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CLINICAL TRIAL

Short-term outcome of primary operated early breast cancer by hormone and HER-2 receptors

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Abstract *Introduction* Prognostic subgroup classification of operable breast cancers using cDNA clustering of breast cancer-related genes resembles the classification based on the combined immunohistochemical (IHC) expression of the hormone and HER-2 receptors. We here report the short-term disease-free interval (DFI) of operable breast cancers by their joint hormone receptor/HER-2 phenotype. *Patients and methods* Short-term follow-up (FU) of a prospective cohort of 1,958 breast-cancer patients primary operated at our institution between 2000 and 2005. Receptors were evaluated using IHC. Steroid receptors were considered positive for any nuclear staining; HER-2 for strong (3+) membrane staining or positive fluorescence in situ hybridization (FISH). Kaplan–Meier (KM) DFI curves were calculated for any relapse defined as a local, regional, contralateral, or distant breast cancer event for the six predefined breast cancer subgroups: ER + PR + HER-2 − (PPN), ER + PR − HER-2 − (PNN), ER + PR + HER-2 + (PPP), ER − PR − HER-2 − (NNN), ER − PR − HER-2 + (NNP), and ER + PR − HER-2 + (PNP). *P*-values were calculated for comparison of the six different survival curves using two possible adaptations for multiple testing. A multivariate model for the receptors predicting DFI did incorporate local and systemic adjuvant therapy. *Results* Median patient age was 57 years (ranges 26–96) and median FU was 3.35 years. Overall, DFI at median FU was 91%; 94% for PPN, 89% for PNN, 86% for NNN, 81% for PPP, 80% for PNP, and 76% for NNP cases. Some receptor subgroups had a significantly better DFI than others based on multiple testing, especially when the PPN group was compared against the four most frequent subtypes. The multivariate model with local and systemic adjuvant therapy confirmed the prognostic value of ER, PR, and HER-2 for short-term DFI. *Conclusion* It is possible to distinguish short-term prognostic breast cancer subgroups only on the basis of ER, PR, and HER-2 even when stratified for local and systemic adjuvant therapy. While gene expression profiles based on microarray data of over hundreds of genes will probably teach us much about breast cancer biology, heterogeneity, and prognosis, we emphasize the important short-term prognostic value of currently used IHC markers for ER, PR, and HER-2.

Keywords Breast cancer · Disease free survival · Disease free interval · HER-2 · Steroid receptors · Estrogen receptor · Progesterone receptor

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Introduction

Worldwide, breast cancer is the most common malignancy among women. Its incidence has been increasing over recent decades, with a recent trend toward lower incidence in Western Countries. This trend can partly be explained by primary (SERMs) and secondary prevention (screening mammography) and a decrease in the use of postmenopausal hormone-replacement therapy [1]. However, breast cancer remains the leading cause of death in women between ages 40 and 55 [2]. The large biological heterogeneity of breast cancers is reflected in the large differences in disease outcome between women with this disease. Although prognostic classification systems of operable breast cancers, for example the Nottingham Prognostic Index (NPI) are important determinants of clinical outcome, they remain insufficient [3]. Even women with a similar NPI and with similar histological tumor type experience differences in disease outcome. Age at diagnosis, a healthy lifestyle, co-morbidity, race, previous use of hormone-replacement therapy, and localization of the tumor within the breast are only a limited listing of known prognostic factors of breast cancer outcome other than the commonly used morphological prognostic factors [4].

Outcome of operable breast cancer is also affected by the presence or absence of hormone receptors and human epidermal growth factor receptor 2 (HER-2) [5]. The prognostic value of these predictive biomarkers can be summarized as follows. Women with an ER-positive tumor have a better prognosis than those with an ER-negative tumor, although this is not true for very young women and not after approximately eight years of follow-up (FU) when survival curves of ER-positive and ER-negative cases cross [5–7]. The combination of ER with PR further refines the short-term prognostic value of ER. Within ER-positive breast cancers, women with negative PR expression do worse than those expressing PR, but data are only of value for the postmenopausal population [8–11]. This is probably related to the fact that a negative PR status stands for alternative signaling through, for example, the epidermal growth factor pathway [12–15]. ER-positive/PR-negative tumors express higher levels of HER-1 and, especially, HER-2 and have more aggressive features than ER/PR-positive tumors [12, 16]. Overexpression of receptors for growth factors such as HER-2 has also been shown to play an important prognostic role, independent of lymph node status, tumor size, or ER-status [17–21].

