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Oestrogen receptors $\beta 1$ and βcx have divergent roles in breast cancer survival and lymph node metastasis

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Background: The expression of oestrogen receptor (ER) α characterises a subset of breast cancers associated with good response to endocrine therapy. However, the clinical significance of the second ER, ER $\beta 1$, and its splice variant ER βcx is still unclear.

Methods: We here report an assessment of ER α , ER $\beta 1$ and ER βcx by immunohistochemistry using quantitative digital image analysis of 340 primary tumours and corresponding sentinel lymph nodes.

Results: No differences were seen in ER levels in primary tumours vs lymph node metastases. ER $\beta 1$ and ER βcx were equally distributed among age groups and tumour histological grades. Loss of ER $\beta 1$ in the primary tumour was strongly associated with poor survival. Its prognostic impact was particularly evident in young patients and in high-grade tumours. The worst outcome was seen in the tumours lacking both ER α and ER $\beta 1$. ER βcx expression in the primary tumour correlated with a higher risk of lymph node metastasis, and with poor survival when expressed in sentinel node lymphocytes.

Conclusions: Our study reveals highly significant although antagonising roles of ER $\beta 1$ and ER βcx in breast cancer. Consequently, we suggest that the histopathological assessment of ER $\beta 1$ is of value as a prognostic and potentially predictive biomarker.

Oestrogen receptor (ER)-mediated signalling has a fundamental role in breast cancer biology. In the majority of breast cancers, generally classified as the luminal subtypes, ER alpha (ER α), one of the members of the ER family, works as a central hub governing tumour cell proliferation and tumour progression (Sorlie *et al*, 2006; Ross-Innes *et al*, 2012).

Clinical trials have confirmed the predictive role of ER α as a biomarker in response to adjuvant endocrine therapy and thereby its association with favourable outcomes. Hence, tamoxifen has been shown to reduce the risk of any recurrence by 39% over a 10-year treatment period (Early Breast Cancer Trialists' Collaborative Group (EBCTCG) *et al*, 2011); however, the prognostic value of

ER α seems to decrease after 5 years (Bentzon *et al*, 2008). This indicates that our understanding of ER signalling in breast cancer is still very limited. Since the discovery of a second ER, ER beta (ER β) in 1996 (Kuiper *et al*, 1996), the general focus has been to decipher its role in biology and its corresponding clinical importance. ER α and ER β are located on different chromosomes but show considerable homology. Both ERs bind tamoxifen and their main ligand, oestradiol, with similar affinity, but the differences within the ligand-binding pockets are significant enough to permit the synthesis of ER α - and ER β -selective ligands (Kuiper *et al*, 1998). In terms of gene regulation, experiments performed on breast cancer cell lines in the presence of oestradiol

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indicate partly overlapping transcriptomes induced by the two ER subtypes (Chang *et al*, 2006). However, experimental studies *in vitro* and during tumour xenograft growth have shown opposing roles of the two receptors in terms of proliferation (Ström *et al*, 2004; Hartman *et al*, 2006). Contributing to the complex picture of ER β signalling is the presence of several splice variants. The full-length ER β is known as ER β 1 whereas the best characterised splice variant in breast cancer is ER β cx (also known as ER β 2; Ogawa *et al*, 1998). We now know that ER β is expressed in the epithelium and stroma of normal as well as malignant mammary gland and mediates oestrogen response. Immunohistochemistry (IHC) studies indicate an association of ER β expression with ER α -positive tumours and/or progesterone receptor (PR) status (Omoto *et al*, 2002; Fuqua *et al*, 2003). However, these studies were in part performed with pan-specific antibodies detecting all ER β splice variants, including ER β cx. Later on, the roles of different ER β splice variants have been dissected by the use of monoclonal C-terminus-targeted ER β antibodies. It now appears that ER β expression is associated with tamoxifen response, particularly within ER α -negative tumours (Gruvberger-Saal *et al*, 2007; Honma *et al*, 2008). Other researchers have been unable to confirm this association and instead report an association between ER β cx and ER α expression and a strong correlation of cytoplasmic ER β cx with poor survival (Shaaban *et al*, 2008). In a large population-based study, Marotti *et al* (2009) could confirm a positive correlation of ER β 1 expression with ER α , but not with survival; similar results were described by Borgquist *et al* (2008). One study has even shown an association of ER β 1 with poor prognosis, but only in lymph node-positive patients (Novelli *et al*, 2008). The majority of studies on larger patient populations have been performed by IHC on tissue microarrays (TMAs), a suboptimal platform for investigating heterogeneously expressed proteins. In the present study, we characterised ER β 1 and ER β cx and re-evaluated ER α expression by IHC of whole tumour sections from 340 patients with archived breast tumours and corresponding sentinel lymph nodes (SLNs).

MATERIALS AND METHODS

Study population and follow-up. The study cohort was identified from the patient registry at the Department of Pathology, Karolinska University Hospital, Stockholm, Sweden. Only patients who had undergone sentinel node biopsy (SNB) from 2001 to 2006 were included. All patients had a preoperative diagnosis of breast cancer and a clinically negative axilla. A subset of the cohort originated from a prospective study evaluating the oncological safety of SNB, the results of which have been published elsewhere (Andersson *et al*, 2012). The surgery was performed at either Karolinska University Hospital, South General Hospital or at Sofiahemmet Hospital, all in the Stockholm area. Routinely, patients were followed up annually for 5–10 years, and then reintroduced into the national mammography-screening programme. All recurrences within the Stockholm area are routinely referred back to the Department of Oncology at Karolinska University Hospital where the study has been performed. Patients who had moved away from the Stockholm County during follow-up were censored at the time of their deregistration.

