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ORIGINAL ARTICLE

Age related influence of triple receptor status on metastatic breast cancer post relapse survival

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Summary

Purpose: *Prognostic f actors in metastatic breast cancer (MBC) differ from those of primary breast cancer. The aim of this study was to identify the clinical significance of combined estrogen and progesterone receptors (ER,PR) and human epidermal growth factor receptor-2 (HER2) status on MBC post relapse survival.*

Methods: *The survival of 109 MBC patients was analyzed according to clinical characteristics and ERIPR status (tested by ligand binding assay) and HER2 status (tested by chromogenic in situ hybridization/CISH).*

Results: *Proper parameters for follow up of MBC patients were patient age, duration of disease free interval (DFI), dominant site of metastasis, number of metastatic sites and ER, PR status. Follow up of MBC patients showed the statistically significant difference in post relapse survival between patients with extreme phenotypes ER+PR+ and ER-PR-. Addition of HER2 status confirmed negative effect of HER2 amplification on MBC post relapse survival resulting in worse prognosis of ER-PR-HER2+ patients. The corresponding triple receptor (ER,PR,HER2) combination repeated the same pattern. In combination with patient age it was shown that difference in post relapse survival between extreme phenotypes (ER+PR+HER2- and ER-PRHER2+) was age related i.e. patients older than 50 years, with ER-PR-HER2+ phenotype, had mortality rate 100% and median survival time 14 months.*

Conclusion: *There is a strong indication for use of combined triple receptor status for follow-up of MBC patients. Based on our results, the worst phenotype was neither triple positive nor triple negative, but the one that most likely reflects the biological background of these biomarkers (ERPR-HER2+). Double and triple receptor status showed repeated pattern of influence on prognosis, but the finding that ER-PR-HER2+ phenotype in an age-restricted subgroup of patients means extremely poor prognosis and a highest mortality rate deserves further consideration regarding therapy efficiency.*

Key words: *ERIPR status, HER2, metastatic breast cancer, survival*

Introduction

MBC is an incurable condition, with survival ranging from few months to several years. This fact underlines the importance for defining prognostic factors of survival in MBC patients. Risk evaluation in MBC cancer is based on parameters different from primary breast cancer, such as hormone receptor status, HER2 status, DFI, number of metastases, sites of metastases and vital organ involvement [1]. A recent study also focused on MBC survival, trying to establish a prognostic model and risk scores for MBC patients based on factors known at the time of first diagnosis and at the time of recurrence [2].

Among these parameters, ER/PR and HER2 are still the only molecular biomarkers accepted in clinical practice and their importance in breast cancer progression is well known. Recent gene expression studies confirmed that breast cancer

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Table 1. Patient clinicopathological characteristics

Characteristics	*N*	*%*
Age, years		
≤50	57	52
>50	46	43
NA	6	5
Menopausal status		
Pre	49	44
Post	54	51
NA	6	5
Estrogen receptor status		
ER-	45	42
ER+	55	50
NA	9	8
Progesterone receptor status		
PR-	53	49
PR+	47	43
NA	9	8
Tumor size		
T1	16	15
T2	57	52
T3	9	8
T4	16	15
NA	11	10
Nodal status		
N-	19	17
N+	81	75
NA	8	8
Distant metastasis (initial)		
M-	90	82
M+	12	11
NA	7	7
Dominant site of metastasis		
Visceral	71	65
Soft tissue	20	18
Bone	12	11
NA	6	6
Number of metastatic sites		
Single	48	44
Multiple	55	50
NA	6	6
HER2 status		
Non amplified	67	61
Amplified	42	49
NA	0	0
Histological types		
IDC	60	55
ILC	27	25
Rest	4	4
NA	18	16
Stage at diagnosis		
I	12	11
II	56	51
III	22	20
IV	12	11
NA	6	7

NA: not available, ICD: invasive ductal carcinoma, ILC: in vasive lobular carcinoma

is heterogeneous disease, with several different phenotypes (based on steroid receptor and HER2 status) and different outcomes [3,4]. ER/PR and HER2 affect the expression of a significant number of genes representing multiple biochemical pathways and there are substantial and complex interactions among them. The consequences of these interactions are not anticipated enough and could be intelligible only in the context of patient survival.

