Clinical Cancer Research

High Estrogen Receptor β Expression Is Prognostic among Adjuvant Chemotherapy–Treated Patients—Results from a Population-Based Breast Cancer Cohort

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Abstract

Purpose: Isoform-specific tumor estrogen receptor β (ERβ) expression may hold prognostic information in breast cancer, especially among endocrine-treated breast cancer patients. The study's purpose was to evaluate ERβ isoform 1 (ERβ1) expression in relation to tumor characteristics, *ESR2* genotypes, and prognosis in different treatment groups.

Experimental Design: A population-based prospective cohort of 1,026 patients diagnosed with primary invasive breast cancer in Lund, Sweden, between October 2002 and June 2012 was followed until June 2014 (median 5 years). Associations between immunohistochemical ERβ1 expression, patient and tumor characteristics, as well as outcome within treatment groups were analyzed.

Results: Tumor ERβ1 expression was available for 911 patients (89%) and was not associated with *ESR2* genotypes. ERβ1 positivity, defined as >75% ($ERβ1_{75}^+$, 72.7%), was positively associated with established favorable tumor characteristics. Overall, $ERβ1_{75}^+$ was associated with lower risk of breast cancer events [HR_{adj} = 0.60; 95% confidence interval (CI), 0.41–0.89]. The magnitude of the association was larger in patients with $ERα^-$ tumors (HR_{adj} = 0.30; 95% CI, 0.12–0.76), compared with $ERα^+$ tumors (HR_{adj} = 0.66; 95% CI, 0.42–1.03). Among the 232 chemotherapy-treated patients, $ERβ1_{75}^+$ tumors were associated with lower risk of breast cancer events compared with $ERβ1_{75}^-$ tumors (HR_{adj} = 0.31; 95% CI, 0.15–0.64). Among the 671 chemonaïve patients, $ERβ1_{75}$ status was not associated with the outcome.

Conclusions: High ERβ1 expression was a favorable prognostic marker in this breast cancer cohort, especially in chemotherapy-treated patients, but not in endocrine therapy–treated patients. These results warrant confirmation, preferably via a biomarker study in a previously conducted randomized trial. *Clin Cancer Res; 23(3); 766–77. ©2016 AACR.*

Introduction

The complexity of estrogen receptor (ER) signaling in breast cancer was further revealed with the discovery of ERβ in the 1990s (1). ERβ is encoded by estrogen receptor gene 2 (*ESR2*), which is highly polymorphic. The majority of the genetic variation can be captured by four haplotype tagging single SNPs (htSNP; ref. 2). We have previously reported that *ESR2* genotypes seem to divide patients into good and poor survivors, depending on the body mass index (BMI) of the patient (3). Whether *ESR2* genotypes are associated with ERβ tumor expression is currently unknown. ERβ is a transcription factor that has been suggested to regulate ERα activity (4) and to have an antiproliferative and tumor-suppressing role (5). ERβ may also have different effects depending on the currently known five different isoform variants expressed at the protein level (6). Highly specific antibodies have been called for, to better characterize the role of ERβ and its variants in breast cancer, with the ultimate aim to develop specific ERβ agonists to improve breast cancer treatment (7).

In terms of outcomes, tumor ERβ expression (total and isoform specific) has been positively associated with favorable prognosis, especially when ERβ was coexpressed with ERα, but also for patients with $ERα^-/ERβ^+$ tumors (8). Contrasting findings of ERβ-driven proliferative effects, foremost in $ERα^-$ tumors, have suggested a differential role for ERβ, depending on breast cancer subtype (9, 10). As the results from clinical studies have been inconsistent, large prospective trials that examine isoform-specific ERβ expression stratified by ERα status have been called for (5). Recently, the first meta-analysis on clinical outcomes in relation to ERβ expression in nonmetastatic breast cancer was published. The ERβ isoforms 1, 2, and 5 (ERβ1, ERβ2/cx, and ERβ5) were assessed at either the protein or mRNA level (11); the main finding was that tumor ERβ1 expression was favorable for disease-free survival (DFS) irrespective of ERα status and was also favorable for overall survival (OS) among patients with $ERα^+$ tumors. ERβ2 was only prognostic for DFS, while ERβ5 was not associated with the

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Translational Relevance

In this large, prospective population-based cohort of primary breast cancer, high tumor expression of estrogen receptor β (ERβ1; >75%) was associated with favorable clinicopathologic characteristics, but not with the previously studied germline *ESR2* genotypes. In chemotherapy-treated patients, high ERβ1 was an independent favorable prognostic marker. In contrast, high ERβ1 expression was not associated with better outcomes in endocrine-treated patients, as has been previously reported by other groups. The results warrant confirmation, preferably via a biomarker study in an already performed randomized controlled trial, to enable evaluation of chemotherapy response in relation to high ERβ1 expression.

outcome. The authors proposed that this prognostic significance of ERβ would suggest new molecular subtypes of hormone-sensitive breast cancer. However, the potential treatment-predictive value of ERβ was not analyzed in the meta-analysis, and the heterogeneity of these retrospective study populations in terms of age and subtypes was pointed out (11).

The beneficial impact of ERβ expression on endocrine treatment response has been repeatedly reported (8, 12–14). Recently, the first results from the Intergroup Exemestane Study highlighted the potential importance of ERβ expression in relation to endocrine treatment response, and also its complexity. Therein, ERβ1 was not prognostic among all endocrine-treated patients. However, the patients with ERα^{+} breast tumors with low, but not with high, ERβ1 expression had a survival benefit from the switch from tamoxifen to exemestane (15).

Furthermore, Wang and colleagues showed that high tumor ERβ1 expression was an independent prognostic marker for DFS and OS in a large retrospective series of triple-negative breast cancer (TNBC) patients and proposed specific ERβ agonists as a potential addition to chemotherapy for these patients (16). The ERβ agonist S-equol is currently being evaluated in a presurgical setting for TNBC patients in a phase 0 clinical trial (ClinicalTrials.gov identifier: NCT0235202).

We hypothesized that ERβ1 expression is prognostic in primary breast cancer irrespective of ERα status and that it can impact clinical outcomes, especially among endocrine-treated patients.

The aim of this study was to elucidate whether tumor ERβ1 expression was associated with established clinicopathologic markers and risk of breast cancer events, both for the overall study population and in different adjuvant treatment groups, in a population-based prospective cohort of primary breast cancer. A secondary aim was to assess whether tumor ERβ1 expression was associated with the previously studied *ESR2* genotypes in this cohort.

Materials and Methods

The study cohort

The BC Blood Study is an ongoing population-based prospective cohort study at the Skåne University Hospital (Lund, Sweden). It explores the impact of genetic and lifestyle factors on prognosis and treatment in primary breast cancer. Patients diagnosed with primary breast cancer are invited to participate at their preoperative visit. Exclusion criteria are a history of cancer in the last 10 years or any history of breast cancer (17).

This study included patients from October 2002 to June 2012 ($N = 1{,}116$). After excluding patients with *in situ* only cancers or who had received preoperative treatment, the final study cohort consisted of 1,026 patients (Fig. 1). Preoperatively, patients filled out questionnaires on lifestyle and medication use. Body measurements were taken and blood samples were collected by a research nurse. For patients with no previous breast surgeries, breast size was measured using plastic cups (18). Clinical information and patient characteristics were retrieved through medical records and combined with information from follow-up questionnaires at 3 to 6 months, as well as 1, 2, 3, 5, 7, 9, and 11 years postoperatively, thus providing information regarding adherence (19).

