001$ ). With a cutoff level of 2.325, high COX-2 expression in ER-negative tumors was associated with a somewhat higher risk for an early event (Log Rank  $p = 0.27$ ), while no association was seen in ER-positive tumors with this cutoff (Log Rank  $p = 0.83$ ). However, patients with ER-positive tumors and maximum COX-2 expression (3.0) had lower risk for an early event than those with lower COX-2 expression (Log Rank  $p = 0.085$ ), adjusted HR 0.40 (0.12–1.28;  $p = 0.12$ ).

**Early events in relation to COX2 genotype, patient characteristics and ER status among chemotherapy-treated patients**

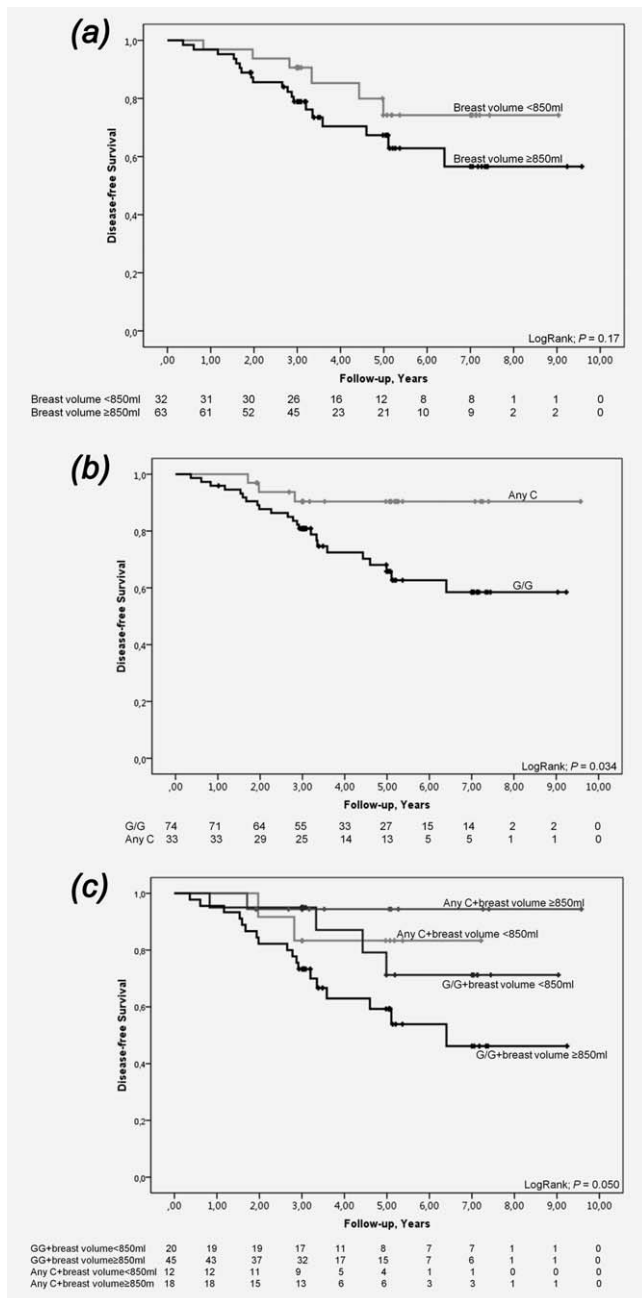
Chemotherapy-treated patients with a breast volume  $\geq 850$  ml did not have an increased risk of early events compared to patients with a breast volume <850 ml (Fig. 3a; Log Rank 1 df;  $p = 0.17$ ). Chemotherapy-treated G/G carriers had an increased risk of early events compared to C-allele carriers, irrespective of ER status (Fig. 3b; Log Rank 1 df;  $p = 0.034$ ).

If *COX2* genotype and breast volume were combined into four groups, the increased risk for early events was confined to patients with a G/G genotype and a breast volume  $\geq 850$  ml (Fig. 3c; Log Rank 3 df;  $p = 0.050$ ). G/G carriers with a breast volume  $\geq 850$  ml had a near 9-fold increased risk of early events compared to C-allele carriers with a breast volume  $\geq 850$  ml in a model adjusting for tumor size, axillary nodal involvement, age, histological grade III and ER status (adjusted HR 8.99, 95% CI 1.14–70.89;  $p = 0.037$ ). In fact, while 18 of 45 (40%) chemotherapy-treated G/G carriers with a breast volume  $\geq 850$  ml had breast cancer events, only seven of the 50 remaining chemotherapy-treated patients experienced early cancer events (14%).