Clinicopathological parameters and data on received adjuvant therapy were extracted from patient medical records. As some of the routine assessments such as PR and proliferation markers changed during the period, cut-offs employed at the time of diagnosis were used. Oestrogen receptor α was because of its central role in this study, however, re-evaluated throughout using IHC. Depending on tumour characteristics and stage of the disease, patients were treated with radiotherapy, chemotherapy and/or endocrine therapy. The occurrence of local, regional or distant relapse, death, breast cancer-specific death and the dates of last

follow-up were collected by assessing the medical records of each patient. Permits were obtained from the regional ethics board at Karolinska Institutet in Stockholm (2012/90-31/2) and from the biobank at Karolinska University Hospital.

Specimen selection and immunohistochemistry. From each patient, one formalin-fixed paraffin tissue block of the primary tumour and one block of the matching SLN were identified. The sections were cut at 4- μ m thickness and mounted. Immunohistochemistry was performed either on an Autostainer (Dako, Glostrup, Denmark; ER β 1) or on IntelliPath FLX (BioCare medical, Concord, CA, USA; ER α and ER β cx) according to the protocols and reagents provided by the manufacturers (BioCare medical and Dako), together with negative and positive controls. Heat-induced antigen retrieval in high pH solution was performed using a PT-linker (Dako) at 97°C for 20 min. The slides were incubated for 30 min at room temperature with the primary monoclonal antibodies. Anti-ER β 1 (clone PPG5/10; Dako) 1:50, anti-ER β cx (clone 57/3; AbD Serotec, Oxford, UK) 1:200 and anti-ER α (clone NCL-L-ER-6F11; Novocastra, Wetzlar, Germany) 1:200 antibodies were used. 3,3'-diaminobenzidine (DAB) was used to detect primary antibody binding and haematoxylin as counterstaining.

Digitalisation of slides and image analysis. To set appropriate intensity cut-offs for the digital scoring, a subset of tumours and SLNs were first scored manually by two independent researchers for ER α , ER β 1 (GR and JH) and ER β cx (GR and GMK) in the following compartments: primary tumour, SLN metastasis, if present, and lymphocytes residing in the SLN. The Allred scoring system was used for the manual scoring. The method has been adapted to evaluate and quantify different proteins using IHC (Fuqua *et al*, 2003; Rosin *et al*, 2012). When the two researchers did not agree on the score, they re-evaluated the section together until an agreement could be reached. All slides were digitally scanned using a Panoramic MIDI or Panoramic 250 Flash (3DHistech, Budapest, Hungary). The Panoramic viewer 1.15.2 software (3DHistech) was used for viewing the scanned images together with the built-in image analysis application, Nuclear-Quant (3DHistech), which has been validated and shown to be reproducible in the detection of ER in breast cancer (Krecsák *et al*, 2011). The software quantifies both the frequency and intensity of nuclear staining with DAB. Detection thresholds for the size, circularity and differences in contrast of the nuclei can be adjusted to distinguish cancer cells from other cell types such as fibroblasts or lymphocytes. The frequency score was based on the Allred system (Harvey *et al*, 1999), where a score between 0 and 5 is given depending on the frequency of positive cells (0% = 0, <1% = 1, 1% to <10% = 2, 10% to <33% = 3, 33% to <66% = 4 and \geq 66% = 5). The cut-off for the intensity (0 to 255) can also be changed to adjust the threshold to divide the nuclei stained into four different scoring categories ('no positive nuclei' = 0, 'low intensity' = 1, 'moderate intensity' = 2 and 'high intensity' = 3). The cut-offs were adjusted in a stepwise manner until the manual scoring and digital scoring matched on most occasions. The specific intensity threshold settings were for ER β 1: 0, \geq 175; 1, <175 and \geq 125; 2, <125 and \geq 80; 3, <80; for ER α : 0, \geq 170; 1, <170 and \geq 120; 2, <120 and \geq 70; 3, <70 and for ER β cx: 0, \geq 177; 1, <177 and \geq 130; 2, <130 and \geq 70; 3, <70. The frequency and intensity scores were then combined into a final score ranging from 0 to 8 (excluding 1). For all sections, three representative areas of invasive tumour were annotated and then subjected to image analysis. Only nuclear ER staining was analysed. When possible, each of the three areas contained at least 1000 nuclei. An average score of 4 or higher was considered as positive ER expression, corresponding to 10% positive cells with weak intensity, a cut-off that has been used in several reports (Mann *et al*, 2001; Honma *et al*, 2008).

Statistics. For descriptive statistics, continuous variables are presented as median (range), while categorical variables are presented as numbers of cases and corresponding percentages. The Pearson's Chi-square test was used to test the hypothesis of equal distribution of ER expression in categorical variables (positive vs negative). For the evaluation of ER status in node-negative vs node-positive patients, the Pearson's Chi-square test was supplemented by additional logistic regression in order to estimate the odds ratio for the presence of metastasis in different ER status groups. For the testing of distribution of ER expression (positive vs negative) in paired samples, such as primary tumours and their corresponding SLNs, the McNemar test was applied for categorical variables.

