High estrogen receptor beta expression is prognostic among adjuvant chemotherapy-treated patients – results from a population-based breast cancer cohort

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List of abbreviations

AI aromatase inhibitors
ALNI axillary lymph node involvement
AR androgen receptor
BMI body mass index
CI confidence interval
DFS disease-free survival
DMFS distant metastasis-free survival
ERα estrogen receptor alpha
ERβ estrogen receptor beta
ERβ1 estrogen receptor beta isoform 1
ERβ2/cx estrogen receptor beta isoform 2 or cx
ERβ5 estrogen receptor beta isoform 5
ESR2 estrogen receptor beta gene
HER2 human epidermal growth factor receptor 2
HR hazard ratio
HT hormonal therapy
htSNP haplotype tagging single nucleotide polymorphism
IQR interquartile range
Ki67 proliferative index
OR odds ratio
OS overall survival
p53 tumor protein p53
PR progesterone receptor
TAM tamoxifen
High Estrogen Receptor Beta Expression in Breast Cancer

TMA tissue microarray
TNBC triple negative breast cancer

Translational relevance

In this large, prospective population-based cohort of primary breast cancer, high tumor expression of estrogen receptor beta (ERβ1) (>75%) was associated with favorable clinicopathological characteristics, but not with the previously studied germline ERβ 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.
Abstract

Purpose: Isoform-specific tumor estrogen receptor beta (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, ERβ 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 five 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 ERβ genotypes. ERβ1 positivity, defined as >75% (ERβ175+, 72.7%), was positively associated with established favorable tumor characteristics. Overall, ERβ175+ was associated with lower risk of breast cancer events (HRadj 0.60; 95% CI 0.41-0.89). The magnitude of the association was larger in patients with ERα negative tumors (HRadj 0.30; 95% CI 0.12-0.76), compared with ERα positive tumors (HRadj 0.66; 95% CI 0.42-1.03). Among the 232 chemotherapy-treated patients, ERβ175+ tumors were associated with lower risk of breast cancer events compared to ERβ175- tumors (HRadj 0.31; 95% CI 0.15-0.64). Among the 671 chemonaïve patients, ERβ175 status was not associated with the outcome.

Conclusion

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.
Introduction

The complexity of estrogen receptor signaling in breast cancer was further revealed with the discovery of estrogen receptor beta (ERβ) in the 1990s (1). ERβ is encoded by estrogen beta receptor gene 2 (ESR2), which is highly polymorphic. The majority of the genetic variation can be captured by four haplotype tagging single nucleotide polymorphisms (htSNPs) (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 estrogen receptor alpha (ERα) activity (4), and to have an anti-proliferative 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, in order to better characterize the role of ERβ and its variants in breast cancer, with the ultimate aim to develop specific ERβ agonists in order 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 co-expressed with ERα, but also for patients with ERα negative (ERα-)/ERβ positive (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). Since 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 non-metastatic breast cancer was published. The ERβ isoforms 1, 2, and 5 (ERβ1, ERβ2/cx, 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 positive (ERα+) tumors. ERβ2 was only prognostic for DFS while ERβ5 was not associated with the 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 were 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 (TAM) to exemestane (15).

Furthermore, Wang et al. 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 pre-surgical 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 clinicopathological 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 ten 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 (Figure 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 3–6 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 were 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 previously described (3).

All patients signed informed consents upon enrollment. 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 µm TMA sections were cut for immunohistochemical semi-automated staining of ERβ1 (Autostainer Plus, Dako, Glostrup, Denmark), using the ERβ1 specific monoclonal antibody clone PPG5/10 (M7292, Dako, dilution 1:20). Semi-quantitative scoring of ERβ1 was performed twice independently by one researcher (KE) blinded to the clinical outcome. In cases where discrepancies occurred, a third scoring was performed (KE+AR) 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 (cut-off at >10% positively stained nuclei), was collected from pathology reports, as previously described (20-22). Human epidermal growth factor receptor 2 (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).