Patients were followed until June 30, 2014. Information on survival and breast cancer events was retrieved from the Swedish National Register on Causes of Death, the Regional Tumor Registry, pathology reports, and patient charts. Local or regional recurrences, contralateral cancers, or distant metastasis were considered as endpoints in DFS analyses. For analyses of distant metastasis–free survival (DMFS) and OS, distant metastasis and death from any cause, respectively, were used as endpoints. Patients were censored at the time of a non–breast cancer–related death or last follow-up.

Genotyping of the *ESR2* htSNPs (rs4986938, rs1256031, rs1256049, and rs3020450) was performed, and haplotypes were constructed as described previously (3).

All patients signed informed consents upon enrolment. The study was approved by the Lund University Ethics Committee (Dnr LU75-02, LU37-08, LU658-09, LU58-12, LU379-12, LU227-13, LU277-15, and LU458-15).

Histopathological analyses

Tumor specimens were retrieved as formalin-fixed paraffin-embedded blocks from which tissue microarrays (TMA) with duplicate 1-mm cores were constructed, as described previously (20). Four-micrometer TMA sections were cut for immunohistochemical semiautomated staining of ERβ1 (Autostainer Plus, Dako), using the ERβ1-specific mAb clone PPG5/10 (M7292, Dako, dilution 1:20). Semiquantitative scoring of ERβ1 was performed twice independently by one researcher (K. Elebro) blinded to the clinical outcome. In cases where discrepancies occurred, a third scoring was performed (K. Elebro + A.H. Rosendahl) to reach consensus. Fractions were assessed as 0%, 1%–10%, 11%–20%, 21%–75%, 76%–100 % of positively stained nuclei, and intensity as none, weak, moderate, or strong nuclear staining intensity, irrespective of cytoplasmic staining. Two cut-off points for positivity were evaluated: >75% and >10% of positively stained nuclei. If the duplicate cores were discordant, the fraction of positively stained nuclei was estimated across both sampled cores.

Information on the clinically established tumor markers, such as ERα and progesterone receptor (PR) expression (cutoff at >10% positively stained nuclei), was collected from pathology reports, as described previously (20–22). HER2 status (amplified/non-amplified) was available for 688 (93.2%) patients as of November 2005, when HER2 assessment was introduced into Swedish clinical routines for patients younger than 70 years of age. Information on histological type and grade, invasive tumor size, and axillary lymph node involvement (ALNI) was retrieved from the patient charts and pathology reports. The TMAs had been previously assessed for androgen receptor (AR) expression (20).

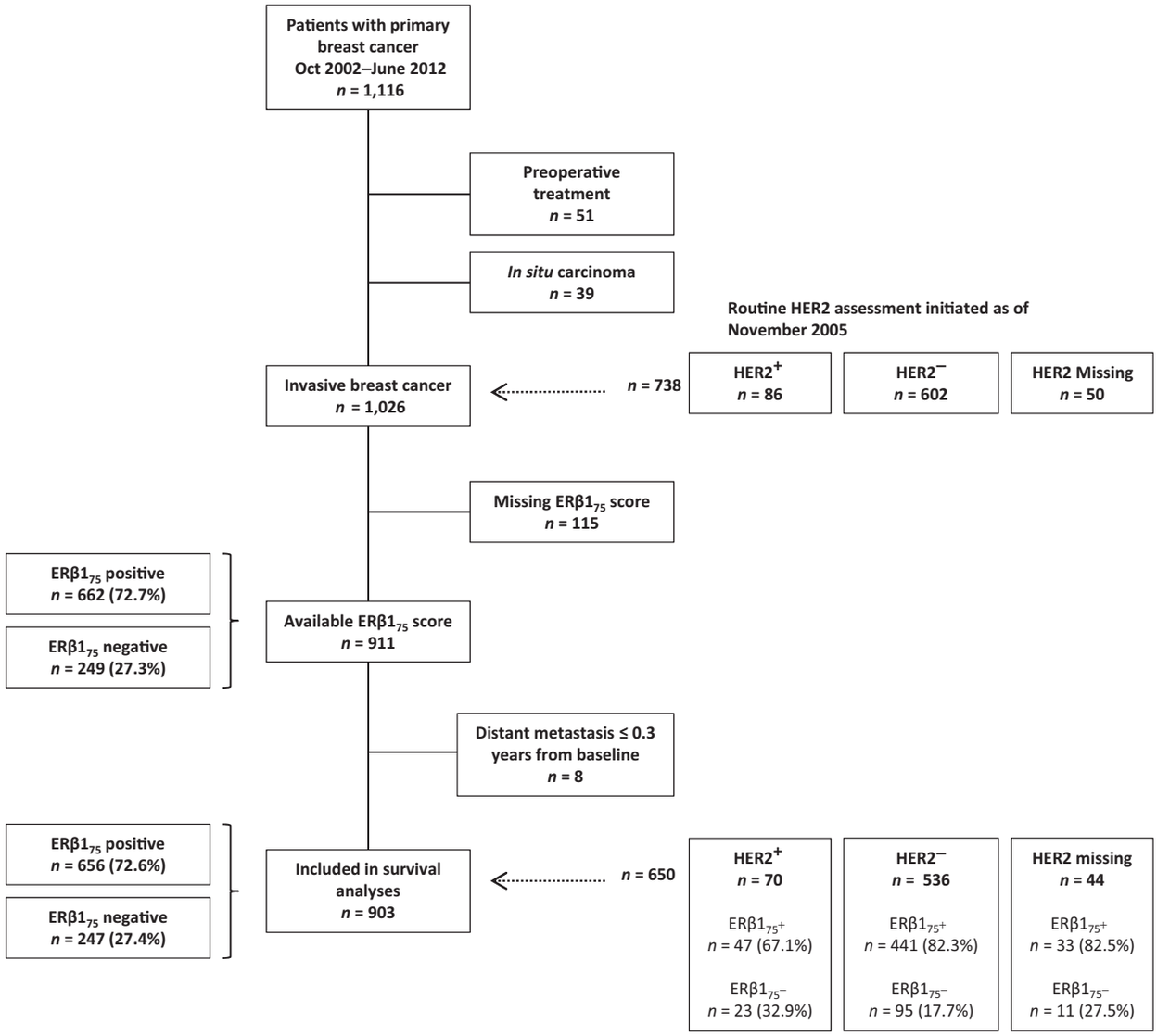

Figure 1.
Flow chart of the study population included in various analyses.

Statistical analyses

The statistical analyses were conducted with the software program SPSS version 22.0 (IBM). Descriptive patient and tumor characteristics were summarized as either continuous variables (median, interquartile range) or categorical (number, percentage) variables, in relation to ERβ1 status (±, or missing ERβ1 status). The potential associations between these variables and ERβ1 status (±) were analyzed by the Mann–Whitney *U* test, or by χ^2 or logistic regression analyses, for which ORs with 95% confidence intervals (CI) are presented. To examine whether there was an effect modification by ERα on the association between AR and ERβ1 expression, a multiplicative interaction variable between AR and ERα was calculated and included in the logistic regression model. Categories were based on either previously studied cutoffs [i.e., BMI ($\geq$25 kg/m^2), total breast size $\geq$850 mL (18)] or dichotomized variables (parous, ever use of oral contraceptive, ever use of hormone therapy, coffee intake $\geq$2 cups/day, current smoking prior to surgery, and alcohol abstainer). Tumor characteristics were categorized as follows: tumor size (invasive $\leq$20 mm, 21–50 mm, $\geq$51 mm, or skin or muscle involvement independent of size), ALNI (0, 1–3, 4+), histologic grade (1, 2, 3), ERα, PR, AR, combinations of ERα and PR status, and HER2 status (amplified/nonamplified). Information on adjuvant treatment by last follow-up and before any event was dichotomized for chemotherapy, radiotherapy, tamoxifen, and aromatase inhibitors (AI). Trastuzumab treatment was incorporated into subgroup analyses of treatments for the patients included as of November 2005.