Estimation of 10-year survival rates was performed using Kaplan–Meier survival analysis. For the analysis of overall survival, follow-up time was calculated from the date of primary surgery until death of any cause or the date of medical record review, as medical records are directly linked to the national death registry. For the analysis of breast cancer-specific survival, follow-up time was from the date of primary surgery until death caused by breast cancer or the last recorded follow-up visit as documented in medical records at the department of oncology. All patients who died with metastasised breast cancer were considered to have died of the disease. For the analysis of disease-free survival, follow-up time was recorded from the date of primary surgery until the date of any relapse or until the last recorded follow-up visit. The influence of ER status on survival was tested using the log-rank test within the Kaplan–Meier model. As endocrine treatment was assumed to strongly affect survival analysis regarding ER expression, analyses were also adjusted for any endocrine treatment by adding this information as strata into the Kaplan–Meier model. For the comparative analysis of the impact of known risk factors on survival rates and their comparison with the impact of ER receptor status, both uni- and multivariable Cox proportional hazard analyses were performed and results are presented as hazard ratios (HRs) with their 95% confidence intervals (CIs). All statistical computations were performed using IBM SPSS Statistics, Version 21. A *P*-value of ≤ 0.05 was considered statistically significant.

RESULTS

Overall, 340 breast cancer patients operated between January 2001 and December 2006 were included. Of these, 322 tumours were stained and analysed for ER α , 316 for ER β 1 and 315 for ER β cx (see Supplementary Figure 1 for representative IHC stainings). Only nuclear staining of ER α , ER β 1 and ER β cx was analysed. Overall, 11 patients had no available tumour results of all three ERs, however, they were still included in the cohort since they had ER data from SLNs. Three patients had missing tumour data on ER α and ER β 1, three patients on ER α and ER β cx, and two patients on ER β 1 and ER β cx. Patient and tumour characteristics are presented in Table 1. Median follow-up for disease-free and breast cancer-specific survival was 81 months (range 0–148). Median follow-up for overall survival was 115 months (range 2–152). Thirty-six patients had died during the follow-up period, 16 of whom had died of breast cancer. Recurrences were found in 35 patients, sometimes multiple. In total, 10 local, 10 regional and 22 distant relapses were recorded. Ten patients developed contralateral breast cancer during the follow-up period, which was not considered a relapse. The Kaplan–Meier estimate of overall survival for the entire cohort was 83.9%, breast cancer-specific survival 92.1% and disease-free survival 81.6%. Adjuvant endocrine treatment had been given to 268 patients (78.8%) and chemotherapy to 132 patients (39.8%).

Oestrogen receptor status in different age groups and tumour histological grades. A cut-off of 10% was used to distinguish ER-positive from ER-negative tumours. As shown in Figure 1A, the percentage of ER α -positive tumours increased significantly with lower Elston–Ellis histological grade ($P < 0.001$). ER β cx positivity followed a similar pattern but without reaching statistical significance ($P = 0.23$). ER β 1, however, had an equal distribution in all tumour grades ($P = 0.771$). Patients were divided into four age groups: < 40 years ($N = 12$), 40–54 years ($N = 104$), 55–64 years ($N = 141$) and ≥ 65 years ($N = 81$), to study age-related changes in ER positivity. As shown in Figure 1B, there was a significant increase in ER α -positive tumours with increasing age ($P = 0.011$). There was a similar but non-significant trend for ER β cx positivity ($P = 0.062$) with increased age category. Again ER β 1 showed a different expression pattern, where the positive tumours were equally distributed among all age groups ($P = 0.22$).

Oestrogen receptor status in primary tumour and synchronous lymph node metastasis. The ER status of primary tumours did not differ significantly compared to their paired synchronous SLN metastases for any of the ERs: ER α ($P = 0.33$), ER β 1 ($P = 1.0$) and ER β cx ($P = 0.13$). However, the proportion of ER β cx-positive tumours was higher in node-positive than node-negative patients ($P = 0.021$; Table 2). This was confirmed by logistic regression, which resulted in an odds ratio of 2.54 (95% CI 1.15–5.61) for synchronous SLN metastasis in ER β cx-positive compared to negative cases. No difference in risk of SLN metastasis was seen in patients with ER α - or ER β 1-positive primary tumours.

ER β 1 expression in the primary tumour strongly affects survival. Ten-year overall survival was significantly lower in women with ER β 1-negative tumours (79.7% vs 91.1%, log rank $P = 0.009$, Figure 2A) with a HR of 2.48 (95% CI 1.23–5.01). The corresponding figures for patients receiving adjuvant endocrine treatment were 88.7% vs 92.0% and for untreated patients 48.6% vs 92.4% (endocrine treatment-adjusted log rank $P = 0.005$). No differences were seen when controlling for different types of endocrine treatment, such as tamoxifen or aromatase inhibitors. When primary tumours were stratified according to histological grades, 10-year overall survival was significantly worse for women with ER β 1-negative tumours of high grade compared to patients with ER β 1-positive high-grade tumours (60% vs 87.8%, log rank $P = 0.008$). There was no significant survival difference with regard to ER β 1 expression within grade 1 and grade 2 tumours. The prognostic potential of ER β 1 was also compared within the four age groups described above. Although the youngest age group included too few patients for subgroup analysis, 10-year overall survival was significantly lower in women with ER β 1-negative tumours aged 40–54 and 55–64 years than in their ER β 1-positive counterparts (80% vs 98.7%, log rank $P = 0.001$ and 84.7% vs 91.5%, log rank $P = 0.042$). No significant survival difference in regard to ER β 1 status was seen in elderly women (> 64 years).