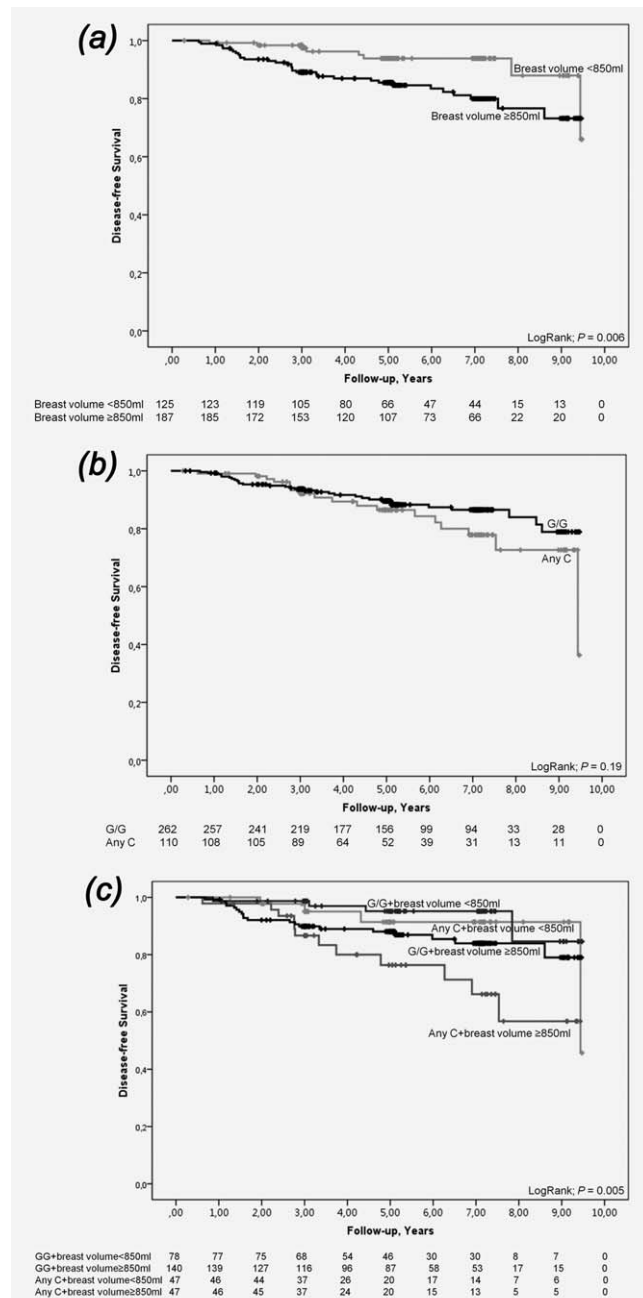
**Early events in relation to COX2 genotype and patient characteristics among patients receiving endocrine treatment**

The impact of *COX2* genotype and body size on early events in endocrine-treated patients with ER-positive tumors was investigated. Endocrine-treated patients with a breast volume  $\geq 850$  ml had an increased risk of early events compared to





**Figure 3.** Kaplan–Meier estimates of breast cancer-free survival among chemotherapy-treated patients in relation to COX2 rs5277 genotype and breast volume. As this is an ongoing cohort, there are fewer patients with longer follow-up times. (a) In relation to breast volume (Log Rank;  $p = 0.17$ ). (b) In relation to COX2 rs5277 genotype (Log Rank;  $p = 0.034$ ). (c) In relation to breast volume and COX2 rs5277 genotype (Log Rank, 3 df;  $p = 0.050$ ). Adjusted HR 8.99 (95% CI 1.14–70.89;  $p = 0.037$ ).



**Figure 4.** Kaplan–Meier estimates of breast cancer-free survival among endocrine-treated patients with ER-positive tumors in relation to COX2 rs5277 genotype and breast volume. As this is an ongoing cohort, there are fewer patients with longer follow-up times. (a) In relation to breast volume (Log Rank;  $p = 0.006$ ). (b) In relation to COX2 rs5277 genotype (Log Rank;  $p = 0.19$ ). (c) In relation to breast volume and COX2 rs5277 genotype (Log Rank, 3 df;  $p = 0.005$ ). Adjusted HR 2.30 (95% CI 1.12–4.75;  $p = 0.024$ ).

endocrine-treated patients with a breast volume <850 ml (Fig. 4a; Log Rank 1 df;  $p = 0.006$ ). C-allele carriers did not have a significantly increased risk of early events compared to G/G carriers (Fig. 4b; Log Rank 1 df;  $p = 0.19$ ). However, C-allele carriers with a breast volume ≥850 ml had an increased risk of early events (Fig. 4c; Log Rank 3 df;  $p =$

0.005). The results were not dependent on whether the patients had received aromatase inhibitors (AI) or tamoxifen. In a multivariate model, C-allele carriers with a breast volume ≥850 ml had a more than 2-fold increased risk of early events compared to GG carriers with a breast volume ≥850 ml (adjusted HR 2.30; 95% CI 1.12–4.75;  $p = 0.024$ ).