The impact of ERβ1 expression on DFS was assessed by Kaplan–Meier curves and the log-rank test. Analyses were performed for ERβ1 status alone and in combination with ERα status. Stratification by various treatment groups was performed; regarding

Table 1. Patient characteristics by ERβ1$_{75}$ status

	All	Missing total	Patients with available tumor ERβ1 status			Missing ERβ1 status
			ERβ1$_{75}^-$	ERβ1$_{75}^+$		
	N = 1,026		n = 249	n = 662		n = 115
	Median (IQR) or %	n	Median (IQR) or %	Median (IQR) or %	P[a] or OR (95% CI) for ERβ1$_{75}^+$	Median (IQR) or %
Patient characteristics						
Age at diagnosis, yrs	61.1 (52.1–68.1)	0	59.6 (51.0–66.6)	61.9 (53.4–68.9)	**0.008**	60.7 (48.3–68.1)
Weight, kg	69.0 (62.0–78.0)	26	70.0 (63.0–79.3)	69.0 (61.0–78.0)	0.23	67.8 (61.3–76.5)
Height, m	1.65 (1.62–1.70)	26	1.65 (1.62–1.70)	1.66 (1.62–1.70)	0.54	1.65 (1.61–1.69)
BMI, kg/m^2	25.1 (22.5–28.3)	28	25.6 (22.9–28.6)	25.0 (22.4–28.3)	0.15	24.6 (22.1–28.2)
Waist–hip ratio, m/m	0.86 (0.81–0.90)	38	0.85 (0.80–0.90)	0.86 (0.81–0.90)	0.55	0.85 (0.80–0.90)
Total breast volume, mL	1,000 (650–1,500)	160	1,000 (700–1,600)	950 (650–1,500)	**0.036**	1,000 (650–1,300)
≥850 mL, %	57.3		63.4	54.7	**0.70 (0.50–0.96)**	58.9
Age at menarche, yrs	13 (12–14)	6	13 (12–14)	13 (12–14)	0.61	14 (13–14)
Parous, %	87.9	1	88.0	87.7	0.98 (0.63–1.53)	88.7
Age at first full-term pregnancy, yrs	25 (22–28)	131	24 (21–28)	25 (22–28)	0.19	25 (22–27)
Ever use of oral contraceptives, %	70.8	1	69.4	70.8	1.07 (0.78–1.48)	73.9
Ever use of HT, %	43.9	3	43.1	44.1	1.04 (0.77–1.40)	44.3
Coffee intake ≥2 cups/day	81.4	4	83.8	80.2	0.78 (0.53–1.15)	83.5
Current smoker prior to surgery, %	20.5	2	24.1	19.2	0.75 (0.53–1.06)	20.0
Alcohol abstainer, %	10.5	7	12.6	10.0	0.78 (0.49–1.22)	8.8

NOTE: Bold letters indicate statistically significant results.

Abbreviations: ERβ1$_{75}$, ERβ1, cutoff for positivity >75%; HT, hormone therapy; IQR, interquartile range.

[a]Mann–Whitney *U* test.

endocrine treatment, analyses were performed within the ERα$^+$ group, with and without chemotherapy, and stratified by type of endocrine treatment and age (</≥50 years). The prognostic importance of ERβ1 alone, or in combination with ERα, was further analyzed by univariable and multivariable Cox regression analyses, yielding HRs with 95% CIs. Adjustments were performed in four models: Model 1: age (continuous) and tumor characteristics (invasive tumor size >20 mm or skin or muscular involvement irrespective of size, grade 3, any ALNI, ERα status); model 2: age, tumor characteristics, BMI, and smoking; model 3: age, tumor characteristics, and treatment (chemotherapy, radiotherapy, tamoxifen, AI); model 4: model 3 with the addition of trastuzumab treatment and restricted to patients included as of November 2005. Patients with tumors without available ERβ1 status ($n = 115$) and patients who were diagnosed with distant metastasis within 0.3 years or closer to inclusion ($n = 8$) were excluded from survival analyses (Fig. 1).

Prior power calculations assuming 900 patients with an accrual interval of 10 years and additional follow-up time of 0.5 years showed that the study was able to detect true HRs between 0.66 and 1.62 if the frequency of ERβ1$^-$ tumors was 10% (and 0.75–1.37 if 25% ERβ1$^-$), with 80% power and α of 5% (power and sample size calculation program, PS, version 3.0, developed by Dupont and Plummer; http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). Nominal *P* values without correction for multiple testing are presented. All statistical tests were two-sided, and *P* values less than 0.05 were considered significant. This report adheres to the REMARK criteria (23).

Results

Patient and tumor characteristics by ERβ1 status

Valid tumor ERβ1 scores were obtained from 911 patients (88.8%). Using the cutoff >75% of positively stained nuclei, 662 patients (72.7%) displayed ERβ1$_{75}$ positive (ERβ1$_{75}^+$) tumors. These patients were older at inclusion and had smaller breast volumes compared with patients with ERβ1$_{75}$ negative (ERβ1$_{75}^-$) tumors. Other patient characteristics, such as anthropometric measures, reproductive factors, and ever use of exogenous hormones, showed no significant associations with ERβ1$_{75}$ status (Table 1). In terms of tumor characteristics, ERβ1$_{75}^+$ was associated with smaller tumor size, lower histologic grade, less axillary lymph node involvement, as well as coexpression of ERα, PR, and AR (Table 2). Tumors that coexpressed ERα and AR were six times more likely to also express ERβ1$_{75}^+$ compared with no expression or expression of one but not both of the other receptors (OR = 6.41; 95% CI, 2.54–16.14; $P_{interaction} < 0.0001$). In the subgroup where HER2 status was available, HER2 amplification was more common in ERβ1$_{75}^-$ tumors compared with ERβ1$_{75}^+$ tumors. The lowest frequency of HER2 amplification was found in tumors that coexpressed ERα and ERβ1$_{75}$ (7.8%). HER2 amplification was most common in ERα$^-$ tumors, irrespective of ERβ1$_{75}$ and/or PR status (30.3%–32.3%; Table 2).

ERβ1 positivity, defined as >10% of positively stained nuclei [ERβ1$_{10}^+$, $n = 839$ (92.1%)], was associated with ERα and AR coexpression (*P*s < 0.0001). ERβ1$_{10}^+$ did not demonstrate significant associations with other tumor markers, such as invasive tumor size, histologic grade, ALNI, PR expression, and HER2 amplification. Furthermore, it was not significantly associated with any patient-related factors, such as anthropometric measures, reproductive factors, or exogenous hormone use.

Tumor ERβ1$_{75}$ and ERβ1$_{10}$ expression was not significantly associated with the four germline ERβ htSNPs or the two haplotypes "any TCAC" or the number of CCGC, either overall or in patients with BMI ≥25 kg/m^2, where two htSNPs and the two haplotypes were differently associated with DFS depending on BMI in our previous report (3).