We also examined 10-year breast cancer-specific survival. This was significantly lower in women with ER β 1-negative tumours (85.4% vs 97.7%, log rank $P = 0.011$; Figure 2B) with a HR of 3.44 (95% CI 1.24–9.49). The corresponding figures for patients given adjuvant endocrine treatment were 92.6% vs 93.9% and for untreated patients 59.9% vs 93.4% (endocrine treatment-adjusted log rank $P = 0.020$). Also for breast cancer-specific survival, no differences were seen when controlling for different types of endocrine treatment. When stratifying by grade, 10-year breast cancer-specific survival was significantly lower in high-grade ER β 1-negative tumours compared with high-grade ER β 1-positive tumours (58.2% vs 88.2%, log rank $P = 0.036$). Again, no difference was seen within lower histological grades. Similarly to overall survival, a lower 10-year breast cancer-specific survival was seen in women aged 40–54 years with ER β 1-negative tumours than in those with ER β 1-positive tumours (76.4% vs 98.1%, log rank

Table 1. Patient and tumour characteristics of 340 breast cancer samples divided according to ERβ1 status and for all patients

Clinicopathological variables	All patients	ERβ1 positive	ERβ1 negative
Characteristics			
Number	340	241 (76.3) ^a	75 (23.7) ^a
Age (years) ^b	58 (23–86)	58 (23–86)	58 (32–82)
Tumour diameter (mm) ^b	15 (1–73)	15 (1–73)	15 (4–50)
T stage (mm)			
T1 (≤20)	224 (66.5)	155 (65.1)	54 (72.0)
T2 (21–50)	103 (30.6)	75 (31.5)	19 (25.3)
T3 (>50)	10 (3.0)	8 (3.4)	2 (2.7)
Missing	3	3	0
Histological type			
Ductal	264 (78.6)	181 (76.4)	62 (82.6)
Lobular	57 (16.9)	45 (19.0)	9 (12.0)
Mixed	9 (2.7)	6 (2.5)	3 (4.0)
Other	6 (1.8)	5 (2.1)	1 (1.3)
Missing	4	4	0
ERα status			
Positive	258 (80.1)	197 (82.4)	52 (71.2)
Negative	64 (19.1)	42 (17.6)	21 (28.8)
Missing	18	3	2
ERβcx status			
Positive	279 (88.6)	210 (90.1)	58 (81.7)
Negative	36 (11.4)	23 (9.9)	13 (18.3)
Missing	25	8	4
PR status			
Positive	249 (73.9)	182 (76.2)	49 (66.2)
Negative	88 (26.1)	57 (23.8)	25 (33.8)
Missing	3	3	1
Elston histological grading			
1	89 (26.4)	60 (25.2)	22 (29.3)
2	171 (50.7)	126 (52.9)	37 (49.3)
3	77 (22.9)	52 (21.8)	16 (21.3)
Missing	3	3	0
Proliferation			
Low	221 (66.4)	159 (67.4)	47 (64.4)
High	112 (33.6)	77 (32.6)	26 (35.6)
Missing	7	236	2
Sentinel lymph node status			
N0	195 (57.4)	134 (55.6)	46 (61.3)
N1	145 (42.6)	107 (44.4)	29 (38.7)
Adjuvant treatment			
Chemotherapy	132 (39.8)	95 (39.4)	30 (40.0)
Radiotherapy	250 (75.3)	182 (77.4)	52 (71.2)
All endocrine treatment	268 (80.7)	195 (83.0)	55 (75.3)
Oestrogen receptor antagonist	152 (44.7)	110 (45.6)	29 (38.7)
Aromatase inhibitor	63 (18.5)	40 (16.6)	20 (26.7)
Combined endocrine treatment	53 (15.6)	45 (18.6)	6 (8.0)

Table 1. (Continued)

Clinicopathological variables	All patients	ERβ1 positive	ERβ1 negative
ERα/PR status			
Positive/positive	207 (67.0)	165 (69.6)	42 (58.3)
Positive/negative	40 (12.9)	31 (13.1)	9 (12.5)
Negative/positive	21 (6.8)	15 (6.3)	6 (8.3)
Negative/negative	41 (13.3)	26 (11.0)	15 (20.8)
Missing	31	4	3
ERα/PR status^c			
Positive/positive	208 (67.5)	186 (68.1)	22 (62.9)
Positive/negative	39 (12.7)	37 (13.6)	2 (5.7)
Negative/positive	22 (7.1)	19 (7.0)	3 (8.6)
Negative/negative	39 (12.7)	31 (11.4)	8 (22.9)
Missing	32	6	1
Abbreviations: ER = oestrogen receptor; PR = progesterone receptor. Data were collected from patient medical records, except for ERα, ERβ1 and ERβcx, which were evaluated within this study using immunohistochemistry. All numbers are cases (%) if not stated otherwise.			
^a Twenty-four patients had missing ERβ1 classification.			
^b Median (range).			
^c Note that data presented below is on ERβcx expression.			

$P=0.001$). There were no differences in breast cancer-specific survival within the two higher age groups (55–64 and >64 years).