Statistical analyses

The statistical analyses were conducted with the software program SPSS version 22.0 (IBM, Chicago, IL, USA). Descriptive patient and tumor characteristics were summarized as either continuous variables (median, interquartile range (IQR)) 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 $\chi^2$ or logistic regression analyses, for which odds ratios (ORs) with 95% confidence intervals (CIs) 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 cut-offs (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 (HT), 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+), histological grade (I, II, III), ERα, PR, AR, combinations of ERα and PR status, and HER2 status (amplified/non-amplified). Information on adjuvant treatment by last follow-up and before any event was dichotomized for chemotherapy, radiotherapy, TAM, and aromatase inhibitors (AI). Trastuzumab treatment was incorporated into sub-group 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 LogRank test. Analyses were performed for ERβ1 status alone, and in combination with ERα status. Stratification by various treatment groups was performed; regarding 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 hazard ratios (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 III, any ALNI, ERα status). Model 2: age, tumor characteristics, BMI, and smoking. Model 3: age, tumor characteristics, and treatment (chemotherapy, radiotherapy, TAM, 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 (Figure 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/).
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 cut-off >75% of positively stained nuclei, 662 patients (72.7%) displayed ERβ175 positive (ERβ175+) tumors. These patients were older at inclusion and had smaller breast volumes compared to patients with ERβ175 negative (ERβ175-) tumors. Other patient characteristics such as anthropometric measures, reproductive factors, and ever use of exogenous hormones showed no significant associations with ERβ175 status (Table 1). In terms of tumor characteristics, ERβ175+ was associated with smaller tumor size, lower histological grade, less axillary lymph node involvement, as well as co-expression of ERα, PR, and AR (Table 2). Tumors that co-expressed ERα and AR were six times more likely to also express ERβ175+ compared to 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 ERβ175- tumors compared to ERβ175+ tumors. The lowest frequency of HER2 amplification was found in tumors that co-expressed ERα and ERβ175 (7.8%). HER2 amplification was most common in ERα- tumors, irrespective of ERβ175 and/or PR status (30.3-32.3%), (Table 2).

ERβ1 positivity, defined as >10% of positively stained nuclei [ERβ110+, n=839 (92.1%)], was associated with ERα and AR co-expression ($P_s < 0.0001$). ERβ110+ did not demonstrate significant associations with other tumor markers such as invasive tumor size, histological 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 ERβ175 and ERβ110 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/m², where two htSNPs and the two haplotypes were differently associated with DFS depending on BMI in our previous report (3).

**Disease-free survival 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 ERβ175+ tumors had approximately two thirds the risk for any breast cancer event compared to patients with ERβ175- tumors (Figure 2A). In the ERα- subgroup, patients with ERβ175+ tumors had one third the risk for an event compared to patients with ERβ175- tumors, and this association remained significant after adjusting for age, tumor characteristics, and adjuvant treatment (Figure 2B). Among patients with ERα+ tumors, ERβ175+ was also prognostically favorable. However, the magnitude of the association was smaller. Patients with ERβ175+ tumors had two thirds the risk for an event compared to patients with ERβ175- tumors and this association was not statistically significant ($P=0.066$) (Figure 2C).

ERβ175 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 ERβ175 remained significant (Table 3, model 3). This association also existed in the subgroup analyses that included treatment with trastuzumab (Table 3, model 4).

In order to further characterize the prognostic role of ERβ175, the combinations of ERα and ERβ175 status were analyzed further. In univariable analyses, patients with tumors
that co-expressed ERα and ERβ175 had the best prognosis and were used as a reference group. Conversely, patients with ERα- and ERβ175- tumors had the worst prognosis. In the multivariable models, patients with ERα- and ERβ175- tumors had significantly worse prognosis across all models (Table 3, models 1-4). The prognosis for patients with discordant ERα and ERβ175 expressing tumors did not significantly differ from patients with tumors that co-expressed ERα and ERβ175. Hence, ERβ175 appeared to distinguish between patients with good or poor prognosis, regardless of ERα status.

ERβ110+ 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 LogRank Ps ≥0.29).

Disease-free survival within treatment groups by ERβ175 status

Since ERβ175 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, ERβ175+ expression was associated with only one third of the risk of any breast cancer event, compared to ERβ175-. This association remained significant after adjusting for age, tumor characteristics, and adjuvant treatment (Figure 3A). The association remained significant in the ERα- subgroup (LogRank P=0.024, HRadj 0.12: 95% CI 0.03-0.51) and in the ERα+ subgroup (LogRank P=0.024, HRadj 0.35: 95% CI 0.14-0.86). ERα status had no impact on prognosis within the chemotherapy-treated group (Figure 3B). Among the 671 chemonaïve patients, there was no significant association between ERβ175 status and DFS (Figure 3C-D). Conversely, ERα was significantly associated with risk for
breast cancer events among chemonaive patients, but not among chemotherapy-treated patients (Figure 3B,D).