DFS by ERβ1 status

Patients were followed for up to 11 years (median follow-up 5.0 years for patients still at risk). In the overall study population, patients with ERβ1$_{75}^+$ tumors had approximately two thirds the risk for any breast cancer event compared with patients with

Table 2. Tumor characteristics by ERβ1$_{75}$ status

	All		Patients with available tumor ERβ1 status			Missing ERβ1 status
		Missing	ERβ1$_{75}^-$	ERβ1$_{75}^+$		
	***N* = 1,026**		***n* = 249**	***n* = 662**	***P* or OR (95% CI) for**	***n* = 115**
	***n* (%)**	***n***	***n* (%)**	***n* (%)**	**ERβ1$_{75}$ positive**	***n***
Tumor characteristics						
Invasive tumor size	1026	0			0.09[a]	
1 (≤20 mm)	740 (72.1)		165 (66.3)	486 (73.4)	Ref.	89
2 (21–50 mm)	269 (26.2)		78 (31.3)	166 (25.1)	**0.71 (0.52–0.97)**	25
3 (>51 mm)	15 (1.5)		6 (2.4)	8 (1.2)		1
4 (skin or muscular involvement independent of size)	2 (0.2)		0 (0.0)	2 (0.3)		0
Axillary lymph node involvement	1024	2			**0.040**[b]	
0	627 (61.2)		134 (54.0)	417 (63.1)	Ref.	76
1–3	306 (29.9)		89 (35.9)	185 (28.0)	**0.69 (0.51–0.92)**	32
≥4	91 (8.9)		25 (0.1)	59 (8.9)		7
Histological grade	1,025	1			**0.001**[b]	
I	252 (24.6)		42 (16.9)	177 (26.7)	Ref.	33
II	511 (49.9)		123 (49.4)	332 (50.2)		56
III	262 (25.6)		84 (33.7)	153 (23.1)	**0.59 (0.43–0.81)**	25
Hormone receptor status						
ERα$^+$	896 (87.5)	1	194 (78.2)	604 (91.2)	**2.90 (1.93–4.34)**	98
PR$^+$	726 (70.9)	1	163 (65.7)	484 (73.1)	**1.42 (1.04–1.94)**	79
AR$^+$	776 (85.0)	113	186 (75.9)	562 (88.4)	**2.41 (1.65–3.52)**	28
in ERα$^-$ subgroup	50 (44.2)	15	27 (51.9)	19 (33.9)	0.48 (0.22–1.03)	4
in ERα$^+$ subgroup	726 (90.9)	97	159 (82.8)	543 (93.6)	**3.05 (1.84–5.03)**	24
Combined ER and PR status	1024				**<0.0001**[a]	
ERα$^-$PR$^-$	122 (11.9)		50 (20.2)	57 (8.6)	Ref.	15
ERα$^-$PR$^+$	6 (0.6)	2	4 (1.6)	1 (0.2)	0.22 (0.02–2.03)	1
ERα$^+$PR$^-$	176 (17.2)		35 (14.1)	121 (18.3)	**3.03 (1.78–5.18)**	20
ERα$^+$PR$^+$	720 (70.3)		159 (64.1)	483 (73.0)	**2.66 (1.75–4.06)**	78
As of November 2005:	688	50[c]				
HER2 amplification[c]						
Among all	86 (12.5)	50	23 (19.5)	49 (9.9)	**0.45 (0.26–0.78)**	14
In ERα$^-$ subgroup	28 (31.8)	1	10 (30.3)	14 (30.4)	1.01 (0.38–2.66)	4
In ERα$^-$ PR$^-$ subgroup	28 (32.9)	0	10 (32.3)	14 (31.1)	0.95 (0.36–2.53)	4
In ERα$^+$ subgroup	58 (9.7)	49	13 (15.3)	35 (7.8)	**0.47 (0.24–0.93)**	10
Treatment by last follow-up[d]	1,026					
Ever chemotherapy	259 (25.2)	0	76 (30.5)	156 (23.6)	**0.70 (0.51–0.97)**	27
Ever radiotherapy	641 (62.5)	0	158 (63.5)	418 (63.1)	0.99 (0.73–1.34)	65
ERα$^+$ only Ever endocrine therapy	694 (77.5)	0	163 (84.0)	465 (77.0)	**0.64 (0.41–0.98)**	66
Ever tamoxifen	528 (58.9)	0	129 (66.5)	347 (57.5)	**0.68 (0.48–0.95)**	52
Ever aromatase inhibitors	345 (38.5)	0	89 (45.9)	224 (37.1)	**0.70 (0.50–0.96)**	32
As of November 2005:	738					
Ever trastuzumab[e]	66 (8.9)	0[e]	18 (14.0)	38 (7.2)	**0.48 (0.26–0.87)**	10

NOTE: Bold letters indicate statistically significant results.

Abbreviation: ERβ1$_{75}$, ERβ1, cutoff for positivity >75%.

[a]χ^2 df, 3.

[b]χ^2 df, 2.

[c]HER2 status routinely analyzed in patients <70 years with invasive tumors as of November 2005. In total, 738 patients were included in the study from November 2005 to June 2012, among which 688 (93.2%) were tested for HER2 status and 50 had missing HER2 status.

[d]Patients may have received more than one type of treatment.

[e]Data on trastuzumab treatment were available for all patients as of November 2005. However, 50 patients (6.8%) had missing HER2 status.

ERβ1$_{75}^-$ tumors (Fig. 2A). In the ERα$^-$ subgroup, patients with ERβ1$_{75}^+$ tumors had one third the risk for an event compared with patients with ERβ1$_{75}^-$ tumors, and this association remained significant after adjusting for age, tumor characteristics, and adjuvant treatment (Fig. 2B). Among patients with ERα$^+$ tumors, ERβ1$_{75}^+$ was also prognostically favorable. However, the magnitude of the association was smaller. Patients with ERβ1$_{75}^+$ tumors had two thirds the risk for an event compared with patients with ERβ1$_{75}^-$ tumors, and this association was not statistically significant ($P = 0.066$; Fig. 2C).

ERβ1$_{75}$ expression and ERα expression were independent prognostic factors of DFS in models adjusted for age, tumor characteristics, and also after further adjustments for BMI and smoking (Table 3, models 1–2). However, in model 3, where adjustment for adjuvant treatments was added, ERα was no longer significant but ERβ1$_{75}$ remained significant (Table 3, model 3). This association also existed in the subgroup analyses that included treatment with trastuzumab (Table 3, model 4).

To further characterize the prognostic role of ERβ1$_{75}$, the combinations of ERα and ERβ1$_{75}$ status were analyzed further. In univariable analyses, patients with tumors that coexpressed ERα and ERβ1$_{75}$ had the best prognosis and were used as a reference group. Conversely, patients with ERα$^-$ and ERβ1$_{75}^-$