ERα-positive tumours were associated with better breast cancer-specific survival (log rank $P=0.048$, Figure 2C) but not overall survival (log rank $P=0.20$). There was no significant association between ERβcx status in the primary tumour and overall or breast cancer-specific survival (Figure 2D and E), and no significant patterns were observed when stratifying for histological grades and age groups. None of the analysed ERs affected disease-free survival.

All potential prognostic variables were tested using univariable Cox regression analysis. Significant univariable factors were entered into a multivariable Cox regression model (Supplementary Table 1). As there were few patients in the youngest age group, no 95% CIs could be calculated for this specific group. In the multivariable model, loss of ERβ1 was associated with worse prognosis with a HR of 2.40 (95% CI 1.16–4.94). Also the loss of PR (HR 3.38, 95% CI 1.76–6.50), older age (>65 years) and advanced nodal stage (HR 3.77, 95% CI 1.54–9.19) remained independent of prognostic factors for overall survival. For breast cancer-specific survival, only ERβ1 (HR = 3.38, 95% CI 1.09–10.45) and advanced nodal stage (HR = 11.64, 95% CI 2.97–45.63) remained independent prognostic factors.

ER marker combinations affect breast cancer-specific and overall survival.

Four combinations of intratumoural ERα and ERβ1 expression were created: ERα +/ERβ1 + ($N=197$), ERα +/ERβ1 – ($N=52$), ERα –/ERβ1 + ($N=42$) and ERα –/ERβ1 – ($N=21$). Differential 10-year overall survival differed significantly between these four groups with 91.4%, 85.4%, 90.0% and 75.6%, respectively (log rank $P=0.050$; Table 3). The same pattern was seen for 10-year breast cancer-specific survival rates of 93.9%, 91.0%, 93.6% and 72.1%, respectively (log rank $P=0.009$). Interestingly, these differences lost their significance when adjusting for any endocrine treatment, however, significance was retained when adjusted only for ER antagonist-containing post-operative therapy (e.g., tamoxifen; log rank $P=0.032$ and 0.031). Corresponding groups were created for ERβ1 and ERβcx ($N=210$ (ERβ1 +/ERβcx +), 23 (ERβ1 +/ERβcx –), 58 (ERβ1 –/ERβcx +) and 13 (ERβ1 –/ERβcx –)); in these groups, 10-year overall survival was 92.0%, 95.5%, 78.1% and 100%, respectively

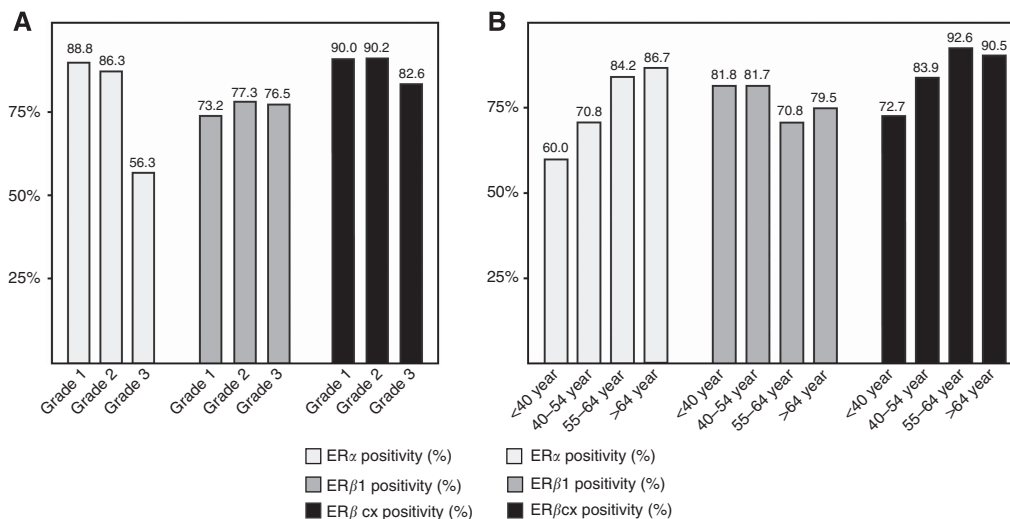


Figure 1. ER expression among different age groups and histological grades. (A) ERα positivity decreased with higher Elston–Ellis histological grade (white; $P < 0.001$, $N = 80, 168$, and 71). ERβcx showed a similar trend, however not significant (black; $P = 0.23$, $N = 80, 163$, and 69). ERβ1 was equally distributed among all three grades (grey; $P = 0.771$, $N = 82, 163$, and 68). (B) Patients were divided into four different age groups according to age at diagnosis, < 40 , $40–54$, $55–64$, and ≥ 65 years. ERα (white) positivity was lowest in the youngest age group and increased with age ($P = 0.011$, $N = 10, 96, 139$, and 75). A similar trend was seen with ERβcx (black), however not reaching significance ($P = 0.062$, $N = 11, 93, 135$, and 73). ERβ1 was equally distributed along all age groups (grey; $P = 0.221$, $N = 11, 93, 137$, and 74). Numbers above bars reflect percentage of positive tumours.