In general, most of these studies were based on the prognostic value of one or two of the hormone receptors but studies focusing on breast cancer prognosis based on the combined expression of ER, PR, and HER-2 are limited. Recently, cDNA microarrays of 496 genes of breast cancer tissue identified different prognostic subgroups based on differences in gene expression profiles [22–24]. This prognostic subgroup classification of breast cancers seems closely, but not completely, related to the immunohistochemical (IHC) classification of tumors according to their combined expression of hormone receptors and HER-2 [22, 25, 26]. The partial inconsistency between IHC "phenotype" and microarray "genotype" is not fully understood and might be partially related to technical issues of receptor determination. Five main prognostic "genotype" subgroups are described. The most frequent is the "luminal A" subgroup which represents in general all ER-positive and/or PR-positive cases; these tumors are HER-2-negative. The second group are breast cancers in the "luminal B" subgroup; they are also ER-positive and/or PR-positive but with a lower expression of both steroid receptors. The HER-2-overexpressing breast cancers that are ER-positive also belong to this subgroup. The "normal-like" subgroup is less well defined but seems to include ER-positive cases not showing HER-2 gene amplification. The fourth group consists of "ER-negative HER-2-overexpressing" breast cancers, in principle characterized by HER-2 gene amplification with an absence of both ER and PR. The last group is called "triple negative" tumors. Although they lack expression of both the female steroid hormone receptors and HER-2, some are also classified as basal like, but such tumors should overexpress HER-1 and/or express high-molecular-weight cytokeratins CK 5.6, CK 14, and/or CK17. The latter are typically expressed in the basal, myoepithelial cells lining the breast duct. This particular group seems to be better defined as time goes by [22–24].

Because of its high cost, the need for frozen tissue, and the absence of a morphological control, it remains hard to use the molecular classification system in the routine clinical setting. IHC markers for ER, PR, HER-2, and CK 5 and 6 have been successfully verified against the gene expression profiles as such in a short-term disease-free survival study [25, 26]. Francis et al. recently showed Kaplan–Meier (KM) survival curves with a reduced breast cancer-specific survival for HER-2-positive tumors [26]. The breast cancer specific survival in these patients correlated with hormone receptor status of the tumors for both HER-2-negative and HER-2-positive tumors. This has been confirmed in various ethnic populations [25–30]. Therefore, they can be used to estimate the prevalence of the five intrinsic subtypes in epidemiological studies without loss of their prognostic significance [22, 25, 31]. IHC of receptors for female steroid hormones and HER-2 remains the most common practice in the primary evaluation of breast cancer. Although there can be several technical reasons for discordant results between centers, hormone receptor determination has not only become routine because it is easy in use—it also has a morphological

control and does have a much lower cost than gene profiling. It is a straightforward technique that uses paraffin-embedded tissue and can therefore be used for retrospective analyses [32].

We here present KM survival curves of primary operated breast cancer patients stratified by the IHC expression of the joint receptor status for ER/PR/HER-2. We also studied the role of joint receptor subgroups, adjusting for adjuvant therapy.

Patients and methods

Cases were selected from our institutional database containing all patients with breast cancer treated at the Multidisciplinary Breast Centre in UZ-Leuven between January 1, 2000 and June 2005. We used our database to retrospectively seek all patients eligible for inclusion. Patients were included after primary surgery for an early invasive breast cancer if clinico-pathological data and updated FU information were available. Women who received preoperative systemic therapy were excluded. In cases of bilateral breast cancer we included the tumor characteristics of the lesion with the highest NPI.

A total of 2,059 patients were included in this study. In 85 patients, information about ER, PR, and/or HER-2 status was incomplete. It was mainly HER-2 status which was missing in patients operated during early 2000. In that year analysis of HER-2 by IHC was introduced into clinical routine in UZ Leuven. After exclusion of the small group of women with an ER-negative/PR-positive tumour, a dataset of 1,958 patients remained (Fig. 1). Patients with incomplete information on tumor variables such as tumor size, grade, or lymph node status were only excluded in function of the analysis performed.

Tumor size was evaluated by the pathologist and the largest diameter from the histopathology report was chosen. If there was more than one lesion, the largest was selected, even if this was biologically (grading, receptor status) not the most aggressive. Tumor grading was performed according to the Ellis and Elston grading system [33]. Lymph nodes were assessed on at least three sections stained with conventional hematoxylin and eosin (HE). Sentinel lymph nodes and lymph nodes from lobular breast cancers classified as negative on HE were also stained with epithelial markers. NPI was computed from the above mentioned tumour characteristics, using the formula NPI = 0.2 × T + N (1–3) + G (1–3) where T is the maximum diameter (in cm), N the number of lymph nodes involved (1 = no axillary lymph nodes involved; 2 = 1–3 axillary lymph nodes involved; 3 = more than 3 axillary lymph nodes involved), and G histological grade (1–3: good, moderate, poorly differentiated). The patients are grouped into three prognostic risk categories for relapse according to the NPI results: 1 = low risk, with NPI equal to or less than 3.4; 2 = medium risk, with NPI between 3.4 and 5.4; 3 = high risk, with NPI over 5.4 [34]. NPI could not be calculated in cases when no axillary lymph node dissection was performed (n = 31).