In terms of endocrine treatment, ERβ175+ was not associated with risk of any breast cancer event among the patients with ERα+ tumors who received TAM and/or AIs (both LogRank \( P \geq 0.25 \)). Among the TAM-treated patients with ERα+ tumors who had also received chemotherapy, a tendency towards better prognosis with ERβ175+ was seen in patients <50 years (LogRank \( P = 0.067 \)), but not in older patients (LogRank \( P = 0.33 \)). Among the chemonaive TAM-treated patients with ERα+ tumors, no association between ERβ175 status and prognosis was seen, irrespective of age (all LogRank \( P \geq 0.35 \)). Among all AI-treated patients, no association between ERβ175 status and prognosis was seen, irrespective of chemotherapy and age.

**Distant metastasis-free survival and overall survival by ERβ175 status**

The prognostic benefit of ERβ175+ compared to ERβ175- was also seen in the analysis of DMFS (LogRank \( P = 0.001 \), HR_{adj} 0.57: 95% CI 0.35-0.93). The association remained significant in the ERα- subgroup (LogRank \( P = 0.010 \), HR_{adj} 0.13: 95% CI 0.03-0.58) but not in the ERα+ subgroup (LogRank \( P = 0.11 \), HR_{adj} 0.69: 95% CI 0.38-1.23). Within specific treatment groups, the benefit of ERβ175+ remained significant in chemotherapy-treated patients (LogRank \( P = 0.015 \), HR_{adj} 0.31: 95% CI 0.13-0.72) but not in the chemonaive group (LogRank \( P = 0.052 \), HR_{adj} 0.69: 95% CI 0.37-1.31). ERβ175+ was not associated with DMFS in patients with ERα+ tumors who received TAM and/or AIs overall, or when stratified by chemotherapy and age (all LogRank \( P \geq 0.14 \)).

Among the 87 patients who died during follow-up, 53 patients (61%) had a reported breast cancer event prior to death. ERβ175+ was associated with lower risk of death.
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(LogRank $P=0.0002$, $HR_{adj} 0.50$: 95% CI 0.32-0.78), and the association was stronger in patients with ER$\alpha$- tumors ($LogRank P=0.015$, $HR_{adj} 0.20$: 95% CI 0.06-0.69) than in patients with ER$\alpha$+ tumors ($LogRank P=0.034$, $HR_{adj} 0.60$: 95% CI 0.36-1.01).

ER$\beta_{175}$+ was associated with a significantly lower risk of death in both chemotherapy-treated patients ($LogRank P=0.014$, $HR_{adj} 0.32$: 95% CI 0.12-0.80) and in chemonaïve patients ($LogRank P=0.006$, $HR_{adj} 0.51$: 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 ER$\alpha$+ tumors, ER$\beta_{175}$+ was associated with lower risk of death in only TAM-treated patients ($LogRank P=0.025$, $HR_{adj} 0.49$: 95% CI 0.26-0.93) and not in AI-treated patients ($LogRank P=0.50$). For TAM-treated patients, this association was driven by the chemonaïve subgroup of patients 50 years or older ($LogRank P=0.034$, $HR_{adj} 0.47$: 95% CI 0.23-0.97), but it was not evident in patients that had received chemotherapy ($LogRank P=0.63$), which is in contrast to the association between ER$\beta_{175}$ and DFS that was observed.
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 promoter methylation, leading to loss of ERβ expression and thus reduced anti-proliferative 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 promoter methylation, which may explain the previously reported association between ESR2 genotypes and anthropometrics (3). However, in the present study, there was no association between the previously studied ESR2 genotypes and tumor-specific ERβ1 expression, irrespective of the cut-off 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 to 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 to 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 present study, unlike AR, ERβ175+ 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 previously reported 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: anti-androgens as a treatment option in the ERα-/AR+ setting, whereas patients with ERα-/ERβ175+ would rather benefit from ERβ agonists. However a triple-signature (6) was not explored in this study.