Table 2. Oestrogen receptor (ER) α, ERβ1 and ERβcx expression in node-positive vs node-negative primary breast cancer		
	Primary tumour node negative	Primary tumour node positive
ERα		
Negative	35 (19.3)	29 (20.6)
Positive	146 (80.7)	112 (79.4)
P-value	0.784	
ERβ1		
Negative	46 (25.6)	29 (21.3)
Positive	134 (74.4)	107 (78.7)
P-value	0.382	
ERβcx		
Negative	27 (15.2)	9 (6.6)
Positive	151 (84.8)	128 (93.4)
P-value	0.018*	

Abbreviation: SLN = sentinel lymph node. The expression of ERβcx was significantly higher in the primary tumours of patients with SLN metastasis. This was not observed for ERα or ERβ. SLN negative $N = 195$, SLN positive $N = 145$. Pearson’s Chi-square test. * $P < 0.05$.

(log rank $P = 0.011$). Rates for 10-year breast cancer-specific survival were 95.9%, 92.3%, 80.0% and 100%, respectively (log rank $P = 0.001$). Adjusting for any endocrine treatment or for ER antagonists retained similar results (log rank $P = 0.006$ and 0.007 for overall survival and $P = 0.002$ and 0.003 for breast cancer-specific survival). Similar analyses of combinations of ERα and ERβcx expression did not render any significant associations with survival.

ERβcx expression in SLN lymphocytes is more common in node positivity and affects overall survival. ERβ1 and ERβcx positivity of lymphocytes residing in the SLN was seen in 202 out of 285 (70.9%) and 116 out of 292 (39.7%) patients, respectively. In

contrast, ERα positivity in these cells was an extremely rare event with only one positive out of 248 cases (0.4%). ERβcx positivity in SLN lymphocytes was more common in node-positive than node-negative patients (57 out of 125 (45.6%) vs 59 out of 167 (35.3%)), even though this did not reach statistical significance ($P = 0.076$). This trend was supported by the fact that patients with ERβcx positivity in their SLN had a higher mean number of axillary lymph node metastases (1.4) than those with ERβcx negativity (0.9; $P = 0.055$). Ten-year overall survival was 93.0% for patients with ERβcx negativity in their SLN lymphocytes, as compared with 84.8% in those with ERβcx SLN lymphocyte positivity (log rank $P = 0.053$, Figure 2F). Interestingly, this finding turned significant when adjusting for adjuvant endocrine therapy (log rank $P = 0.039$). There was no effect of ERβcx SLN lymphocyte status on breast cancer-specific survival or on disease-free survival.

DISCUSSION

Our analysis revealed that ERβ1 positivity within the primary tumour is an independent marker for good outcome, more powerful than ERα, which is classically associated with increased survival after adjuvant endocrine therapy. ERβ1 expression remained an independent prognostic marker for both overall and breast cancer survival in a multivariable Cox regression model, which strengthens the prognostic value of the receptor. The number of events in our cohort during follow-up period was small due to the generally good prognosis of breast cancer today and considering that included patients were clinically node negative. It has been shown, however, that survival analysis can be reliable with even as little as five events per variable within the Cox regression model. This is especially evident when the association is plausible and hypothesised *a priori* (Vittinghoff and McCulloch, 2007). When performing survival analysis in breast cancer while studying ERs it is important with sufficient follow-up due to the risk of late recurrences. The follow-up for breast cancer-specific survival was shorter than for overall survival due to their definitions specified in the Statistics section. However, since any recurrent breast cancer