## Discussion

The present study investigated the prognostic value of the rs5277 *COX2* polymorphism in relation to early events in an ongoing cohort of breast cancer patients. The most important finding was that while there was no overall impact of this germline polymorphism on early events or on tumor *COX-2* expression, there was a strong interaction between being a *COX2* rs5277 C-allele carrier and ER status on the risk of early breast cancer events. We also identified two subgroups of patients with regard to treatment response: G/G carriers with breast volume  $\geq 850$  ml who responded poorly to chemotherapy regardless of ER status and C-allele carriers with ER-positive tumors and a breast volume  $\geq 850$  ml who responded poorly to endocrine-treatment but responded well to chemotherapy. To our knowledge, the effect of *COX2* polymorphisms on breast cancer survival and treatment response in relation to tumor ER status has not been previously investigated.

Whether the *COX2* rs5277 polymorphism is directly associated with inflammation is unclear. The *COX2* genotype was not associated with tumor *COX-2* expression. The rs5277 SNP may be associated with other unmeasured inflammatory patient characteristics, and the association between rs5277 and the risk of early events is driven by other mediators of inflammation, such as NF- $\kappa$ B.<sup>17</sup> Two of three<sup>23,26,27</sup> studies have reported increased risks of inflammation-associated cancers for rs5277 G/G carriers. In the present study, G/G carriers had an increased risk of early events if they had ER-negative tumors; but not if they had ER-positive tumors. In two previous studies, *COX-2*-mediated inflammation was associated with a poor outcome in ER-negative but not ER-positive breast cancer.<sup>11,15</sup> We hypothesize that the G/G genotype increases inflammation and the impact of the SNP on survival and treatment response is modulated by nongenetic host factors and tumor ER status (Fig. 5). This could be mediated through linkage disequilibrium to an unknown, functional SNP. G/G carriers with ER-negative tumors had an increased risk of early events compared to C-allele carriers with ER-negative tumors. ER-negative tumors may be more sensitive to *COX-2* induced Akt-pathway activation,<sup>15</sup> which raises the threshold for apoptosis<sup>38</sup> and leads to more aggressive tumors.<sup>11,15</sup>