In a study by Honma et al., 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 TAM, 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-/ERβ175+ tumors seemed to have good prognosis, on a level comparable to the prognosis for patients with ERα+/ERβ175+ tumors. We concluded that patients with double negative (ERα-/ERβ175-) 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-/ERβ175+ breast cancer would be a likely candidate patient population to target with ERβ agonists, as tested in an ongoing clinical trial.
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(ClinicalTrials.gov identifier: NCT02352025). Additionally, a recent phase 2 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 underway (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 TAM-treated group (32). In the randomized controlled MA12-trial, TAM-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 et al., in which patients received adjuvant chemotherapy and/or endocrine therapy, results were similar to our findings (34). Since 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 ERβ175 expression on prognosis among patients who received adjuvant chemotherapy, some of whom also received TAM 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 TAM 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, ERβ175 expression was an independent prognostic marker, foremost in ERα- disease. In OS analyses by treatment groups, an association between ERβ175 expression and response to TAM but not to AIs was observed in the subgroup of chemonaïve patients ≥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 TAM treatment. Thus, in this cohort, the additional assessment of ERβ1 did not seem to improve the prediction of endocrine response to either AIs or TAM, 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 present 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 et. al. (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 possible 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 non-participation 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 present study, assessment of Ki67 was not incorporated since 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 et al., 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 pre-treatment ERβ expression was associated with lower proliferation rates and better pathological response in the post-treatment 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 was reported by a study on ERα+ breast cancer cell lines where ERβ expression was associated with chemotherapy resistance, whereas TAM 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 present 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 inter-laboratory 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 cut-offs 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 cut-offs, such as >20% (12,46,49,50) or higher (33,34,36), have also been applied. One highly-cited study applied cut-offs for ERβ1+ that resulted in highly skewed distributions; >95% of the patients had ERβ1+ tumors and although there was a tendency towards 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 cut-offs that ultimately suggested significant prognostic effects on outcome, yet which displayed few, if any, associations with established clinicopathological characteristics (12,34,36,49), while others reported only associations between ERβ1+ and established markers (32).

In the present study, we tried to address the above-mentioned issues by choosing a cut-off 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 ERβ175+ of 73%. We chose to also report the null findings
for the cut-off >10%, since that cut-off has also been commonly used. Finally, we decided not to include intensity in our score, in order 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, in order to evaluate chemotherapy response in relation to high ERβ1 expression.

Acknowledgements and funding

We wish to thank our research nurses Anette Ahlin Gullers, Monika Eberhard Mészaros, Maj-Britt Hedenblad, Karin Henriksson, Anette Möller, Helén Thell, Jessica Åkesson, and Linda Ågren. We also wish to thank Erika Bågeman, Maria Henningson, and Maria Hjertberg for data entry, Björn Nodin and Elise Nilsson for TMA construction, Kristina Lövgren for immunohistochemical staining, and Catarina Blennow for sectioning, as well as breast pathologist Anna Ehinger (AE) for help with histopathological assessments.
References


33. Yan Y, Li X, Blanchard A, Bramwell VH, Pritchard KI, Tu D, et al. Expression of both estrogen receptor-beta 1 (ER-beta1) and its co-regulator steroid receptor RNA activator protein (SRAP) are predictive for benefit from tamoxifen therapy in patients


Table 1. Patient characteristics by ERβ175 status.

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>All</th>
<th>Missing total</th>
<th>Patients with available tumor ERβ1 status</th>
<th>Missing ERβ1 status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 1026</td>
<td></td>
<td>ERβ175 negative</td>
<td>ERβ175 positive</td>
</tr>
<tr>
<td>Age at diagnosis, yrs</td>
<td>61.1 (52.1-68.1)</td>
<td>0</td>
<td>59.6 (51.0-66.6)</td>
<td>61.9 (53.4-68.9)</td>
</tr>
<tr>
<td>Weight, kgs</td>
<td>69.0 (62.0-78.0)</td>
<td>26</td>
<td>70.0 (63.0-79.3)</td>
<td>69.0 (61.0-78.0)</td>
</tr>
<tr>
<td>Height, m</td>
<td>1.65 (1.62-1.70)</td>
<td>26</td>
<td>1.65 (1.62-1.70)</td>
<td>1.66 (1.62-1.70)</td>
</tr>
<tr>
<td>BMI, kgs/m²</td>
<td>25.1 (22.5-28.3)</td>
<td>28</td>
<td>25.6 (22.9-28.6)</td>
<td>25.0 (22.4-28.3)</td>
</tr>
<tr>
<td>Waist-Hip Ratio, m/m</td>
<td>0.86 (0.81-0.90)</td>
<td>38</td>
<td>0.85 (0.80-0.90)</td>
<td>0.86 (0.81-0.90)</td>
</tr>
<tr>
<td>Total breast volume, mL</td>
<td>1000 (650-1500)</td>
<td>160</td>
<td>1000 (700-1600)</td>
<td>950 (650-1500)</td>
</tr>
<tr>
<td>≥850mL, %</td>
<td>57.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at menarche, yrs</td>
<td>13 (12-14)</td>
<td>6</td>
<td>13 (12-14)</td>
<td>13 (12-14)</td>
</tr>
<tr>
<td>Parous, %</td>
<td>87.9</td>
<td>1</td>
<td>88.0</td>
<td>87.7</td>
</tr>
<tr>
<td>Age at first full term pregnancy, yrs</td>
<td>25 (22-28)</td>
<td>131</td>
<td>24 (21-28)</td>
<td>25 (22-28)</td>
</tr>
<tr>
<td>Ever use of oral contraceptives, %</td>
<td>70.8</td>
<td>1</td>
<td>69.4</td>
<td>70.8</td>
</tr>
<tr>
<td>Ever use of HT, %</td>
<td>43.9</td>
<td>3</td>
<td>43.1</td>
<td>44.1</td>
</tr>
<tr>
<td>Coffee intake ≥2 cups/day</td>
<td>81.4</td>
<td>4</td>
<td>83.8</td>
<td>80.2</td>
</tr>
<tr>
<td>Current smoker prior to surgery, %</td>
<td>20.5</td>
<td>2</td>
<td>24.1</td>
<td>19.2</td>
</tr>
<tr>
<td>Abstainer, %</td>
<td>10.5</td>
<td>7</td>
<td>12.6</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*Mann-Whitney U test.
Bold letters indicate statistically significant results.
Abbreviations: BMI = body mass index; CI = confidence interval; ERβ175 = estrogen receptor beta 1, cut-off for positivity >75%; HT = hormone therapy; IQR = interquartile range; OR = odds ratio.
Table 2. Tumor characteristics by ERβ175 status.