For routine clinical use, breast cancer tissue was examined by IHC for the expression of ER, PR, and HER-2 using antibodies NLC-ER-6F11, NCL-PR-312, and CB11, respectively, all from Novocastra Laboratories (Newcastle-on-Tyne, UK). Since 2005, highly sensitive rabbit monoclonal antibodies have been in use for the assessment of ER and PR expression (SP1 and SP2 respectively; Labvision, Fremont, CA, USA).

Briefly, 4 μm thick paraffin sections were cut. Heat-induced epitope retrieval was carried out in a calibrated water bath (95–99°C) and antibody complexes were visualized by use of EnVision+ (DakoCytomation, Glostrup, Denmark) and diaminobenzidine. The latter antibodies are highly sensitive and SP1 has recently been proposed as an improved standard for ER IHC assessment in breast cancer [35].

Stainings were scored using the semi-quantitative Allred score considering both the proportion of stained tumour cell nuclei (scored on a 0–5 scale) and staining intensity (scored

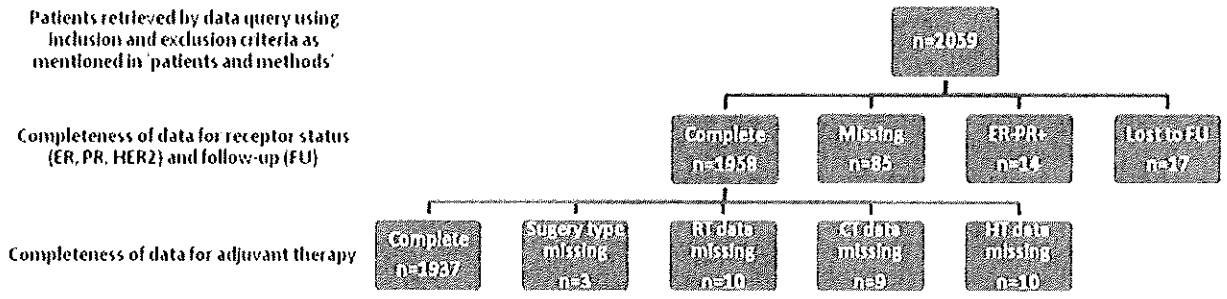


Fig. 1 Flow of patients through the study. Overlap of excluded patients is possible, for example, patients lost to FU could also be those with missing data or those with an ER–PR+ tumor

on a 0–3 scale) [36, 37]. A total sum of more than 2 was considered as positive for ER and PR status. The semi-quantitative *H*-score was used before 2003. This was calculated by summing the products of the percentage of cells stained (0–100%) by staining intensity (0–3+). For example, a specimen with 10% of cells staining 3+, 20% of cells staining 2+, 10% of cells staining 1+, and 50% of cells unstained would have a complete *H*-score of $(3 \times 10) + (2 \times 20) + (1 \times 10) = 80$. For steroid receptors, any nuclear staining in invasive tumour cells was considered positive, both using Allred- or *H*-score.

HER-2 immunostaining was scored according to the standardized HercepTest scoring system in which HER-2 expression is scored on a 0 to 3+ scale. Score 0 represents absence of staining whereas score 3+ indicates strong membranous HER-2 staining. We have previously shown that women with a HER-2/neu DAKO score 3+ were all HER-2 FISH-positive [38]. In the case of intermediate scoring (score 2+), underlying *HER-2* gene copy number was investigated by fluorescence in situ hybridization or FISH (PathVision; Vysis, Downers Grove, IL, USA) for the presence of *HER-2* gene amplification. Only when the latter was present in score 2+ cases was HER-2 status considered positive.

Surgical treatment consisted in wide local excision plus axillary dissection followed by whole breast radiotherapy (RT) plus a boost on the tumour bed, or modified radical mastectomy when breast-conserving surgery was not indicated. In our institution, sentinel node biopsy became standard treatment for cT1N0 patients in June 2003; since then, completion axillary dissection was carried out only in cases of metastatic sentinel node. Chest wall RT following mastectomy was given to patients with T3 or T4 tumors, with positive lymph nodes or with positive tumour section margins. Irradiation to the internal mammary chain was performed only in cases of axillary lymph node involvement or medial tumour sites. This was implemented first in the EORTC trial and subsequently as routine in this subset of patients.