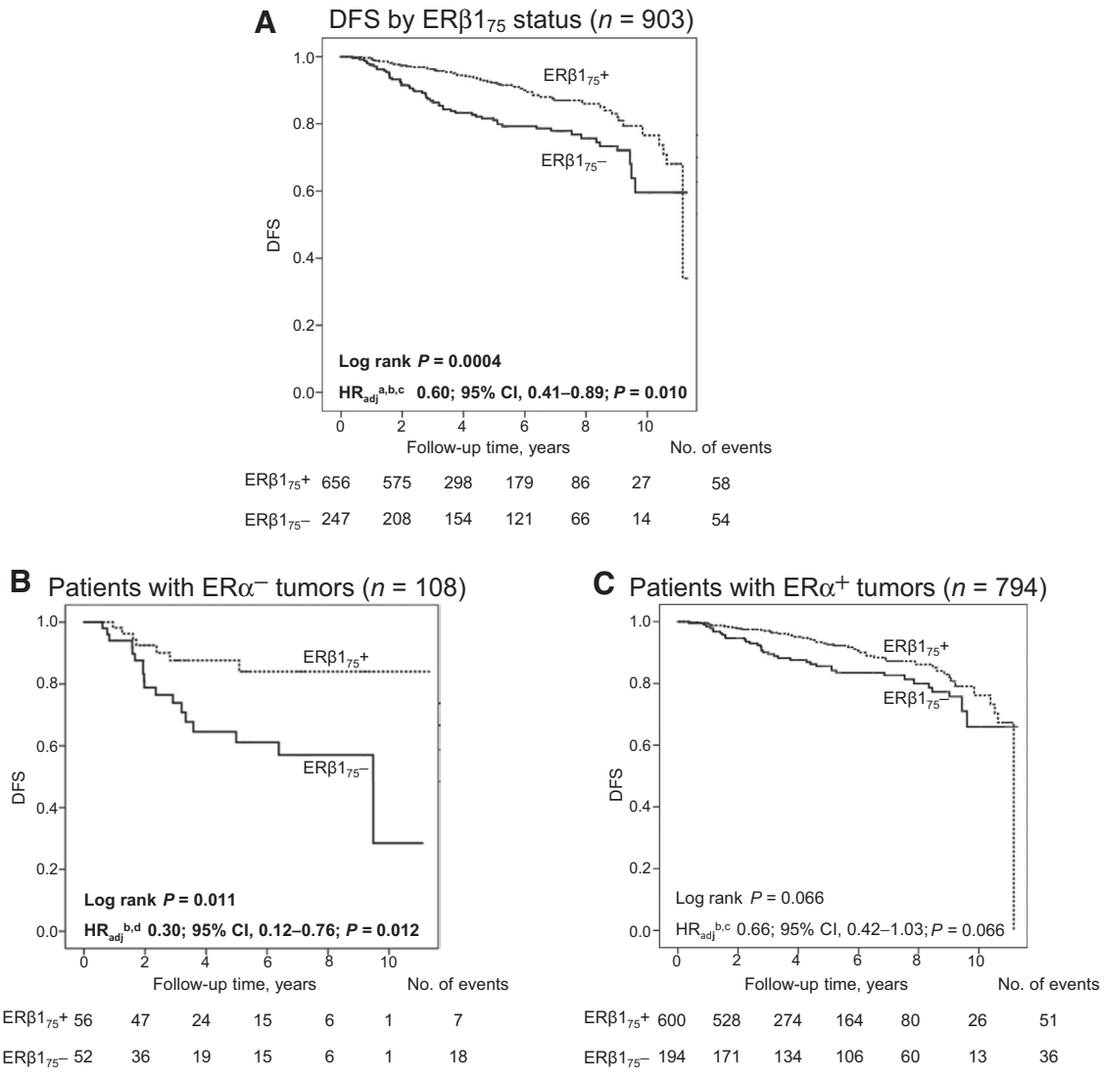

Figure 2.

The prognostic role of ERβ1$_{75}$, alone and in combination with ERα. **A-C,** Kaplan–Meier estimates of DFS for all patients ($n = 903$) by ERβ1$_{75}$ status (**A**), patients with ERα$^-$ tumors ($n = 108$) by ERβ1$_{75}$ status (**B**), patients with ERα$^+$ tumors ($n = 794$) by ERβ1$_{75}$ status (**C**). Because this is an ongoing cohort, the number of patients at each follow-up decreased. Bold letters indicate statistically significant results. HRs are presented with 95% confidence intervals (CI) and are adjusted for [a]ERα status (±), [b]invasive tumor size (<21 mm vs. ≥21 mm, or skin or muscular involvement independent of size), axillary lymph node involvement (yes/no), tumor grade 3 (yes/no), age (continuous), and adjuvant treatment ([c]radiotherapy yes/no, chemotherapy yes/no, tamoxifen yes/no, AIs yes/no, [d]radiotherapy yes/no, chemotherapy yes/no).

tumors had the worst prognosis. In the multivariable models, patients with ERα$^-$ and ERβ1$_{75}{}^-$ tumors had significantly worse prognosis across all models (Table 3, models 1–4). The prognosis for patients with discordant ERα and ERβ1$_{75}$-expressing tumors did not significantly differ from patients with tumors that coexpressed ERα and ERβ1$_{75}$. Hence, ERβ1$_{75}$ appeared to distinguish between patients with good or poor prognosis, regardless of ERα status.

ERβ1$_{10}{}^+$ was not associated with DFS, overall or when stratified by ERα status, nor was it associated with DFS in patients who received tamoxifen, AI, and/or chemotherapy (all log-rank Ps ≥ 0.29).

DFS within treatment groups by ERβ1$_{75}$ status

As ERβ1$_{75}$ but not ERα remained a prognostic factor after adjusting for risk factors and adjuvant treatment (Table 3), further analyses that stratified by treatment type were performed.

First, stratification by adjuvant chemotherapy was performed. Among the 232 chemotherapy-treated patients, ERβ1$_{75}{}^+$ expression was associated with only one third of the risk of any breast cancer event, compared with ERβ1$_{75}{}^-$. This association remained significant after adjusting for age, tumor characteristics, and adjuvant treatment (Fig. 3A). The association remained significant in the ERα$^-$ subgroup (log-rank $P = 0.024$; HR$_{adj} = 0.12$; 95% CI, 0.03–0.51) and in the ERα$^+$ subgroup (log-rank $P = 0.024$;

Table 3. DFS by ERβ1$_{75}$, ERα, and combinations of ERα and ERβ1$_{75}$ status

	Total	Events	Missing	Crude HR		Adjusted HR							
						Model 1		Model 2		Model 3		Model 4[a]	
Tumor status	***n***	***n***	***n***	**HR (95% CI)**	***P***	**HR$_{adj}$[b] (95% CI)**	***P***	**HR$_{adj}$[b,c] (95% CI)**	***P***	**HR$_{adj}$[b,d] (95% CI)**	***P***	**HR$_{adj}$[b,d,e] (95% CI)**	***P***
All	903											650	
ERβ1$_{75}$ status													
ERβ1$_{75}^{+}$	656	58	0	Ref.		Ref.		Ref.		Ref.		Ref.	
ERβ1$_{75}^{-}$	247	54		**1.93 (1.33–2.81)**	**0.001**	**1.63 (1.12–2.39)**	**0.011**	**1.60 (1.09–2.34)**	**0.016**	**1.66 (1.13–2.44)**	**0.010**	**2.06 (1.13–3.76)**	**0.018**
ERα status													
ERα^{+}	794	87	1	Ref.		Ref.		Ref.		Ref.		Ref.	
ERα^{-}	108	25		**2.58 (1.65–4.03)**	**<0.0001**	**1.92 (1.14–3.24)**	**0.014**	**1.79 (1.05–3.04)**	**0.032**	1.32 (0.66–2.64)	0.43	1.54 (0.56–4.24)	0.40
Combinations of ERα and ERβ75 status													
ERα^{+} ERβ1$_{75}^{+}$	600	51	1	Ref.		Ref.		Ref.		Ref.		Ref.	
ERα^{+} ERβ1$_{75}^{-}$	194	36		**1.56 (1.02–2.41)**	**0.042**	1.43 (0.93–2.21)	0.11	1.41 (0.91–2.18)	0.12	1.49 (0.96–2.32)	0.078	1.96 (0.95–4.04)	0.067
ERα^{-} ERβ1$_{75}^{+}$	56	7		1.57 (0.71–3.47)	0.26	1.31 (0.57–3.01)	0.53	1.24 (0.53–2.87)	0.62	0.99 (0.39–2.51)	0.98	1.42 (0.42–4.84)	0.58
ERα^{-} ERβ1$_{75}^{-}$	52	18		**4.72 (2.75–8.08)**	**<0.0001**	**3.50 (1.92–6.39)**	**<0.0001**	**3.17 (1.73–5.84)**	**0.0002**	**2.44 (1.16–5.16)**	**0.019**	**3.28 (1.06–10.19)**	**0.040**

NOTE: Events and missing data in the adjusted models: 111 events in model 1–3, missing; 3, 31, and 3 respectively. In model 4; 48 events, 1 missing. Bold letters indicate statistically significant results.