For that reason, besides the relative contribution of the available clinicopathological parameters known at the time of diagnosis and parameters related to metastatic disease, we tried to evaluate in particular the influence of the so called "triple receptor status" i.e. combined steroid receptor (ER and PR) and HER2 on the MBC post relapse survival. Determination of the hierarchy of the breast cancer phenotypes (based on the triple receptor status) in relation to their corresponding prognostic value could help to better characterization of an individual's prognosis. This is especially important for MBC post relapse survival when identification of patients who are at poorest risk could improve their clinical management.

Methods

This study included 109 MBC patients, initially diagnosed between 1991 and 2001 at different stages of disease. Patients were included in the study between 2002 and 2004 and were followed for post relapse survival (whether they were initially diagnosed with MBC or they had recurrent disease with visceral/nonvisceral involvement). Patient clinicopathological characteristics are given in Table 1. On primary diagnosis 75% of the patients had positive lymph node status and 11 % had distant metastasis. Except for the patients who were diagnosed at advanced disease stage, 10 % didn't receive any kind of adjuvant treatment and the remaining received different kinds of adjuvant therapy (chemo and hormonal), alone or in combination. Metastatic patients received chemotherapy (CMF: cyclophosphamide, methotrexate, 5-fluorouracil, and FAC: 5-fluorouracil, doxorubicin, cyclophosphamide) and/or hormonal therapy (tamoxifen). However, assessment of treatment efficacy in relation to triple-receptor status was not investigated in this study, because we wanted to analyze MBC post relapse survival rather than progression-free interval.

Histopathological classification of the primary tumor tissue samples was done on hematoxylin/eosin stained slides according to standard histopathological criteria. All of the samples were fixed in neutral buffered formalin and embedded in paraffin. Serial sections of 4 μm thicknesses on Superfrost/Plus microscope slides were obtained from representative tissue blocks, baked 4h in 60°C and processed for CISH, according to the manufacturer's instructions. Steroid receptor status ER and

PR) was determined by ligand-binding assay i.e. in the cytosol of primary breast cancer tissue using dextran-coated (DCC) method. Cut-off value for the qualitative classification of positive receptor status was 10 fmol/mg per cytosol protein for ER and 20 fmol/mg per cytosol protein for PR.

CISH

Tissue slides were deparaffinized in xylene (2xl0 min) and washed in 100% ethanol (3x3 min). When dry, the slides were incubated for 15 min at 96°C (92-100°C) in Spot-Light Tissue Heat Pretreatment Buffer (Zymed Inc. San Francisco, CA), cooled down for 20 min and washed in PBS for 2x3 min. Tissue was covered with 100 µl of Spot-Light Tissue Pretreatment Enzyme (Zymed Inc.) and incubated at 37°C for 10 min. Then, the slides were washed in phosphate-buffered saline (PBS) and dehydrated in 70, 85, 95%, and 100% ethanol for 2 min each. The slides were denaturized in denaturation buffer (containing formamide, 20 x standard saline citrate/SSC, and dd H_2O) at 78°C in water bath. Digoxigenin-labeled probe (Spot-Light HER-2 DNA probe, Zymed Inc.) was denaturized in the same way. After dehydration, 16 µl probes were added on each slide, covered with coverslip and incubated overnight at 37°C. After incubation, slides were washed in 0.5 x standard saline citrate for 5 min at 78°C and treated with quenching solution (containing hydrogen peroxide and absolute methanol). After washing in PBS/ Tween 20 for 3x2 min, Spot-Light CISH Detection Kit (Zymed Inc.) was used for chromogenic visualization.

Hybridization results were evaluated in 40x and l00x magnification (Olympus BX51 microscope). One to 5 gene copies per nucleus were defined as no amplification, while more than 6 gene copies per nucleus or large gene copy clusters in > 50% of tumor cells as amplification.