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>All</th>
<th>Missing</th>
<th>Patients with available tumor ERβ1 status</th>
<th>Missing ERβ1 status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ERβ175 negative</td>
<td>ERβ175 positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Invasive tumor size</td>
<td>1026</td>
<td>0</td>
<td>1026</td>
<td></td>
</tr>
<tr>
<td>1 (≤20 mm)</td>
<td>740 (72.1)</td>
<td>249</td>
<td>165 (66.3)</td>
<td>486 (73.4)</td>
</tr>
<tr>
<td>2 (21-50 mm)</td>
<td>269 (26.2)</td>
<td>66</td>
<td>78 (31.3)</td>
<td>166 (25.1)</td>
</tr>
<tr>
<td>3 (&gt;51 mm)</td>
<td>15 (1.5)</td>
<td>2</td>
<td>6 (2.4)</td>
<td>8 (1.2)</td>
</tr>
<tr>
<td>4 (skin or muscular involvement independent of size)</td>
<td>2 (0.2)</td>
<td>0</td>
<td>0 (0.0)</td>
<td>2 (0.3)</td>
</tr>
<tr>
<td>Axillary lymph node involvement</td>
<td>1024</td>
<td>2</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>627 (61.2)</td>
<td>115</td>
<td>134 (54.0)</td>
<td>417 (63.1)</td>
</tr>
<tr>
<td>1-3</td>
<td>306 (29.9)</td>
<td>56</td>
<td>89 (35.9)</td>
<td>185 (28.0)</td>
</tr>
<tr>
<td>≥4</td>
<td>91 (8.9)</td>
<td>5</td>
<td>25 (0.1)</td>
<td>59 (8.9)</td>
</tr>
<tr>
<td>Histological grade</td>
<td>1025</td>
<td>1</td>
<td>1025</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>252 (24.6)</td>
<td>44</td>
<td>42 (16.9)</td>
<td>177 (26.7)</td>
</tr>
<tr>
<td>II</td>
<td>511 (49.9)</td>
<td>80</td>
<td>123 (49.4)</td>
<td>332 (50.2)</td>
</tr>
<tr>
<td>III</td>
<td>262 (25.6)</td>
<td>81</td>
<td>84 (33.7)</td>
<td>153 (23.1)</td>
</tr>
<tr>
<td>Hormone receptor status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα+</td>
<td>896 (87.5)</td>
<td>1</td>
<td>896</td>
<td>194 (78.2)</td>
</tr>
<tr>
<td>PR+</td>
<td>726 (70.9)</td>
<td>1</td>
<td>726</td>
<td>163 (65.7)</td>
</tr>
<tr>
<td>AR+</td>
<td>776 (85.0)</td>
<td>1</td>
<td>776</td>
<td>186 (75.9)</td>
</tr>
<tr>
<td>in ERα- subgroup</td>
<td>50 (44.2)</td>
<td>15</td>
<td>50</td>
<td>27 (51.9)</td>
</tr>
<tr>
<td>in ERα+ subgroup</td>
<td>726 (90.9)</td>
<td>97</td>
<td>726</td>
<td>159 (82.8)</td>
</tr>
<tr>
<td>Combined ER and PR status</td>
<td>1024</td>
<td>2</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>ERα-PR-</td>
<td>122 (11.9)</td>
<td>50</td>
<td>122</td>
<td>50 (20.2)</td>
</tr>
<tr>
<td>ERα-PR+</td>
<td>6 (0.6)</td>
<td>2</td>
<td>6</td>
<td>4 (1.6)</td>
</tr>
<tr>
<td>ERα+PR-</td>
<td>176 (17.2)</td>
<td>2</td>
<td>176</td>
<td>35 (14.1)</td>
</tr>
<tr>
<td>ERα+PR+</td>
<td>720 (70.3)</td>
<td>159</td>
<td>720</td>
<td>159 (64.1)</td>
</tr>
<tr>
<td>As of November 2005:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2 amplification¹</td>
<td>688</td>
<td>50³</td>
<td>688</td>
<td>23 (19.5)</td>
</tr>
<tr>
<td>Among all</td>
<td>688</td>
<td>50³</td>
<td>688</td>
<td>23 (19.5)</td>
</tr>
<tr>
<td>in ERα- subgroup</td>
<td>28 (31.8)</td>
<td>1</td>
<td>28</td>
<td>10 (30.3)</td>
</tr>
<tr>
<td>in ERα- PR- subgroup</td>
<td>28 (32.9)</td>
<td>0</td>
<td>28</td>
<td>10 (32.3)</td>
</tr>
<tr>
<td>in ERα+ subgroup</td>
<td>58 (9.7)</td>
<td>49</td>
<td>58</td>
<td>13 (15.3)</td>
</tr>
<tr>
<td>Treatment by last follow-up⁶</td>
<td>1026</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ever chemotherapy</td>
<td>259 (25.2)</td>
<td>0</td>
<td>259</td>
<td>76 (30.5)</td>
</tr>
<tr>
<td>ever radiotherapy</td>
<td>641 (62.5)</td>
<td>0</td>
<td>641</td>
<td>158 (63.5)</td>
</tr>
<tr>
<td>ever endocrine therapy</td>
<td>694 (77.5)</td>
<td>0</td>
<td>694</td>
<td>163 (64.0)</td>
</tr>
<tr>
<td>ever tamoxifen</td>
<td>528 (58.9)</td>
<td>0</td>
<td>528</td>
<td>129 (46.5)</td>
</tr>
<tr>
<td>ever aromatase inhibitors</td>
<td>345 (38.5)</td>
<td>0</td>
<td>345</td>
<td>89 (49.5)</td>
</tr>
<tr>
<td>As of November 2005:</td>
<td>738</td>
<td>0⁺</td>
<td>738</td>
<td>18 (14.0)</td>
</tr>
<tr>
<td>ever trastuzumab⁵</td>
<td>66 (8.9)</td>
<td>0⁺</td>
<td>66</td>
<td>18 (14.0)</td>
</tr>
</tbody>
</table>