Patients received systemic adjuvant therapy that was considered appropriate for the time of treatment (rule based) and several patients participated in clinical studies with endocrine or chemotherapeutic modalities. All other patients were treated according to international guidelines and consensus conferences (rule based). Endocrine therapy (HT) was prescribed if ER and/or PR expression were present. Although tamoxifen 20 mg/day for five years was the standard HT, many postmenopausal women also received an oral aromatase-inhibitor for a period of five years. Anthracycline-based chemotherapy (CT) was given if patients were classified as intermediate or high risk for relapse but endocrine sensitivity, NPI, and age at diagnosis were also important when deciding upon systemic adjuvant therapy. Only a few patients ($n = 10$, included in the HERA-trial) were treated with adjuvant trastuzumab, which was, therefore, not taken into account.

Using the electronic patient files, FU status was updated (range 1–2,484 days) for most of the patients. If patients did not have FU in Gasthuisberg, Leuven, treating general physicians were contacted by telephone to obtain the latest FU status. Our objective was to provide FU data for all patients up until June 2006. Only 17 patients defined as "lost to FU" had no FU at all and were excluded from this study. We applied the reporting REcommendations for tumour MARKer prognostic studies (REMARK) [39]

Statistical analysis

The clinical and pathologic characteristics of women in this study are reported as frequencies, medians, and ranges. Statistical analysis was performed by ESAT, and SAS 9.1.3 service pack 4 was used to run all statistical analysis.

The primary outcome was disease-free interval (DFI) by joint receptor status and defined as the length of time from the date of surgery to a first predefined breast-cancer event. Events included local (ipsilateral or regional) recurrence of breast cancer, contralateral disease, and distant metastasis. The event time of patients without relapse was censored at last FU or death. We thus evaluated only breast cancer-specific DFI. Overall survival was not calculated. No patients died of breast cancer without first having had a relapse.

We defined the following six subgroups based on joint receptor status and excluding ER-negative/PR-positive tumors: ER + PR + HER-2 − (PPN), ER + PR − HER-2 − (PNN), ER + PR + HER-2 + (PPP), ER − PR − HER-2 − (NNN), ER − PR − HER-2 + (NNP), and ER + PR − HER-2 + (PNP). Median NPI and NPI risk group distribution were calculated for each joint receptor status.

The non-parametric KM method, allowing for right censoring, was used to visualize the survival curves. Different types of right censoring occurring in this study were: patients without relapse at the end of the study time, patients lost to FU, and patients who died a non-breast-cancer-related death. The KM method incorporates the censored data by changing the risk set at every event time. Data points having an event or censoring time which is less than the survival time at which point the survival is calculated were excluded from the risk set.

The log-rank test was used for comparison between different survival curves. At each event time a (2 × 2) table is constructed. The recurrence rates between the two groups are compared, conditional on the number of patients at risk in the group. The tables are then combined using the Cochran–Mantel–Haenszel test. If the constructed tables

are independent, the log-rank-statistic will have an approximate χ^2 distribution with one degree of freedom. Correction for multiple testing was performed using the step-down Bonferroni correction as proposed by Holm et al. [40] and the step-up method introduce by Benjamini and Hochberg [41].

Results

Median FU was 41 months (1–2,484 days). Tables 1 and 2 summarize the clinico-pathological features and local and systemic adjuvant therapy of all patients by the predefined joint receptor subtype. In the overall study population ($n = 1958$), median age at diagnosis was 57 years and most (65.19%) women were in a postmenopausal state. Analysis of receptor-prevalence showed ER staining in 87.47% of all cases, whereas PR and HER-2 were positive in 75.18% and 12.16%, respectively. Half (47.80%) of the patients presented with an intermediate NPI risk at diagnosis (median NPI 4.30; Table 1), most patients were node-negative (61.90%) and grade-1 tumors were not frequently seen (13.79%).

Most women (57.51%) had breast conservative surgery and 84.13% received postoperative RT. Data on adjuvant systemic therapy were complete for all modalities for 1,937 patients; 1,808 (93.34%) did receive it. For HT data were available for 1,948 patients and 1,571 of these (80.23%) received an anti-oestrogen for five years which was tamoxifen, ovarian suppression and tamoxifen, or an oral aromatase inhibitor, but some postmenopausal patients participated in clinical trials comparing tamoxifen with an oral aromatase inhibitor (BIG 1-98, IES, TEAM). Of all patients with complete data on adjuvant systemic therapy 690 patients (38.16%) received adjuvant CT, most likely in an anthracycline-based protocol which in 454 cases (25.11%) was followed by an adjuvant HT (Table 2).