Statistics

Time of diagnosis of metastatic disease (whether patients primarily diagnosed as metastatic or not) was taken as starting point for MBC post relapse survival. Survival curves for MBC were constructed according to the Kaplan-Meier method and compared with the log-rank test. Associations between parameters were analyzed by the Chi-square test. P value less than 0.05 was considered as statistically significant.

Table 2. Mortality and survival of patients stratified according to clinicopathological characteristics

Characteristics	*N*	*Mortality*		*Median survival time,*	*Log rank*
		N	*%*	*months*	*p*
Age (years)					0.02
≤50	54	23	43	44	
>50	45	25	55	26	
Menopausal status					0.06
Pre	47	22	47	44	
Post	52	26	50	26	
ER status					0.01
ER-	43	26	60	23	
ER+	52	19	36	44	
PRstatus					0.008
PR-	49	28	57	26	
PR+	46	16	35	57	
Tumor site					0.6
T1	15	6	40	44	
T2, T3, T4	80	40	50	29	
Nodal status					0.3
N-	18	7	39	57	
N+	79	39	49	29	
Distatnt metastasis (initial)					0.06
M-	87	39	45	43	
M+	12	8	67	17	
Dominant site of metastasis					0.01
Visceral	69	34	49	30	
Soft tissue	20	7	35	31	
Bone	11	7	63	29	
Number of metastatic sites					0.01
Single	46	19	41	65	
Multiple	54	29	54	42	
Stage					0.08
I+II	65	27	41	44	
III+IV	34	21	62	29	

Results

The median follow-up period for the cohort at the time of analysis was 5 years (from the time of primary diagnosis). The median MBC post relapse survival for the whole group was 21 months. According to survival analysis, among available clinicopathological parameters as relevant for follow up of MBC patients were age, DFI, dominant site of metastasis, number of metastatic sites and steroid receptor status (ER as well as PR) (Table 2). Treatment of MBC cases (antioestrogen, anthracycline-based and CMF) did not affect post relapse survival (treated vs not treated), although survival of responders vs non responders in each treatment subgroup was significantly different (Table 3). Furthermore, combined steroid receptor status (Table 4, Figure 1) showed that there was statistically significant difference in case of extreme phenotypes (ER-PR- and ER+PR+). When each of these receptors was assessed in relation to HER2 status, statistically significant difference Was noticed in MBC

Table 3. Mortality and median survival according to the kind of treatment in metastatic disease

Kínds of treatment	*N*	*Mortality*		*Median survival time, months*	*Log rank, p*
		N	*%*		
TAM					0.3
No	62	25	40	44	
Yes	38	23	60	29	
CMF					0.5
No	45	23	51	29	
Yes	55	25	45	43	
FAC					0.6
No	71	35	49	42	
Yes	29	13	45	57	
Response to TAM					<0.001
PD	9	8	89	17	
CB	28	13	46	44	
Response to CMF					0.02
PD	15	11	73	18	
CB	40	14	35	54	
Response to FAC					0.02
PD	8	6	75	14	
CB	20	7	28	65	

TAM: tamoxifen, CMF: cyclophosphamide/methotrexate/5-fluorouracil, FAC: 5-fluorouracil/doxorubicin/cyclophosphamide, CB: clinical benefit, PD: progressive disease

Table 4. Mortality and median survival according to ER and PR status

Steroíd receptor status	*N*	*Mortality*		*Median survival time, months*	*Log rank, p*
		N	*%*		
ER- PR -	34	21	62	21	0.1
ER- PR+	8	3	37	33	
ER + PR -	14	6	43	44	0.6
ER + PR+	40	13	32	57	
ER- PR -	34	21	62	21	0.1
ER + PR -	14	6	43	44	
ER - PR +	8	3	37	33	0.8
ER + PR +	40	13	32	57	
ER - PR +	8	3	37	33	0.5
ER + PR.	14	6	43	44	
ER - PR.	34	21	62	21	0.008
ER + PR +	40	13	32	57	

post relapse survival again only in case of extreme phenotypes i.e. ER-HER2+ vs ER+HER2- (Tables 5 and 6). Also, analysis of the effect of the three receptor status i.e. ER-PR- HER2+ vs ER+PR+HER2- on post relapse survival showed the same result (Table 7, Figure 2).