Abbreviations: ERβ1$_{75}$, ERβ1, cutoff for positivity >75%; HR$_{adj}$, adjusted HR.

[a]Patients included as of November 2005, $n = 650$.

[b]Adjusted for age (continuous), invasive tumor size (<21 mm vs. ≥21 mm or skin or muscular involvement independent of size), axillary lymph node involvement (yes/no) and tumor grade 3 (yes/no). Adjusted for ERα status (±) in ERβ1$_{75}$ only analysis, and for ERβ1$_{75}$ status (±) in ERα only analysis.

[c]Adjusted for BMI ≥ 25.0 kg/m^2 (yes/no) and preoperative current smoking (yes/no).

[d]Adjusted for treatment; tamoxifen, AIs, chemotherapy, and radiotherapy.

[e]Adjusted for trastuzumab treatment.

HR$_{adj}$ = 0.35; 95% CI, 0.14–0.86). ERα status had no impact on prognosis within the chemotherapy-treated group (Fig. 3B). Among the 671 chemonaïve patients, there was no significant association between ERβ1$_{75}$ status and DFS (Fig. 3C and D). Conversely, ERα was significantly associated with risk for breast cancer events among chemonaïve patients, but not among chemotherapy-treated patients (Fig. 3B and D).

In terms of endocrine treatment, ERβ1$_{75}^{+}$ was not associated with risk of any breast cancer event among the patients with ERα^{+} tumors who received tamoxifen and/or AIs (both log-rank *P*s ≥ 0.25). Among the tamoxifen-treated patients with ERα^{+} tumors who had also received chemotherapy, a tendency toward better prognosis with ERβ1$_{75}^{+}$ was seen in patients <50 years (log-rank $P = 0.067$), but not in older patients (log-rank $P = 0.33$). Among the chemonaïve tamoxifen-treated patients with ERα^{+} tumors, no association between ERβ1$_{75}$ status and prognosis was seen, irrespective of age (all log-rank *P*s ≥ 0.35). Among all AI-treated patients, no association between ERβ1$_{75}$ status and prognosis was seen, irrespective of chemotherapy and age.

DMFS and OS by ERβ1$_{75}$ status

The prognostic benefit of ERβ1$_{75}^{+}$ compared with ERβ1$_{75}^{-}$ was also seen in the analysis of DMFS (log-rank $P = 0.001$; HR$_{adj}$ = 0.57; 95% CI, 0.35–0.93). The association remained significant in the ERα^{-} subgroup (log-rank $P = 0.010$; HR$_{adj}$ = 0.13; 95% CI, 0.03–0.58) but not in the ERα^{+} subgroup (log-rank $P = 0.11$; HR$_{adj}$ = 0.69; 95% CI, 0.38–1.23). Within specific treatment groups, the benefit of ERβ1$_{75}^{+}$ remained significant in chemotherapy-treated patients (log-rank $P = 0.015$; HR$_{adj}$ = 0.31; 95% CI, 0.13–0.72) but not in the chemonaïve group (log-rank P = 0.052; HR$_{adj}$ = 0.69; 95% CI, 0.37–1.31). ERβ1$_{75}^{+}$ was not associated with DMFS in patients with ERα^{+} tumors who received tamoxifen and/or AIs overall, or when stratified by chemotherapy and age (all log-rank *P*s ≥ 0.14).

Among the 87 patients who died during follow-up, 53 patients (61%) had a reported breast cancer event prior to death. ERβ1$_{75}^{+}$ was associated with lower risk of death (log-rank $P = 0.0002$; HR$_{adj}$ = 0.50; 95% CI, 0.32–0.78), and the association was stronger in patients with ERα^{-} tumors (log-rank $P = 0.015$; HR$_{adj}$ = 0.20; 95% CI, 0.06–0.69) than in patients with ERα^{+} tumors (log-rank $P = 0.034$; HR$_{adj}$ = 0.60; 95% CI, 0.36–1.01).

ERβ1$_{75}^{+}$ was associated with a significantly lower risk of death in both chemotherapy-treated patients (log-rank $P = 0.014$, HR$_{adj}$ = 0.32; 95% CI, 0.12–0.80) and in chemonaïve patients (log-rank $P = 0.006$; HR$_{adj}$ = 0.51; 95% CI, 0.30–0.86). Among the 23 chemotherapy-treated patients who died, 87% had a reported breast cancer event prior to death. Among the 64 chemonaïve patients who died, 52% had a reported breast cancer event prior to death.

Among patients with ERα^{+} tumors, ERβ1$_{75}^{+}$ was associated with lower risk of death only in tamoxifen-treated patients (log-rank $P = 0.025$, HR$_{adj}$ = 0.49; 95% CI, 0.26–0.93) and not in AI-treated patients (log-rank $P = 0.50$). For tamoxifen-treated patients, this association was driven by the chemonaïve subgroup of patients 50 years or older (log-rank $P = 0.034$, HR$_{adj}$ = 0.47; 95% CI, 0.23–0.97), but it was not evident in patients that had received chemotherapy (log-rank $P = 0.63$), which is in contrast to the association between ERβ1$_{75}$ and DFS that was observed.