During the study FU, we observed 185 breast-cancer-related events (9.43%) in our cohort. The worst reported events were an ipsilateral relapse in 17 patients (0.87%), a

Table 1 Descriptive statistics for subgroups and overall study population

Variables	Combined steroid/HER-2 receptor subgroup						All	*P*-value
	NNN $n = 174$, 8.89%	NNP $n = 91$, 4.64%	PNN $n = 189$, 9.65%	PNP $n = 32$, 1.63%	PPN $n = 1357$, 69.31%	PPP $n = 115$, 5.87%	$n = 1958$, 100%	
Median age at diagnosis[a]	55.00	56.00	61.00	57.00	57.00	50.00	57.00	<0.0001
Tumour grade								
Missing	1 (0.57)	1 (1.10)	–	–	1 (0.07)	–	3 (0.15)	<0.0001
Grade 1	1 (0.57)	1 (1.10)	31 (16.40)	–	233 (17.17)	4 (3.48)	270 (13.79)	
Grade 2	13 (7.47)	12 (13.19)	78 (41.27)	11 (34.38)	753 (55.49)	29 (25.22)	896 (45.76)	
Grade 3	159 (91.38)	77 (84.62)	80 (42.33)	21 (65.63)	370 (27.27)	82 (71.30)	789 (40.30)	
Nodal status								
Missing	5 (2.87)	3 (3.30)	–	2 (6.25)	16 (1.18)	–	26 (1.33)	<0.0007
Negative	110 (63.22)	48 (52.75)	114 (60.32)	21 (65.63)	867 (63.89)	52 (45.22)	1212 (61.90)	
Positive	59 (33.91)	40 (43.96)	75 (39.68)	9 (28.13)	474 (34.93)	63 (54.78)	720 (36.77)	
pT Stage								
Missing	1 (0.58)	–	–	–	1 (0.07)	–	1 (0.05)	0.1499
0	–	–	–	–	1 (0.07)	–	1 (0.05)	
1	75 (43.10)	40 (43.96)	93 (49.21)	18 (56.25)	702 (51.73)	48 (41.74)	976 (49.85)	
2	79 (45.40)	42 (46.15)	81 (42.86)	14 (43.75)	528 (38.91)	57 (49.57)	801 (40.91)	
3	20 (11.49)	9 (9.89)	15 (7.94)	–	120 (8.84)	10 (8.70)	174 (8.89)	
4	–	–	–	–	5 (0.37)	–	5 (0.26)	
Median NPI[a]	4.60	4.87	4.34	4.45	3.90	4.70	4.30	<0.0001
NPI group								
Missing	6 (3.45)	5 (5.49)	–	2 (6.25)	18 (1.33)	–	31 (1.58)	<0.0001
<3.4	7 (4.02)	3 (3.30)	57 (30.16)	5 (15.63)	516 (38.03)	13 (11.30)	601 (30.69)	
3.4–5.4	114 (65.52)	56 (61.54)	89 (47.09)	19 (59.38)	598 (44.07)	60 (52.17)	936 (47.80)	
≥5.4	47 (27.01)	27 (29.67)	43 (22.75)	6 (18.75)	225 (16.58)	42 (36.52)	390 (19.92)	

[a] Kruskal–Wallis test (Wilcoxon-based test), whereas χ^2 test for other analysis

Table 2 Adjuvant therapy for all predefined tumour subgroups

Local and systemic adjuvant therapy	Subgroup total %	Combined steroid/HER-2 receptor subgroup						All	*P*-value
		NNN	NNP	PNN	PNP	PPN	PPP		
Surgery	ME	76 (43.68)	61 (67.03)	84 (44.44)	17 (53.13)	538 (39.59)	53 (46.09)	829 (42.30)	<0.0001
	BCS	98 (56.32)	29 (31.87)	105 (55.56)	15 (46.88)	817 (60.12)	62 (53.91)	1126 (57.45)	
Adjuvant RT	Yes	147 (84.48)	71 (78.02)	157 (83.07)	27 (84.38)	1146 (84.45)	100 (86.96)	1648 (84.13)	0.6429
	No	27 (15.52)	19 (20.88)	32 (16.93)	4 (12.50)	203 (14.96)	15 (13.04)	300 (15.31)	
Adjuvant CT	Yes	123 (70.69)	67 (73.63)	59 (31.22)	18 (56.25)	351 (25.87)	72 (62.61)	690 (35.24)	<0.0001
	No	49 (28.16)	23 (25.27)	130 (68.78)	13 (40.63)	1001 (73.77)	43 (37.39)	1259 (64.30)	
Adjuvant HT	Yes	10 (5.75)	4 (4.40)	160 (84.66)	24 (75.00)	1267 (93.37)	106 (92.17)	1571 (80.23)	<0.0001
	No	164 (94.25)	85 (93.41)	29 (15.34)	7 (21.88)	83 (6.12)	9 (7.83)	377 (19.25)	
Adjuvant CT + HT	Yes	4 (2.30)	3 (3.30)	41 (21.69)	11 (34.38)	331 (24.39)	64 (55.65)	454 (23.19)	<0.0001
	No	170 (97.70)	86 (94.51)	148 (78.31)	20 (62.50)	1020 (75.17)	51 (44.35)	1495 (76.35)	