Table 5. Mortality and median survival according to combined ER and HER2 status

	N	*Mortality*		*Median survival time, months*	*Log rank, p*
		N	*%*		
ER - HER2 -	21	12	57	30	0.2
ER - HER2 +	22	14	64	19	
ER + HER2 -	35	13	37	44	0.7
ER + HER2 +	17	6	35	14	
ER - HER2 -	21	12	57	30	0.2
ER + HER2 -	35	13	37	44	
ER - HER2+	22	14	64	19	0.06
ER + HER2 +	17	6	35	24	
ER - HER2 +	22	14	64	19	0.003
ER + HER2 -	35	13	37	44	
ER - HER2 -	21	12	57	30	0.4
ER + HER2 +	17	6	35	24	

Table 6. Mortality and median survival according to combined PR and HER2 status

	N	*Mortality*		*Median survival time, months*	*Log rank, p*
		N	*%*		
PR - HER2 -	24	14	58	42	0.2
PR - HER2 +	25	14	56	21	
PR + HER2-	31	10	32	57	0.5
PR + HER2 +	15	6	40	43	
PR - HER2 -	24	14	58	42	0.05
PR + HER2 -	31	10	32	57	
PR - HER2 +	25	14	56	21	0.2
PR + HER2 +	15	6	40	43	
PR - HER2 +	25	14	56	2	0.005
PR + HER2 -	31	10	32	57	
PR - HER2 -	24	14	58	42	0.6
PR + HER2 +	15	6	40	43	

However, since age, DFI, number of metastatic sites and sites of metastasis seem to be strong prognostic indicators for the group as whole, we analyzed the correlations between these parameters and the triple receptor status (for the extreme phenotypes ER-PR-HER2+ and ER+PR+HER2-). There was no statistically significant correlation between triple receptor status and site of metastasis (x^2, p=0.09) although 89% of ER-PR-HER2+ patients had visceral metastasis. Also, no statistically significant correlations were registered between triple receptor status and number of metastatic sites (x^2, p=0.3) although 67% of ERPR-HER2+ pa-

Table 7. Mortality and median survival according to combined ER,PR and HER2 status

	N	*Mortality*		*Median survival time, months*	*Log rank, p*
		N	*%*		
ER-PR-HER2-	17	9	53	30	0.1
ER-PR-HER2+	17	12	70	18	
ER+ PR+ HER2-	28	9	32	57	0.6
ER+ PR+HER2+	10	4	40	30	
ER-PR-HER2-	17	9	53	30	0.2
ER+PR+HER2-	28	9	32	57	
ER+PR+HER2+	10	4	40	30	0.1
ER-PR-HER2+	17	12	70	18	
ER-PR-HER2+	17	12	70	18	<0.001
ER+PR+HER2-	28	9	32	57	
ER-PR-HER2-	17	9	53	30	0.5
ER+PR+HER2+	10	4	40	30	

Table 8. Mortality and median survival according to double (ER,PR) and triple receptor status in different age subgroups

	N	*Mortality*		*Median survival time, months*	*Log rank, p*
		N	*%*		
≤50 years					0.05
ER-PRER+	15	8	53	22	
PR+	26	9	35	57	
>50 years					0.1
ER-PRER+	19	14	74	17	
PR+	11	4	36	44	
≤50 years					0.2
ER-PR-HER2+	8	3	37	21	
ER+PR+HER2-	19	6	31	57	
>50 years					0.003
ER-PR-HER2+	9	9	100	14	
ER+PR+HER2-	8	3	37	44	