¹Chi Square 3 df. ²Chi Square 2 df.
³HER2 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.
⁴Data on trastuzumab treatment was available for all patients as of November 2005. However, 50 patients (6.8%) had missing HER2 status. Bold letters indicate statistically significant results.
Abbreviations: CI = confidence interval; df = degree of freedom; ERα = estrogen receptor alpha; ERβ175 = estrogen receptor beta 1, cut-off for positivity >75%; HER2 = human epidermal growth factor-2; OR = odds ratio; PR = progesterone receptor.
### Table 3. Disease-free survival by ERβ175, ERα and combinations of ERα and ERβ175 status.

<table>
<thead>
<tr>
<th>Tumor status</th>
<th>Total Events</th>
<th>Missing</th>
<th>Crude HR (95% CI)</th>
<th>HRadj (95% CI)</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Adjusted HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>903</td>
<td>650</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ175 status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ175 +</td>
<td>656</td>
<td>58</td>
<td>1.93 (1.33-2.81)</td>
<td>1.60 (1.09-2.34)</td>
<td>1.66 (1.13-2.44)</td>
<td>2.06 (1.13-3.76)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ175 -</td>
<td>247</td>
<td>54</td>
<td>2.58 (1.65-4.03)</td>
<td>1.92 (1.14-3.24)</td>
<td>1.79 (1.05-3.04)</td>
<td>1.32 (0.66-2.64)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα +</td>
<td>794</td>
<td>87</td>
<td>1.56 (1.02-2.41)</td>
<td>1.43 (0.93-2.21)</td>
<td>1.41 (0.91-2.18)</td>
<td>1.49 (0.96-2.32)</td>
<td>1.96 (0.95-4.04)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα -</td>
<td>108</td>
<td>25</td>
<td>4.72 (2.75-8.08)</td>
<td>3.50 (1.92-6.39)</td>
<td>3.17 (1.73-5.84)</td>
<td>2.44 (1.16-5.16)</td>
<td>3.28 (1.06-10.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combinations of ERα and ERβ175 status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα +</td>
<td>600</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERβ175 -</td>
<td>194</td>
<td>36</td>
<td>1.57 (0.71-3.47)</td>
<td>1.31 (0.57-2.87)</td>
<td>1.24 (0.39-2.51)</td>
<td>0.99 (0.42-4.84)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα -</td>
<td>52</td>
<td>18</td>
<td>4.72 (2.75-8.08)</td>
<td>3.50 (1.92-6.39)</td>
<td>3.17 (1.73-5.84)</td>
<td>2.44 (1.16-5.16)</td>
<td>3.28 (1.06-10.19)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Patients included as of November 2005, n=650
*Adjusted for age (continuous), invasive tumor size (<21 mm versus ≥21 mm or skin or muscular involvement independent of size), axillary lymph node involvement (yes/no) and tumor grade III (yes/no). Adjusted for ERα status (+/-) in ERβ175 only analysis, and for ERβ175 status (+/-) in ERα only analysis.
*Adjusted for body mass index ≥25.0 kg/m2 (yes/no) and preoperative current smoking (yes/no).
*Adjusted for treatment; tamoxifen, aromatase inhibitors, chemotherapy, and radiotherapy.
*Adjusted for trastuzumab treatment.