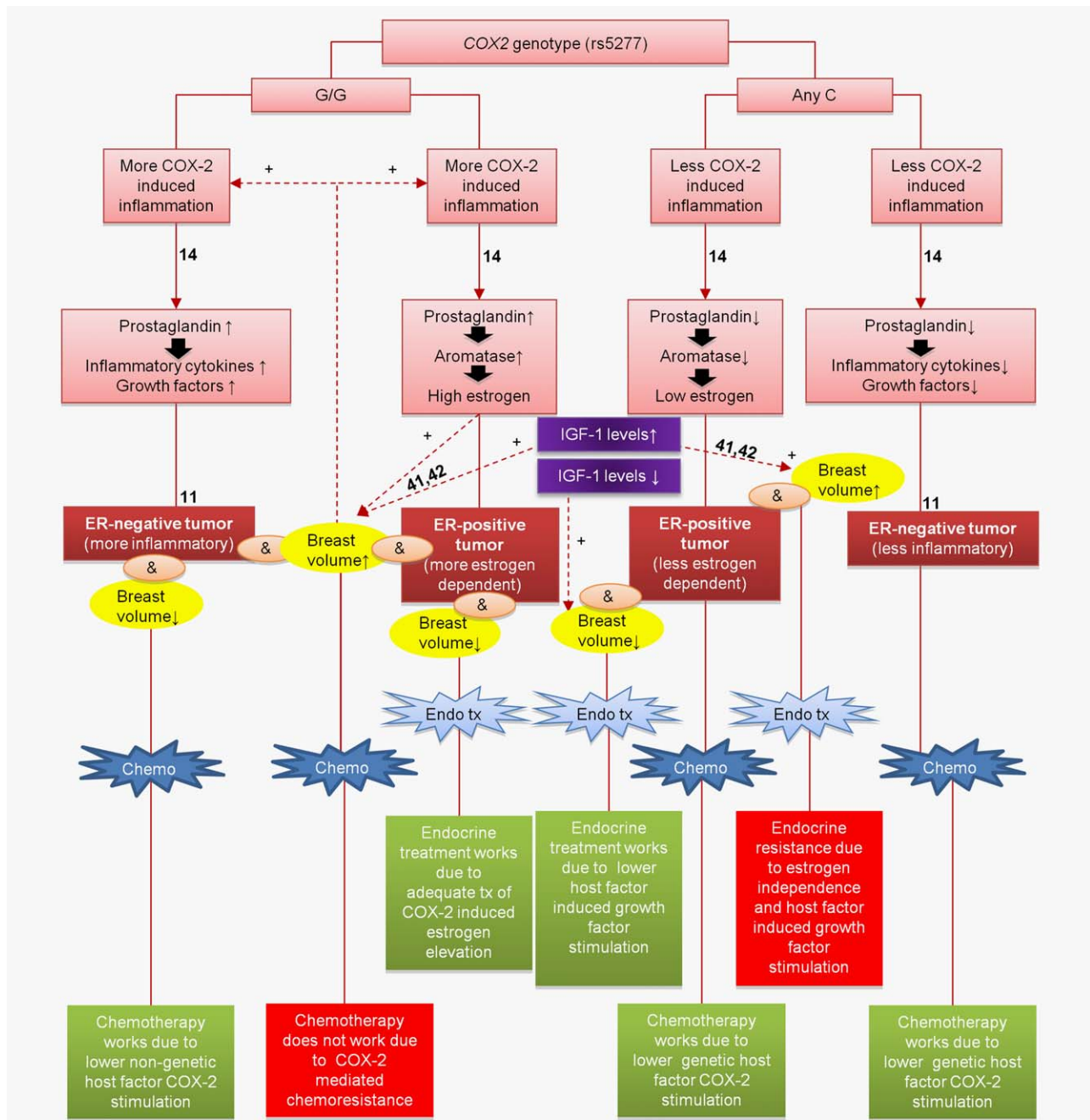
G/G carriers had a poor response to chemotherapy regardless of their tumor ER status, but the increased risk was confined to G/G carriers with a breast volume  $\geq 850$  ml. A large breast volume has been associated with local inflammation, increased *COX-2* levels and an increased risk of early events.<sup>14,18,32</sup> In the present study, breast volume was not significantly associated with tumor *COX-2* expression (data not shown). The majority of patients with ER-negative tumors received chemotherapy. Chemotherapy-treated patients with ER-positive tumors had larger tumor sizes, increased lymph node involvement and a more advanced histological grade than C-allele carriers with ER-positive tumors who did not receive chemotherapy. The patients received different kinds

of polychemotherapy; most were treated with an anthracycline-based regimen with or without a taxane. Hence, both ER-negative and ER-positive tumors required aggressive treatment, and *COX-2*-mediated chemotherapy-resistance would result in an increased risk of early events regardless of their ER status.

*COX*-inhibition has been associated with prolonged disease-free survival among breast cancer patients, and is believed to be particularly valuable for ER-negative tumors.<sup>11,13</sup> G/G carriers with ER-negative tumors or who have received chemotherapy may benefit most from *COX*-inhibition treatment. A previous study showed no difference in response to aspirin-treatment for the rs5277 SNP.<sup>22</sup> In the present study, preoperative *COX*-inhibitor use was not associated with early events, and removing *COX*-inhibitor users from the survival analyses did not affect the results (data not shown). In observational studies, patient characteristics may differ between treatment users and nonusers.<sup>39</sup> It is possible that the *COX*-inhibitor users in this cohort were more prone to chronic inflammation than nonusers, thus using anti-inflammatory medication more often.

C-allele carriers with ER-positive tumors responded poorly to endocrine treatment but well to chemotherapy. The increased risk of early events was confined to patients with a large breast volume. If C-allele carriers have a lower *COX-2* activity, local estrogen levels may be depressed because *COX-2* stimulates aromatization of estrogens.<sup>4,14</sup> ER-positive tumors that develop in breasts with lower estrogen levels may be more aggressive and more often endocrine treatment-resistant.<sup>40</sup> In a previous study of this cohort, breast volume was an independent predictor of disease-free survival among breast cancer patients with ER-positive tumors.<sup>32</sup> We there hypothesized that the results could be explained by the association between higher IGF-I levels and larger breast volume.<sup>41,42</sup> IGF-I signaling is associated with resistance to endocrine treatment in ER-positive tumors.<sup>43,44</sup> Hence, a large breast volume could strengthen estrogen-independence among C-allele carriers with ER-positive tumors and decrease disease-free survival.<sup>45</sup> The effect of high *COX-2*-activity in ER-positive tumors may be neutralized by endocrine treatment.<sup>10</sup> In the present study, patients with ER-positive tumors had significantly higher tumor *COX-2* expression than patients with ER-negative tumors. The majority of patients with ER-positive tumors received endocrine treatment. Moreover, patients with ER-positive tumors and either maximum *COX-2* expression or G/G genotype had lower risk for early events, which merits further study in an extended cohort. Hence, endocrine treatment may have improved the prognosis for the G/G carriers with ER-positive tumors.