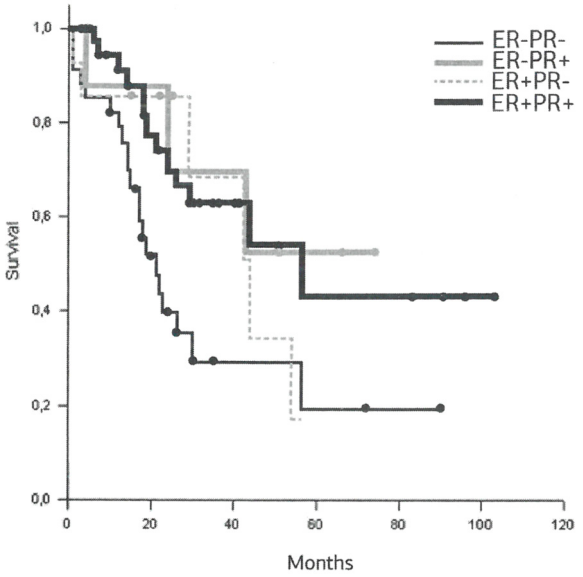

Figure 1. Metastatic breast cancer post relapse survival curves according to combined ER and PR status. P values (log rank) are given in Table 4.

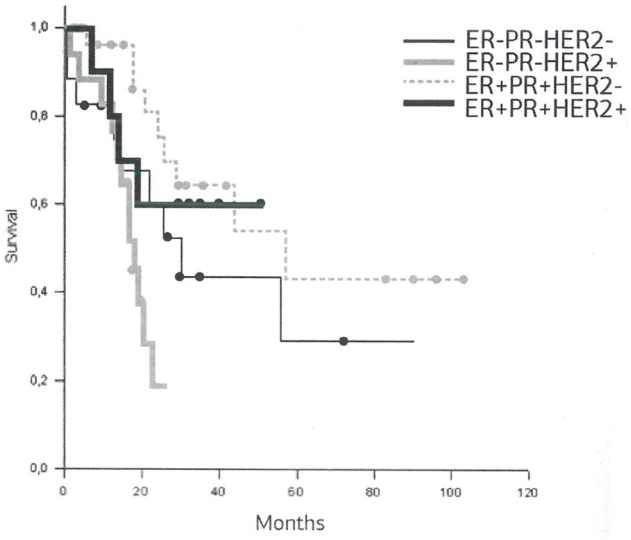

Figure 2. Metastatic breast cancer post relapse survival curves according to combined ER, PR and HER2 status. P values (log rank) are given in Table 7.

tients had multiple metastases. DFI was strongly influenced by triple receptor status (x^2, p=0.005) showing that the majority of patients who had DFI >24 months were ER+PR+HER2-(82%). DFI was not affected by age at the time of diagnosis (x^2, p=0.l). Although no statistically significant correlation was noticed between distribution of extreme phenotypes in relation to different age subgroups (x^2, p=0.2), patients older than 50 years were more likely to be ER+PR+HER2- (68%).

Furthermore, survival analysis of extreme double and triple receptor subgroups (ER-PR- and ER+PR+, ER-PR-HER2+ and ER+PR+HER2-) in different age subgroups (≤50 and >50) showed significant impact on MBC post relapse survival only for patients older than 50 years (Table 8, Figure 3). For patients in this subgroup (ER-PR-HER2+), mortality was 100% and the median survival time was 14 months. The previous observation is also confirmed otherwise, when patients with E-PR• HER2+ phenotype are stratified by age (≤50 and >50 years) (Figure 4).