Missing patients present missing data (for example, for NNN tumors we have data on HT for all 174 patients, but for CT only for 172 patients. Thus one should read: 70.69% of NNN received CT, 28.16% did not, and the remaining 1.15% (not shown) data are missing). RT, radiotherapy; CT, chemotherapy; HT, endocrine therapy; ME, mastectomy; BCS, breast conservative surgery

contralateral breast cancer in 23 patients (1.17%), and distant metastases in 145 patients (7.39%).

The KM curves showed, in order from good to worse survival, the following order: PPN, PNN, NNN, PPP, PNP, NNP (Fig. 2 and Table 3). PPN tumours, the most prevalent group (69.36% of all patients), presented with a superior DFI and lowest median NPI compared with other subgroups (Tables 1 and 3; Fig. 2). Compared with the overall study population, PPN tumours were also more often low-grade (Table 1). HER-2 overexpression in ER/PR-positive tumors resulted in a significantly ($P < 0.0001$) worse DFI and a higher median NPI ($P < 0.0001$ Wilcoxon rank sum test). These PPP tumors constituted a younger age group ($P < 0.0001$ Wilcoxon rank sum test) than PPN tumors (median age 50.00 versus 57.00 years). PPP tumors were also of a higher grade ($P < 0.0001$ (χ^2)) and were more frequently node-positive ($P < 0.0001$ (χ^2)) as was reflected in their median NPI score and NPI risk

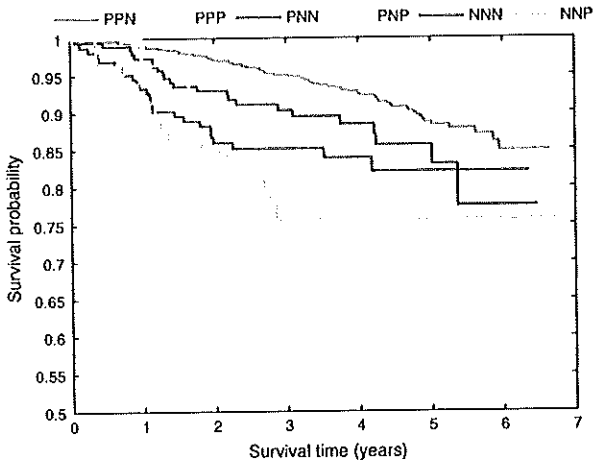


Fig. 2 KM curve depicting DFI of joint receptor subgroups

Table 3 DFI and mean NPI for subgroups

Subgroup	DFI (%)	Order	NPI	Order
NNN	85.65	3	4.95	4
NNP	75.81	6	5.04	6
PNN	89.50	2	4.33	2
PNP	80.42	5	4.61	3
PPN	93.63	1	4.11	1
PPP	81.47	4	4.98	5

group distribution. The impact of HER-2 overexpression on short-term DFI was more prominent in lymph node-positive cases ($P = 0.0002$ (χ^2)). We noticed this finding in all three ER/PR subgroups, but it was only significant in tumors expressing both ER and PR.

Hormone receptor-positive tumors only staining for ER made up 11.29% of all cases. Their DFI was significantly ($P = 0.0082$, log-rank test) worse than ER/PR-positive tumors. HER-2-negative tumors within this subgroup (PNN) had a non-significantly better ($P = 0.30$, log-rank test) DFI (Fig. 2). PNP tumors represented the smallest subgroup (1.63% of all patients) with the smallest median lesion size (22.00 mm).

Taking only ER and PR into account, the 13.53% of breast cancers with a double-negative hormone receptor status had the worst DFI compared with PP and PN tumors. The HER-2-positive subgroup in double hormone receptor-negative tumors had an even worse DFI ($P = 0.22$). ER/PR-negative tumors were more often high grade than tumors belonging to any other subgroup ($P < 0.0001$ (χ^2)). There were no age ($P = 0.6324$, Wilcoxon rank sum test) or NPI ($P = 0.4010$, Wilcoxon rank sum test) differences, nor in the proportion of high grade tumors ($P = 0.2808$ (χ^2)) comparing NNN with NNP lesions.