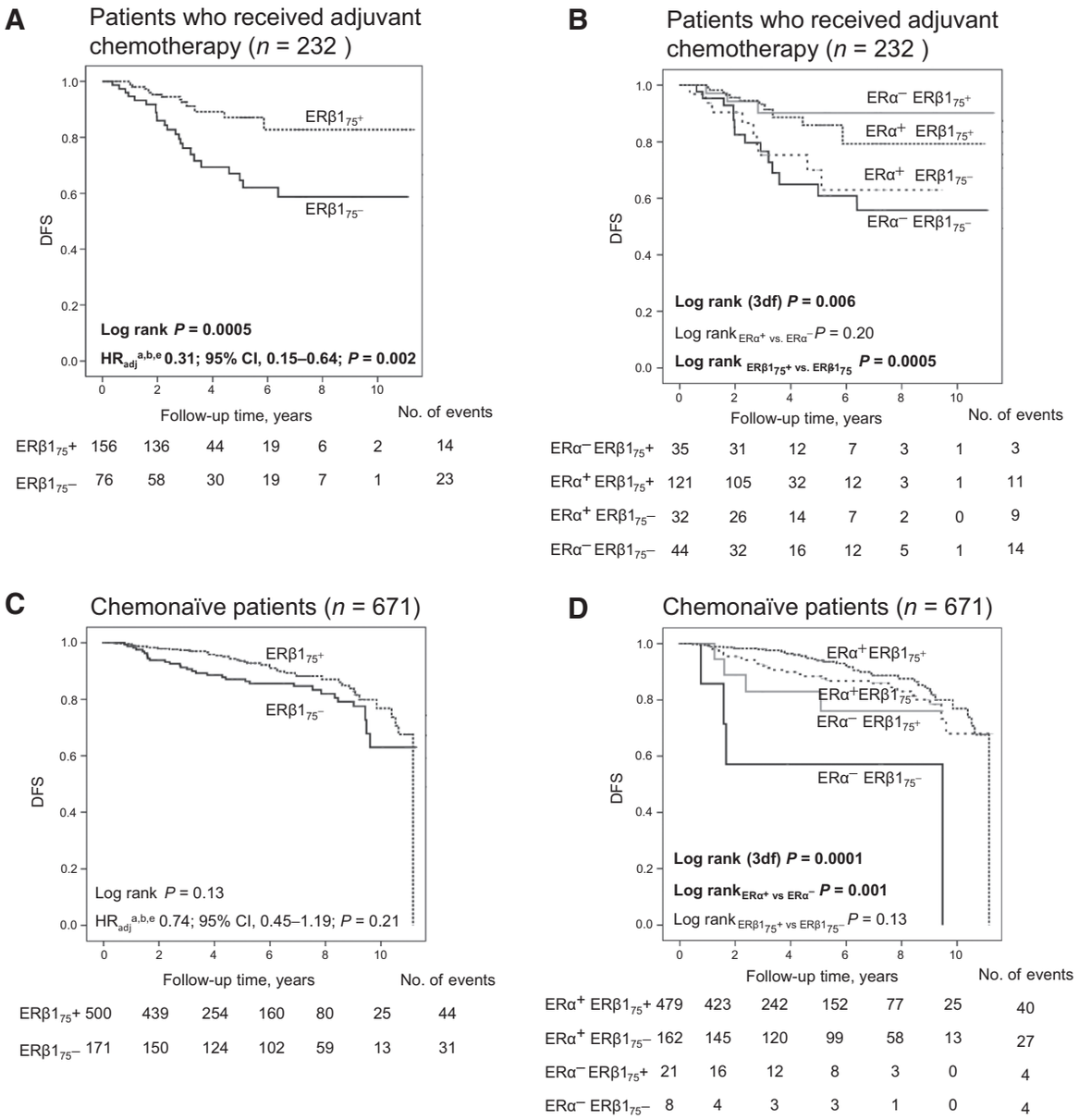

Figure 3.

A–D, DFS by ERβ1$_{75}$ status, alone and in combination with ERα, among patients who received adjuvant chemotherapy (**A** and **B,** respectively; $n = 232$), and chemonaïve patients (**C** and **D,** respectively, $n = 671$). Because this is an ongoing cohort, the number of patients decreased with each follow-up. Bold letters indicate statistically significant results. HRs are presented with 95% CI and adjusted for [a]ERα status (±), [b]invasive tumor size (<21 mm vs. ≥21 mm, or skin or muscular involvement independent of size), axillary lymph node involvement (yes/no), tumor grade 3 (yes/no), age (continuous); and adjuvant treatment ([c]radiotherapy yes/no, tamoxifen yes/no, AIs yes/no).

Discussion

In this study, high tumor ERβ1 expression was associated with favorable clinicopathological characteristics, but not with the previously studied *ESR2* genotypes. High tumor ERβ1 expression was identified as an independent favorable prognostic marker in breast cancer, especially for patients who received adjuvant chemotherapy. Previous reports of ERβ1 as a predictor of endocrine therapy response could not be confirmed in this cohort.

ERβ has high expression in normal breast tissue, and loss of ERβ expression is considered an early event in breast cancer progression (24). One possible mechanism for ERβ downregulation is promotor methylation, leading to loss of ERβ expression and thus reduced antiproliferative effects (5). Our group previously reported that the association between BMI and prognosis was dependent on *ESR2* genotypes and that the key to understanding these results may be ERβ promotor methylation, which may explain the previously reported association between *ESR2*

genotypes and anthropometrics (3). However, in the current study, there was no association between the previously studied *ESR2* genotypes and tumor-specific ERβ1 expression, irrespective of the cutoff used. It is possible that the germline *ESR2* genotypes affect ERβ expression or signaling on a systemic level that is not reflected in the tumor-specific ERβ expression. In addition, ERβ1 expression and anthropometrics were not associated. Further studies are needed to understand how germline genotypes might be associated with the tumor expression of the corresponding protein.

We could confirm our hypothesis that patients with high tumor ERβ1 expression had a better prognosis compared with patients with low ERβ1 expression. The association remained significant in analyses adjusted for ERα expression. The magnitude of the association was larger within the ERα$^-$ population. This may be explained by the shift of ERβ transcriptional binding sites that occurs in the absence of ERα (25) and was recently discussed in a review and meta-analysis (26). Another tentative mechanistic explanation may be the more pronounced ligand-independent actions and basal activity of ERβ compared with that of ERα (27). Previous results from this cohort suggested that the prognostic role of AR in breast cancer was dependent on the ERα status of the tumor (20). Similar hypotheses have been proposed for ERβ (10), and an *in vitro* study suggested ERβ to be the link between AR and ERα interactions (28). However, in the current study, unlike AR, ERβ$1_{75}$$^+$ was prognostically beneficial irrespective of ERα expression. In line with this finding, the association between ERβ and AR was dependent on ERα status, and the interaction was significant. To our knowledge, this has not been reported previously and merits further studies. If verified, these divergent prognostic results for AR and ERβ in patients with ERα$^-$ tumors would suggest opposite targeted treatment strategies for each: antiandrogens as a treatment option in the ERα$^-$/AR$^+$ setting, whereas patients with ERα$^-$/ERβ$1_{75}$$^+$ would rather benefit from ERβ agonists. However, a triple signature (6) was not explored in this study.

In a study by Honma and colleagues, patient outcome was analyzed by several ERβ antibodies, and the authors suggested that ERβ1 should be added to ERα and PR assessment in clinical routine (14). Therein, all patients received tamoxifen, also some patients with ERα- tumors, and ERβ1 was a prognostic marker irrespective of ERα status, which is in line with our findings. A recent meta-analysis also supports this finding (11). Furthermore, patients with ERα$^-$/ERβ$1_{75}$$^+$ tumors seemed to have good prognosis, on a level comparable with the prognosis for patients with ERα$^+$/ERβ$1_{75}$$^+$ tumors. We concluded that patients with double-negative (ERα$^-$/ERβ$1_{75}$$^-$) tumors had inferior prognosis in all adjusted models and thus remain a prognostically vulnerable group, with few targeted treatment options, for whom closer surveillance may be indicated.

The subgroup of patients with ERα$^-$/ERβ$1_{75}$$^+$ breast cancer would be a likely candidate patient population to target with ERβ agonists, as tested in an ongoing clinical trial (ClinicalTrials.gov identifier: NCT02352025). In addition, a recent phase II trial indicated that estradiol treatment might be beneficial in a selected ERβ$^+$ TNBC population (29). One *in vitro* study reported that ERβ agonists reduced cell invasion and the metastatic potential of TNBC (30). Also, new ways of directing ligands to nuclear hormone targets are under way (31), which was recently suggested as a future possibility for ERβ targeting (10).