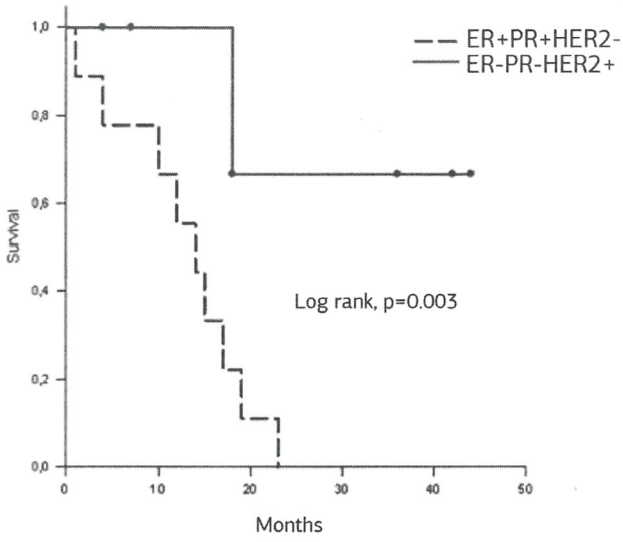

Figure 3. Metastatic breast cancer post relapse survival curves according to combined ER, PR and HER2 status for patients older than 50 years.

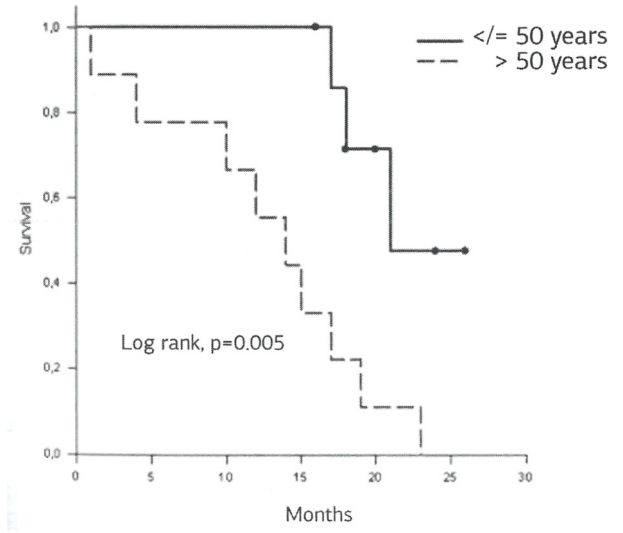

Figure 4. Metastatic breast cancer post relapse survival curves for ER-PR-HER2+ patients stratified by years of age (s50, > 50).

Discussion

There are many controversies regarding possible breast cancer phenotypes, but the widely accepted concept is the one that is based on gene expression profiles. Gene expression profiles formed a new classification systems aimed to clarify relations between breast cancer biology and prognosis [5]. According to this classification there are 4 breast cancer phenotypes: luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), HER2 (ER-PR-HER2+) and basal like (ER-PR-HER2-) [6]. Triple receptor status is in focus in recent years. Many recent studies indicated that the worse prognosis among these different breast cancer phenotypes have the so called triple-negative phenotype (ER-PR-HER2-) [7,8] or even triple-positive phenotype [9]. However, none of these reports studied MBC post relapse survival. It is well known that ER-PR- phenotype correlates with more aggressive behavior. HER2 amplification/overexpression induces its "oncogenic action". Hyperactivation of HER2 leads to deregulation of the cell cycle and increases the proliferation that provides selective advantage to the tumor cells. The negative impact of ER-PR-HER2+ phenotype on patient's prognosis is in agreement with a model in which breast cancer cell growth and tumor progression switches from steroid hormone to growth factor dependence, supported by the general inverse correlation between ER/PR and HER2 expression.

Based on our results, among the classical clinicopathological parameters determined at the time of initial diagnosis (Table 2), age and steroid receptor status seem to be significant prognostic parameters for MBC post relapse survival. It has been also confirmed that DFI has significant impact on the course of metastatic disease as well as the dominant site of metastasis and the number of metastatic sites. HER2 alone didn't show prognostic significance, except when related to ER and PR status (Tables 4 and 5). There was a strongly indicative pattern of the prognostic significance of these biomarkers that is repeated in expected combinations. Analysis of ER/PR phenotype combinations showed that the only statistically significant difference in MBC post relapse survival was between double negative (ER-PR-) and double positive (ER+PR+) phenotype with the median survival time 21 vs 57 months (Table 3). This means that both receptors have the same prognostic significance, further indicating the significant role of PR that is not only marker of ER function. This is also in agreement with a recent study which showed that breast cancer patients with double negative steroid receptor status have the highest mortality in comparison to other subgroups [10]. Addition of HER2 status to ER or PR status separately, showed that HER2 exerts its negative effect on prognosis resulting in statistically significant difference in MBC survival between extreme phenotypes (i.e. ER-HER2+ and ER+HER2- with median survival time 19 vs 44 months and PR-HER2+ and PR+HER2- with median survival time 21 vs 57 months). It is in agreement with the already