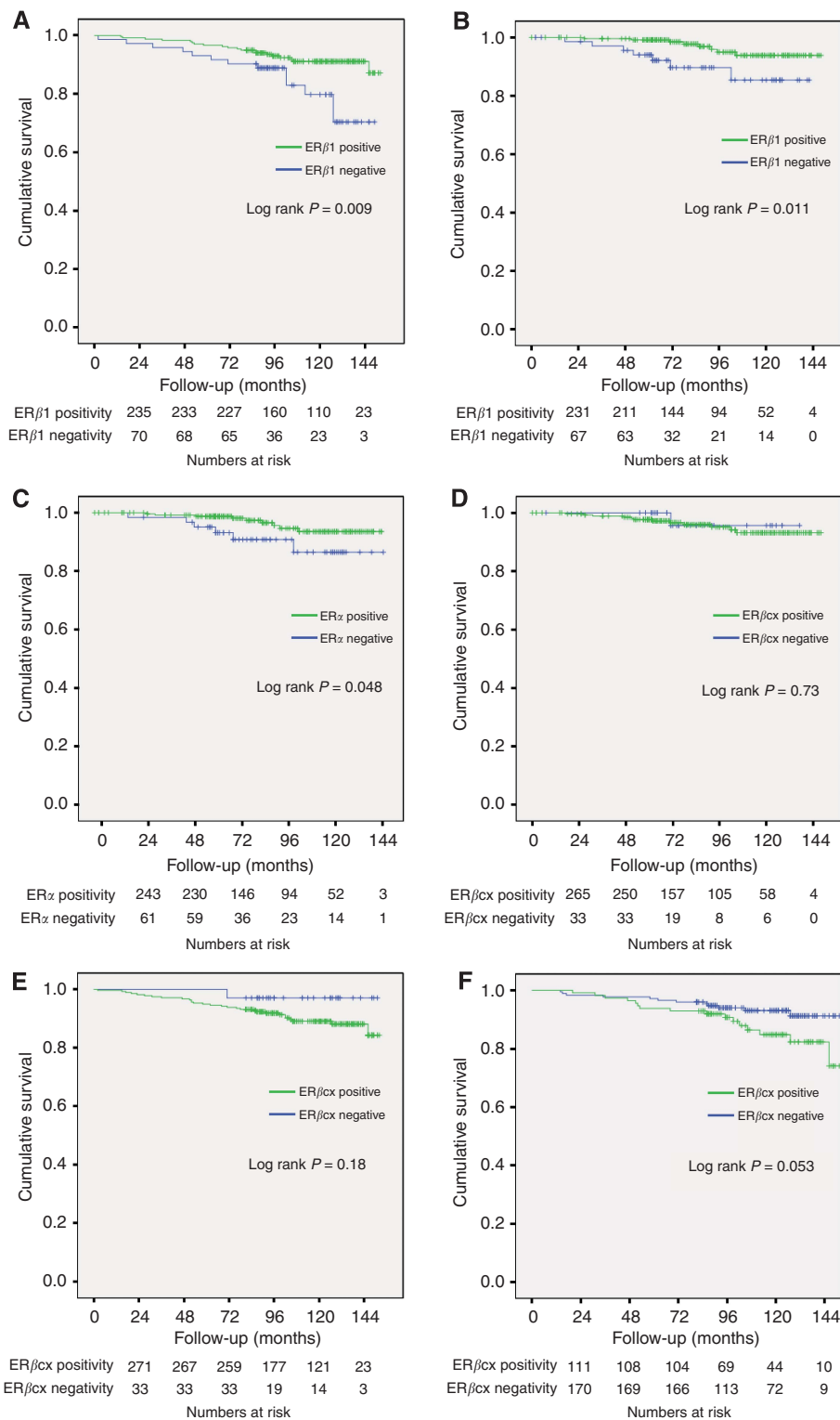


Figure 2. Kaplan–Meier plots of overall survival and breast cancer-specific survival for ER expression in primary tumour and sentinel node lymphocytes. (A) There was a higher overall survival in patients with ERβ1-positive tumours (green) compared to ERβ1-negative (blue; log rank $P = 0.009$). (B) The same was seen for breast cancer-specific survival for ERβ1-positive tumours (log rank $P = 0.011$). (C) Breast cancer-specific survival was higher in patients with ERα-positive tumours (log rank $P = 0.048$). (D) Breast cancer-specific survival did not differ between patients with ERβcx-positive or -negative tumours (log rank $P = 0.73$). (E) There was a trend towards better overall survival in patients with ERβcx-negative tumours; however, this did not reach statistical significance (log rank $P = 0.18$). (F) Patients with ERβcx-negative lymphocytes in the sentinel node showed a non-significant trend towards better overall survival (log rank $P = 0.053$). Numbers at risk at each time point are given below each subfigure.

cases were referred back to the department where this study was performed, it is unlikely that such cases were missed. Thus, follow-up for breast cancer-specific survival and disease-free survival is

probably underestimated. When we stratified patients for endocrine treatment, the survival was similarly high in patients with ERβ1-positive tumours. This is somewhat contradicting when

Table 3. Cross-tabulation of ER co-expression in regard to OS and BCSS

	ER β 1 positive	ER β 1 negative
10-year OS		
ER α positive (%)	91.4	85.4
ER α negative (%)	90.0	75.6
P-value	0.050*	
10-year BCSS		
ER α positive (%)	93.9	91.0
ER α negative (%)	93.6	72.1
P-value	0.009*	
	ER β cx positive	ER β cx negative
10-year OS		
ER β 1 positive (%)	92.0	95.5
ER β 1 negative (%)	78.1	100
P-value	0.011*	
10-year BCSS		
ER β 1 positive (%)	95.9	92.3
ER β 1 negative (%)	80.0	100
P-value	0.001*	
10-year OS		
ER α positive (%)	90.4	100
ER α negative (%)	84.6	90.0
P-value	0.283	
10-year BCSS		
ER α positive (%)	94.7	100
ER α negative (%)	86.0	85.7
P-value	0.093	

Abbreviations: BCSS = breast cancer-specific survival; ER = oestrogen receptor; OS = overall survival. The highest 10-year OS/BCSS was seen in ER α +/ER β 1+ tumours. Either ER α +/ER β 1- or ER α -/ER β 1+ was also associated with better prognosis, compared to double negative tumours (ER α -/ER β 1- that had the worst prognosis for both OS and BCSS. For ER β and ER β cx co-expression, the worst 10-year OS/BCSS was seen in patients with ER β 1-/ER β cx+ tumours, compared to ER β 1+/ER β cx+, ER β 1+/ER β cx- and ER β 1-/ER β cx-. No significant differences in 10-year OS or BCSS were seen when comparing the co-expression of ER α and ER β cx. *P<0.05.

compared with the results of Honma *et al* (2008), where the increase in survival was only seen in ER β 1-positive patients treated with tamoxifen for >2 years. Our group of endocrine-treated women, however, received tamoxifen and/or aromatase inhibitors usually for 5 years, which may perhaps explain the differences in survival. When we examined survival regarding the co-expression of ER α and ER β 1, patients who were either double or single positive had an equally good prognosis, meaning that ER β 1 is a potential biomarker for distinguishing between patients with good or bad prognosis in ER α -negative tumours, generally considered as a group of patients with poor prognosis. ER β 1 and ER β cx co-expression was associated with survival in a similar manner, again the lowest survival was seen in the ER β 1-negative tumours. Intriguingly, these tumours were also ER β cx-positive. ER β cx is thought to affect ER function through negative regulation by heterodimerisation, mainly with ER α , causing degradation of the receptor complex and therefore a decrease of ER α level (Zhao *et al*, 2007). This mechanism may result in a completely ER-negative tumour (ER α - and ER β 1-). Therefore, this tumour could be less sensitive to endocrine therapy and perhaps more responsive to chemotherapy. As described earlier it is important to remember that the stratification and subgroup analysis of the co-expression data is performed on a small cohort and with few events, which merits caution when interpreting these results. However, we believe that the findings are both clinically and biologically relevant, but need further validation.