Events and missing data in the adjusted model: 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: CI = confidence interval; ERα = Estrogen receptor alpha; ERβ175 = estrogen receptor beta 1, cut-off for positivity >75%; HRadj = adjusted hazard ratio.
Figure legends

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

Figure 2. The prognostic role of ERβ175, alone and in combination with ERα. Kaplan-Meier estimates of disease-free survival for: A) all patients (n = 903) by ERβ175 status, B) patients with ERα negative tumors (n = 108) by ERβ175 status, C) patients with ERα positive tumors (n = 794) by ERβ175 status, and D) combinations of ERα and ERβ175 status. Because this is an ongoing cohort, the number of patients at each follow-up decreased. Bold letters indicate statistically significant results. Hazard ratios are presented with 95% confidence intervals (CI) and are adjusted for: aERα status (+/-); binvasive tumor size (<21 mm versus ≥21 mm, or skin or muscular involvement independent of size), axillary lymph node involvement (yes/no), tumor grade III (yes/no), age (continuous); and adjuvant treatment (cradiotherapy yes/no, chemotherapy yes/no, tamoxifen yes/no, aromatase inhibitors yes/no; dradiotherapy yes/no, chemotherapy yes/no).

Figure 3. Disease-free survival by ERβ175 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. Hazard ratios are presented with 95% confidence interval (CI) and adjusted for: aERα status (+/-); binvasive tumor size (<21 mm versus ≥21 mm, or skin or muscular involvement independent of size), axillary lymph node involvement (yes/no), tumor grade III (yes/no), age (continuous); and adjuvant treatment (cradiotherapy yes/no, tamoxifen yes/no, aromatase inhibitors yes/no).
Additional material provided:

Figure 1

Figure 2

Figure 3
Patients with primary breast cancer Oct 2002-June 2012 $n = 1116$

- Preoperative treatment $n = 51$
- In situ carcinoma $n = 39$

Invasive breast cancer $N = 1026$

- Available ERβ₁ seventy-five score $n = 911$
  - ERβ₁ seventy-five positive $n = 662 (72.7\%)$
  - ERβ₁ seventy-five negative $n = 249 (27.3\%)$

- Missing ERβ₁ seventy-five score $n = 115$

- Distant metastasis ≤ 0.3 years from baseline $n = 8$
  - ERβ₁ seventy-five positive $n = 656 (72.6\%)$
  - ERβ₁ seventy-five negative $n = 247 (27.4\%)$

- Included in survival analyses $n = 903$
  - ERβ₁ seventy-five positive $n = 650$
    - ERβ₁ seventy-five + $n = 47 (67.1\%)$
    - ERβ₁ seventy-five - $n = 23 (32.9\%)$
  - ERβ₁ seventy-five negative $n = 247 (27.3\%)$

Routine HER2 assessment initiated as of November 2005

- HER2 + $n = 86$
- HER2 - $n = 602$
- HER2 missing $n = 50$

Figure 1
Figure 2

A Disease-free survival by ERβ1.75 status (n=903)

<table>
<thead>
<tr>
<th>Follow-up, Years</th>
<th>No. of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>656 575 298 179 86 27</td>
</tr>
<tr>
<td>1</td>
<td>528 36 19 15 6 1</td>
</tr>
<tr>
<td>3</td>
<td>194 171 134 106 60 13</td>
</tr>
<tr>
<td>5</td>
<td>194 171 134 106 60 13</td>
</tr>
<tr>
<td>7</td>
<td>194 171 134 106 60 13</td>
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<tr>
<td>9</td>
<td>194 171 134 106 60 13</td>
</tr>
<tr>
<td>10</td>
<td>194 171 134 106 60 13</td>
</tr>
</tbody>
</table>