Table 4 Calculated *P*-values for comparison of the survival curves

Test	*P*-value (log-rank test)	*P*-value (Holm)
PPN–NNP	<0.0001	<0.0001
PPN–NNN	<0.0001	0.0007
PPN–PPP	0.0001	0.0008
PPN–PNN	0.0146	0.1022
PNN–NNP	0.0295	0.1770
NNN–NNP	0.2240	1.0000
PNN–PPP	0.2660	1.0000
PNN–NNN	0.3375	1.0000
PPP–NNP	0.3383	1.0000
NNN–PPP	0.8372	1.0000

The adaptation for multiple testing according to Holm is given

Table 4 presents the calculated *P*-values for comparison of the survival curves. No comparisons were made with the PNP curve, given the low number of such tumors. The calculated *P*-values are subject to multiple testing. Therefore the significance level of $P = 0.05$ is no longer valid. We leave it up to the reader to define a significance level. A possible adaptation for multiple testing (according to Holm) is also given, indicating a significant difference for the first three tests and a non-significant difference for the last five. For more details we refer to the work of Holm and Benjamini [40, 41].

Examination of a multivariate Cox model with ER, PR, and HER2 as main effects, all significant in univariate analysis, indicated that the effect of ER is no longer significant after inclusion of PR (Table 5). To adjust for treatment, the variables indicating whether the patient received chemotherapy and hormonal therapy where included in a model with PR and HER2 (Table 6). The effect of PR and HER2 remained significant after adjusting for treatment. ER status was overruled in the multivariate model by PR but remained a significant prognostic factor in interaction with HT. Furthermore, both ER and PR were found to have a significant interaction with CT. Since the interaction effects of ER–CT and PR–CT are strongly correlated, we excluded the ER–CT interaction, knowing that all PR+ tumours in this study are ER+ and most PR-tumours are ER+ (Tables 1, 5 and 6).

Discussion

We confirmed that breast cancer subgroups based only on their combined IHC expression of ER, PR, and HER-2 have a prognostic impact on the short-term DFI of primary operable breast cancers. We have also shown that there is no important interaction of currently applied local and systemic therapies on the prognostic importance of these IHC subtypes. In a multivariate model with given therapy, PR and HER2 expression or amplification remained independent prognostic factors. We chose not to include NPI in this model, because the treatment is decided on the value of the

Table 5 Multivariate Cox model including three main effects: ER, PR, and HER2

Variable	Parameter estimate	Standard error	Chi-square	*P*-value	Hazard ratio	95% Hazard ratio confidence limits	
ER	−0.22454	0.24463	0.8425	0.3587	0.799	0.495	1.290
PR	−0.48085	0.20902	5.2925	0.0214	0.618	0.410	0.931
HER-2	0.66757	0.18488	13.0375	0.0003	1.949	1.357	2.801

Table 6 Multivariate Cox analyses were performed to check for statistically significant effects of ER, PR, and HER-2, after adjustment for therapy

Variable	Parameter estimate	Standard error	Chi-square	*P*-value	Hazard ratio	95% Hazard ratio confidence limits	
PR	−0.69730	0.23084	9.1248	0.0025	0.498	0.317	0.783
HER-2	0.52384	0.18718	7.8322	0.0051	1.688	1.170	2.437
CT	−0.43454	0.24893	3.0473	0.0809	0.648	0.398	1.055
HT	0.86821	0.46804	3.4409	0.0636	2.383	0.952	5.963
ER * HT	−1.45379	0.48842	8.8598	0.0029	0.234	0.090	0.609
PR * CT	0.94133	0.31450	8.9587	0.0028	2.563	1.384	4.748
ME versus BCS	0.49216	0.15261	10.4004	0.0013	1.636	1.213	2.206

Significant interaction effects were found for ER and CT, PR and CT, and ER and HT. Because the ER–CT and PR–CT interaction effects were strongly correlated, we excluded the ER–CT interaction. There is an independent significant statistical effect for PR and HER-2, after adjusting for therapy

NPI. While gene expression profiles based on microarray data of over hundreds of genes are teaching us a lot about the biology, heterogeneity, and prognosis of breast cancer, we emphasized that early relapse in patients with an operable breast cancer also depends on increased expression and amplification of steroid-receptors and HER-2.

Prevalence of combined receptor subgroups in our study differs from those reported in the literature. The frequency of HER-2-over-expression (12.16%) is low compared with other series but analysis and definitions used in this series were based on current ASCO guidelines [26, 42–44]. Although reports state that CB11 might not be the best predictive antibody for HER2 testing we were able to clearly show its prognostic importance when overexpressed [45, 46]. Our lower than usually presented proportion of HER-2 overexpression may also be explained by the inclusion of a consecutive series of primary operated breast cancers leaving out locally advanced operable breast cancers. In a recently reported series from Sweden considering 5,043 consecutive cases, 13.3% of the samples were HER-2 amplified [47].