In a previous study, about 16% of Caucasian descendants were *COX2* rs5277 SNP minor allele C carriers; the allele was less frequent among African American, Asian and Hispanic descendants (4, 3 and 11%, respectively).<sup>22</sup> Similar distributions have been observed in both cancer and noncancer



**Pathways already explored in previous studies**

- 14 Subbaramaiah *et al*, 2012
- 11 Witton *et al*, 2004
- 41 Jernström *et al*, 2005
- 42 Hartmann *et al*, 1998

Figure 5. The figure shows the hypothesized mechanisms of how COX2 rs5277 genotype impacts the risk of early events differently depending on host factors and ER status of the tumor. References to the steps that have been previously explored are indicated. Endo tx = endocrine treatment.

populations.<sup>46</sup> Most breast cancer patients have ER-positive tumors.<sup>47</sup> Hence, in some ethnic subgroups, a considerable number of women diagnosed with ER-positive breast cancer

are C-allele carriers. Due to the suggested endocrine therapy resistance in this subgroup, these patients may require personalized treatment. Interestingly, there were no events

among the 14 chemotherapy-treated C-allele carriers with ER-positive tumors, despite significantly larger tumor sizes, increased lymph node involvement and more advanced histological grade compared to C-allele carriers with ER-positive tumors who were not treated with chemotherapy. Hence, chemotherapy could be valuable in this subgroup of patients.

Our study is population-based and the included patients were similar to nonincluded patients regarding age and hormone receptor-status. SNP genotyping is considered reliable in the present study as over 10% of the samples were run in duplicate with a concordance of 100%. In the present study, the vast majority of patients were ethnic Swedes. Studies of the association between rs5277 polymorphism and breast cancer prognosis in other ethnic groups are warranted.

Patients were stratified according to age (<50 or ≥50 years) instead of reported menopausal status. Patients who used hormone replacement therapy may have had hormone-induced bleedings and may therefore have been misclassified as premenopausal. Also, patients who had had their uterus, but not their ovaries, removed before menopause may also have been misclassified as postmenopausal. Since the follow-up time was only 5 years, the long-term effects of COX2 genotypes on the risk of breast cancer events could not be evaluated. The estimated failure rates in the previous power calculation exceeded the observed failure rates, which may have influenced the power in our study. In addition, our study assessed numerous variables, and some of the findings may be due to chance. The results must be confirmed in independent cohorts.

The germline COX2 genotype was not associated with COX-2 expression in the tumors. However, we did not have access to urinary levels of tetranor-prostaglandin E2 metabolite to evaluate effects of COX-2 activity on systemic inflammation. Similarly, aromatase expression would have enabled evaluation of local effects. This would have been of value to test the hypothesis presented in Figure 5.

In the present study, breast volume in relation to breast cancer prognosis was assessed rather than mammographic density. While mammographic density is a strong risk factor for breast cancer, its impact on breast cancer prognosis is

unclear.<sup>48</sup> Women with large breasts are more likely to have a decreased mammographic density, and mammographic density seems to be less of a breast cancer risk factor for women with larger breasts.<sup>19</sup> Dense matter in large breasts may be constitutionally different from that in smaller breasts.<sup>19</sup> Furthermore, breast volume is closely related to other inflammatory patient characteristics such as BMI,<sup>49</sup> suggesting that it may be part of an inflammatory body constitution. Also, breast volume seems to have effects on breast cancer prognosis and inflammation independent of BMI and WHR.<sup>32,50</sup>

In conclusion, there was a strong interaction between having any COX2 rs5277 C-allele and ER status on the risk of early events. Two subgroups of patients were identified: G/G carriers with a large breast volume who responded poorly to chemotherapy regardless of ER status and C-allele carriers with ER-positive tumors and a large breast volume who responded poorly to endocrine treatment but responded well to chemotherapy. Additional treatment with COX-inhibitors may be valuable for chemotherapy-treated G/G carriers with a large breast volume. In addition, C-allele carriers with ER-positive tumors and a large breast volume may benefit from chemotherapy as a mono-therapy or in addition to endocrine treatment. The high risk for early events in certain subgroups of patients suggests that the COX2 genotype in combination with body measurements may provide a tool for identifying patients in need of more personalized treatment.

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