LogRank $P=0.0004$

$HR_{adj}^{a+b+c} = 0.60$; 95% CI 0.41-0.89; $P=0.010$

B Patients with ERα negative tumors (n=108)

<table>
<thead>
<tr>
<th>Follow-up, Years</th>
<th>No. of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>56 47 24 15 6 1 7</td>
</tr>
<tr>
<td>1</td>
<td>56 47 24 15 6 1 7</td>
</tr>
<tr>
<td>3</td>
<td>56 47 24 15 6 1 7</td>
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<tr>
<td>5</td>
<td>56 47 24 15 6 1 7</td>
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<td>7</td>
<td>56 47 24 15 6 1 7</td>
</tr>
<tr>
<td>9</td>
<td>56 47 24 15 6 1 7</td>
</tr>
<tr>
<td>10</td>
<td>56 47 24 15 6 1 7</td>
</tr>
</tbody>
</table>

LogRank $P=0.011$

$HR_{adj}^{b+d} = 0.30$; 95% CI 0.12-0.76; $P=0.012$

C Patients with ERα positive tumors (n=794)

<table>
<thead>
<tr>
<th>Follow-up, Years</th>
<th>No. of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>600 528 274 164 80 26 51</td>
</tr>
<tr>
<td>1</td>
<td>600 528 274 164 80 26 51</td>
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<tr>
<td>3</td>
<td>600 528 274 164 80 26 51</td>
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<tr>
<td>5</td>
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<td>7</td>
<td>600 528 274 164 80 26 51</td>
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<td>9</td>
<td>600 528 274 164 80 26 51</td>
</tr>
<tr>
<td>10</td>
<td>600 528 274 164 80 26 51</td>
</tr>
</tbody>
</table>

LogRank $P=0.066$

$HR_{adj}^{a+c} = 0.66$; 95% CI 0.42-1.03; $P=0.066$
Figure 3

A. Patients who received adjuvant chemotherapy (n=232)

Disease-free survival

LogRank $P = 0.0005$

HR$_{adj}^{a+b+e} = 0.31$: 95% CI 0.15-0.64; $P=0.002$

Follow-up time, Years  No. of events
ER$\beta_{175}^+$ 156 136 44 19 6 2 14
ER$\beta_{175}^-$ 76 58 30 19 7 1 23

B. Patients who received adjuvant chemotherapy (n=232)

Disease-free survival

LogRank (3df) $P=0.006$

LogRank $ER\alpha^- vs ER\alpha^+$ $P=0.20$

LogRank $ER\beta_{175}^+ vs ER\beta_{175}^-$ $P=0.0005$

Follow-up time, Years  No. of events
ER$\alpha^- ER\beta_{175}^+$ 35 31 12 7 3 1 3
ER$\alpha^+ ER\beta_{175}^+$ 121 105 32 12 3 1 11
ER$\alpha^+ ER\beta_{175}^-$ 32 26 14 7 2 0 9
ER$\alpha^- ER\beta_{175}^-$ 44 32 16 12 5 1 14

C. Chemonaive patients (n=671)

Disease-free survival

LogRank $P=0.13$

HR$_{adj}^{a+b+e} = 0.74$: 95% CI 0.45-1.19; $P=0.21$

Follow-up time, Years  No. of events
ER$\beta_{175}^+$ 500 439 254 160 80 25 44
ER$\beta_{175}^-$ 171 150 124 102 59 13 31

D. Chemonaive patients (n=671)

Disease-free survival

LogRank (3df) $P=0.0001$

LogRank $ER\alpha^- vs ER\alpha^+$ $P=0.001$

LogRank $ER\beta_{175}^+ vs ER\beta_{175}^-$ $P=0.13$

Follow-up time, Years  No. of events
ER$\alpha^- ER\beta_{175}^+$ 479 423 242 152 77 25 40
ER$\alpha^+ ER\beta_{175}^+$ 162 145 120 99 58 13 27
ER$\alpha^- ER\beta_{175}^-$ 21 16 12 8 3 0 4
ER$\alpha^- ER\beta_{175}^+$ 8 4 3 3 1 0 4
High estrogen receptor beta expression is prognostic among adjuvant chemotherapy-treated patients - results from a population-based breast cancer cohort

Karin Elebro, Signe Borgquist, Ann H Rosendahl, et al.

Clin Cancer Res  Published OnlineFirst November 3, 2016.

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