ER β 1 seems to be present in tumours in patients from all age groups, whereas ER α expression is usually less common in younger women. Most interestingly, the negative prognostic significance of the lack of ER β 1 expression in the primary tumour is particularly strong in younger age groups and in tumours of high histological grades. Therefore, ER β should be considered a valuable prognostic biomarker and perhaps a therapeutic target in younger women in whom triple-negative breast cancer (absence of ER α , PR and HER2) is more common. Tamoxifen and other current endocrine therapies such as aromatase inhibitors, however, are designed to treat ER α -expressing cancers and might not be the optimal therapy for targeting ER β 1. Our data suggest that the examination of ER β 1 status should have an additional prognostic value during routine pathological examination of breast cancer, but not as a biomarker for endocrine responsiveness within ER α -negative cases as described elsewhere (Gruvberger-Saal *et al*, 2007; Honma *et al*, 2008). Further research is needed to identify the ideal ER β 1-targeting therapy and to validate our findings.

Although several prospective investigations on ER β isoforms in breast cancer have been performed, the potential association with clinicopathological parameters and survival remains unclear. The expression of the different ER β isoforms is a result of alternative splicing at the C-terminus; consequently, antibodies raised against the N-terminus will inevitably quantitate the total ER β level. This may cause a false view of the isoform-specific expression. In our study, we analysed ER β 1 expression with a widely cited, well-validated C-terminal monoclonal antibody (Skliiris *et al*, 2002; Carder *et al*, 2005; Weitsman *et al*, 2006; Novelli *et al*, 2008). As mentioned, ER β has been described as an anti-proliferative, tumour-suppressive receptor within breast cancer cells *in vitro* (Hartman *et al*, 2009). Consequently, it has been hypothesised that ER β should be downregulated during breast cancer progression (Roger *et al*, 2001). However, paired primary tumours and corresponding metastatic lesions are extremely scarce materials and the hypothesis is thus hard to prove. Often, the presence of locoregional lymph node metastases are used as a surrogate parameter, as they are one of the strongest risk factors for breast cancer death and distant metastases (Fisher *et al*, 1983; Andersson *et al*, 2010). In a study on 50 patients, Gschwantler-Kaulich *et al* (2011) observed that compared to the primary tumour, there was a reduction in both ER α and ER β levels in axillary lymph node metastases. In our analysis, ER status differed between primary tumour and corresponding lymph node metastases in several patients, even though we could not identify any changes in the overall pattern of ER status. This discrepancy is probably explained by the fact that Gschwantler-Kaulich *et al* used TMAs with 1 mm cores while our analysis was based on whole sections. It is evident that intratumoural ER levels are heterogeneously expressed, hence, IHC on TMAs may not be an optimal method for ER assessment of breast cancer specimens. Furthermore, to reduce variation and bias in our study, all IHC stainings were assessed by computer-assisted image analysis (see Materials and Methods section) in a blinded manner. This method, if correctly performed, is able to reduce both intra- and interobserver variation in biomarker assessments (Krecsák *et al*, 2011). Our analysis further showed that patients with ER β cx-positive primary tumours had an increased risk of lymph node metastasis. Nonetheless, the ER status of the corresponding lymph node metastasis did not correlate to outcome.

Oestrogen receptors, expressed in lymphocytes, are important in the maturation of B cells and play a crucial role in the peripheral immune system; this effect is mediated by both ER α and ER β (Shim *et al*, 2006; Hill *et al*, 2011). Within the stroma of mammary gland and tumour adjacent tissue, ER β is the predominating ER (Speirs *et al*, 2002). In the majority of our patients, we observed that lymphocytes within the SLNs express ER β 1 (70.9%) while a minority expressed ER β cx (39.4%) and <1% expressed ER α . We found that lymphocytes within lymph nodes containing metastatic

breast cancer cells express higher levels of ER β cx. In patients treated with endocrine therapy, ER β cx-positive SLN lymphocytes indicated a poor prognosis and were furthermore associated with shorter 10-year overall survival. Our data imply that ER β cx within SLN lymphocytes may govern a yet unknown mechanism of breast cancer progression. Since the lymphocytes expressed little ER α , the tumorigenic function could perhaps be mediated through heterodimerisation and inhibition of ER β 1.

In summary, this study indicates that ER β 1 and the ER β splice variant ER β cx have several important roles during breast tumourigenesis. In the primary tumour, ER β 1 was associated with good outcome and probably has a tumour-suppressive function. ER β cx, however, seems to play the most important role in regional lymph nodes where its presence in lymphocytes correlated to overall survival in breast cancer patients through an as yet unknown mechanism. The analysis of ER β 1 and ER β cx by IHC provides useful clinical information, especially for younger women and tumours of high histological grade. Further research is needed to understand how to pharmaceutically target the individual ER subtypes in breast cancer patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JH, JdB, JB and JF designed research; GR, JH, JdB and GMK performed research; JdB and GR analysed data; JdB, GR and JH wrote the paper; JH led the project.

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