A number of clinical studies have showed ERβ expression, either as pan-specific ERβ or as different isoforms, to be related to good prognosis and response to endocrine treatment (9–11). Contrasting results from large cohorts have also been reported; the Nurses' Health Study included 2,170 breast cancer patients with tumors of different molecular subtypes (32): It reported no association between ERβ1 expression and breast cancer–specific survival, either overall or within the tamoxifen-treated group (32). In the randomized controlled MA12-trial, tamoxifen-treated patients with ERβ1$^+$ tumors and who previously received chemotherapy had better survival than patients with ERβ1$^-$ tumors, especially if the tumor was ERα$^-$/ERβ1$^+$ (33). In the cohort presented by Nakopoulou and colleagues, in which patients received adjuvant chemotherapy and/or endocrine therapy, results were similar to our findings (34). As many of the clinical studies were observational studies and patients often received both chemotherapy and endocrine therapy (35, 36), it is somewhat surprising that associations between ERβ and chemotherapy have been rarely discussed (7, 24).

The main finding in this study was the impact of ERβ1_{75} expression on prognosis among patients who received adjuvant chemotherapy, some of whom also received tamoxifen and/or AIs. Thus, we performed stratified analyses according to age, chemotherapy, and type of endocrine treatment for all three endpoints in patients with ERα$^+$ tumors. However, we could not confirm our hypothesis that ERβ1 has an endocrine response–predictive role. The minor finding on tamoxifen response in relation to DFS in one single subgroup appeared to be driven by chemotherapy. For DMFS, the prognostic findings were similar to the findings for DFS. In analysis of OS, ERβ1_{75} expression was an independent prognostic marker, foremost in ERα$^-$ disease. In OS analyses by treatment groups, an association between ERβ1_{75} expression and response to tamoxifen but not to AIs was observed in the subgroup of chemonaïve patients $\geq$50 years. Our interpretation of this finding was that these patients more often die from other causes than their breast cancer, rather than reflecting improved response to tamoxifen treatment. Thus, in this cohort, the additional assessment of ERβ1 did not seem to improve the prediction of endocrine response to either AIs or tamoxifen, which suggests a role for ERβ1 in hormone-independent settings. We could not assess endocrine response among patients with ERα$^-$/ERβ$^+$ tumors, which has previously been described (12).

The strength of the study was that it was a prospective, population-based study with a wide variety of baseline and follow-up information and with high follow-up (37). As with all observational studies, the current study has built-in limitations, such as changes in treatment regimens over time and differences in the selection of treatment and how they are combined. This may account for the null finding on endocrine treatment and also limits the possibilities of comparing our result with previous randomized controlled trials, such as the study by Speirs and colleagues (15). Although Speirs and colleagues reported ERβ1 to be prognostic among patients who received switch treatment, they did not detect a prognostic benefit of ERβ1 expression in their overall population, in which all women received endocrine treatment. This is in line with our findings. The follow-up period was relatively short, especially given that ERα$^+$ tumors tend to relapse late, which may be one reason why any findings may have been more pronounced in patients with ERα$^-$ tumors. There was no question on ethnicity in the questionnaire for this study, but the majority of the study participants were of Swedish origin. The

main reason for nonparticipation was the lack of available research nurses (17). The age and frequency of ERα$^+$ in the cohort is similar to that of the Southern Sweden breast cancer population (18), indicating that the cohort is representative. Furthermore, the tumor analyses were based on TMAs, and even though some tumor cores were missing, we found no indication of bias. In the current study, assessment of Ki67 was not incorporated as Ki67 was not introduced into Swedish clinical routine until March 2009; however, it would be of interest to assess in future studies.

Our results regarding chemotherapy were in accordance with the recent study by Wang and colleagues, in which high ERβ1 tumor expression was an independent prognostic marker for chemotherapy-treated patients with TNBC tumors without endocrine treatment or trastuzumab (16). The finding was also supported by a neoadjuvant study, in which high pretreatment ERβ expression was associated with lower proliferation rates and better pathologic response in the posttreatment samples (38). An *in vitro* study suggested that the association might be explained by a chemosensitizing effect of ERβ in tumor protein p53 (p53)-mutant TNBC cell lines (39). Contrasting results were reported by a study on ERα$^+$ breast cancer cell lines where ERβ expression was associated with chemotherapy resistance, whereas tamoxifen response was independent of ERβ expression (40). Another study reported a chemosensitizing effect of ERβ5 expression, irrespective of the ERα and p53 status of the cell line (41). In the current study, p53 status was not available for analysis, and the response to chemotherapy was observed irrespective of ERα expression.

Some of the discrepancies between the results from the clinical and functional ERβ studies have been related to the different ERβ isoforms, as well as interlaboratory differences (5, 42). Also, there has been a lack of cancer cell models with reliable ERβ expression (8). A recent review that addressed clinical outcome in relation to ERβ expression focused exclusively on studies that used the validated antibodies *ppg5/10* (42–44) and *57/3*, directed at ERβ1 and ERβ2, respectively (7). ERβ1, the wild-type isoform, has ligand-binding ability and has been described as the only fully functional isoform (45). We therefore chose to address the prognostic effect of ERβ using the *ppg5/10* ERβ1-specific antibody that does not recognize and stain for ERα or ERβ2.

The immunohistochemical analysis of tumor ERβ1 expression has been far from standardized and merits further attention. The cutoffs used to define positivity have been described in many ways, including not defined, as "distinct nuclear staining" (32), or more commonly defined as >10% of positively stained nuclei (14, 34, 35). Also, scoring systems based on combinations of fraction and intensity have been commonly applied (16, 33, 46–48). Higher cutoffs, such as >20% (12, 46, 49, 50) or higher (33, 34, 36), have also been applied. One highly cited study applied cutoffs for ERβ1$^+$ that resulted in highly skewed distributions; >95% of the patients had ERβ1$^+$ tumors, and although there was a tendency toward a beneficial effect, it was reported as a null finding (46). A dose–response effect has been observed, either by grouped fractions (34) or by groups of stronger staining intensity (12). ERβ positivity has also been defined by moderate or stronger intensity, thereby excluding the weakly stained cases (12, 36, 49). Some previous studies have applied cutoffs that ultimately suggested significant prognostic effects on outcome, yet which displayed few, if any, associations with established clinicopathologic characteristics (12, 34, 36, 49), whereas others reported only associations between ERβ1$^+$ and established markers (32).

In the current study, we tried to address the above-mentioned issues by choosing a cutoff for which we could observe both associations with established clinicopathological characteristics and prognostic impact, as has been done previously (14, 16, 48). The recent meta-analysis reported ERβ1$^+$ of 67% across studies, in spite of varying cut-off point definitions (11), and we reported ERβ1$_{75}^+$ of 73%. We chose to also report the null findings for the cutoff >10%, as that cutoff has also been commonly used. Finally, we decided not to include intensity in our score to reduce variability.

In conclusion, this study provides support for high tumor ERβ1 expression as a marker of good prognosis in breast cancer, especially among chemotherapy-treated patients, but not in endocrine therapy–treated patients. The results warrant confirmation, preferably in an already performed randomized controlled trial, to evaluate chemotherapy response in relation to high ERβ1 expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Elebro, S. Borgquist, C. Ingvar, H. Jernström
Development of methodology: H. Jernström
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Elebro, S. Borgquist, A.H. Rosendahl, M. Simonsson, K. Jirström, C. Ingvar, H. Jernström
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Elebro, S. Borgquist, C. Ingvar, H. Jernström
Writing, review, and/or revision of the manuscript: K. Elebro, S. Borgquist, A.H. Rosendahl, A. Markkula, M. Simonsson, K. Jirström, C. Rose, C. Ingvar, H. Jernström
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Elebro, A. Markkula, M. Simonsson, K. Jirström, H. Jernström
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