The proportion of breast cancers defined as ER-positive (87.47%) or PR-positive (75.18%) is higher than literature-reported proportions for ER (75%) and PR (55%) [7, 14, 48, 49]. Improved laboratory sensitivity for detecting ER, different quantification techniques, and dichotomous cut-off values for assessing ER and PR account for important interlaboratory variability [36, 50, 51]. In our study, all IHC data were interpreted in one institute and validated by one pathologist (MD) which is a strength. The use of very sensitive monoclonal rabbit antibodies urged us to consider any nuclear staining for ER or PR as positive when using this biomarker for prognostic purposes. This approach has recently been described as appropriate for predictive and possibly also for prognostic indications although a quantitative measure for both ER and PR may be better [52].

The quantitative instead of qualitative values of steroid receptors may improve them for breast cancer prognosis. However, different antibodies and cut-offs for steroid receptor evaluation made quantification impossible as this study was retrospective. The qualitative approach for steroid receptors made it possible to separate short-term DFI curves for ER/PR-positive, ER-positive/PR-negative, ER-positive/PR-negative and ER/PR-negative tumors, and this was true within each HER-2 status. Although nobody knows exactly the best cut-off for ER or PR for predictive purposes, Schnitt et al. recently proposed considering "any nuclear staining" as a positive predictor for anti-estrogens [52].

Rather than only using the joint ER/PR-expression as a prognostic factor, DFI was here presented by six predefined breast cancer subgroups combining ER/PR status with HER-2 status. Our outcome data as presented in Fig. 2 confirmed what others have recently published and clearly separates some prognostic subgroups using only ER, PR, and HER2. We however, presented data from a large number of breast cancer patients, all treated in one center, including almost 2,000 patients; this is, as far as we are aware, the largest series reported in the literature. Another strength of our study is that we did take local and systemic therapy into account, which was not done by others [25].

In our series, women with a triple negative tumour presented with a better short-term DFI compared with women with any of the three HER-2-positive subgroups; the literature frequently emphasizes the poor prognosis of this subgroup [48, 53]. HER-2 as a poor prognostic marker is already now being affected by adjuvant trastuzumab therapy and triple negative tumors may, anyway, eventually become the subgroup with the worst DFI. Also, patients with a triple negative breast cancer suffer from more limited treatment options and exhibit inherent aggressive tumour characteristics as expressed by their high median NPI score [48, 49] (Tables 1 and 2).

We noticed a worse DFI for HER-2-positive tumours, irrespective of hormone receptor status (Fig. 2). We confirm, as previously reported by others, that the impact of HER-2 overexpression on short-term DFI was more prominent in lymph node-positive cases ($P = 0.0002$ (χ^2)). We noticed this finding in all three ER/PR subgroups, but it was only significant in tumors expressing both ER and PR. It has, however, been suggested that as more data become available in the literature, and with longer FU, the prognostic role of HER-2 will probably become independent of nodal status [22, 26, 27, 53]. A weakness in our study is the median FU period which consisted of 3.5 years only and which allows us only short-term DFI interpretations. Others have referred to ER and PR as only being short-term prognostic factors whereas little data are available on HER-2 as a prognostic factor beyond five years of FU [6, 54, 55].

Youth is a well recognized risk factor in breast cancer especially for early relapse. We chose not to evaluate the prognostic impact of age at diagnosis in this study. We were able to confirm that women with a triple-positive phenotype were younger than women with any other breast cancer phenotype. Huang et al. have already reported that the inverse association between HER-2 and PR only appears after age 45 [11]. This not only implies that the PR status cannot be used to predict HER-2 signalling in young breast cancer patients, as opposed to the elder women, but also that women with a triple-positive breast cancer tend to be younger than women with any other breast cancer phenotype (Table 1). Women with a triple-positive breast cancer were also more likely lymph node-positive [56] which reflected their worse DFI outcome compared with any other ER-positive group, although the difference between PPP and PNP was not calculated, as already stated (Fig. 2; Tables 3 and 4).

In conclusion, our data and within a short-term FU, clearly confirmed the possibility of separating prognostic subgroups based on joint receptor status for ER, PR, and HER-2. All three receptors remain significant factors in multivariate analysis taking adjuvant therapy into account. While gene expression profiles based on microarray data of over hundreds of genes will probably teach us a lot about the biology, heterogeneity, and prognosis of breast cancer, we emphasize the prognostic value of current routinely used IHC markers in casu ER, PR, and HER-2.

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