known prognostic significance of these parameters. Negative steroid receptor status and HER2 amplification significantly decrease the median survival time of such patients. The triple receptor status also showed the same simple pattern, i.e. in biological terms, negative prognostic impact of ER-PR-HER2+ phenotype. However, the addition of HER2 status didn't have significant effect on increase or decrease of median survival time. The lowest median survival time (19 months) was seen in the ER-PR-HER2+ subgroup in comparison with ER+PR+HER2- (57 months, same as for ER+PR+).

Hormone receptor negative breast cancers are generally thought to be more aggressive and addition of HER2 amplification is a parameter of poorer prognosis *per se*. This is in accordance with recent studies that also found that patients with ER-PR-HER2+ had more aggressive disease and shorter overall survival [11,12]. It is reasonable to assume that different phenotypes have distinct tumor biology and progression pathways. According to this, a recent study based on different gene expression modules related to key biological processes in breast cancer revealed that proliferation is the strongest parameter of clinical outcome in the ER+HER2- subgroup and that immune response and tumor invasion seem to be the main parameters associated with prognosis in the ERHER2+ subgroup [13]. If this is so, it is in agreement with the indisputable significance of ER-PRHER2+ phenotype in MBC.

Triple receptor status could be influenced by age as it is confirmed by some recent studies [14-16]. Comparing the survival curves of ER-PR- (Figure 1) patients with undefined HER2 status, ER-PR-HER2+ (Figure 2) and ER-PR-HER2+ related to age (Figure 3) it is clear that it is always the same subgroup of patients that contribute to the poorer prognosis of the whole group i.e. patients older than 50 years contribute mostly to decreased post relapse survival of ER-PR- or ER-PR-HER2+. These findings confirm that the biology of breast cancer is significantly affected by patient's age, meaning that some parameters that determine different breast cancer phenotypes are age-related. This is clear from Table 8 where patients belonging to different age subgroups (<50, ≥50 years) are stratified by double and triple receptor status. This is supported by the fact that when patients with ERPR-HER2+ phenotype are stratified by age (Figure 4) the worse prognosis is seen in patients older than 50 years (Log rank, p=0.005). This finding indicates the role of HER2 as additional parameter to the well known significance of ER, PR status in MBC. HER2 amplification is frequently associated with shorter DFI and worse overall survival in patients with early stage breast cancer. However, according to some reports, its value in MBC is still uncertain and it seems not to be significant or at least independent predictor of outcome in metastatic disease [17]. In our study of 12 patients with bone metastasis (data not shown), only 1 had HER2 amplification, indicating that patients without HER2 amplification have more indolent disease course, since patients with bone metastasis have longer median survival time [18].

These differences in post relapse survival of chosen phenotypes, based on this simple classification, may be of great importance for defining subgroups with more or less aggressive disease course, since for MBC patients any improvement is desirable. Anyway, it was shown that ER and PR retain important prognostic value in MBC and that HER2 could have additional significance. We wanted to emphasize that it is possible to predict MBC post relapse survival from data that we know at the beginning of the disease i.e. characteristics of the primary tumors, considering the lack of such studies in comparison to reports on primary breast cancer. Since there is no doubt that the biology of the primary tumor affects the course of metastatic disease, in this study we wanted to evaluate in particular the importance of triple receptor status for MBC post relapse survival. Based on our results, the worst phenotype was neither triple positive nor triple negative, but the one that most likely reflects the biological background of these biomarkers. Moreover, these differences in the prognostic value of different phenotypes could be age-related. Identification of ER-PRHER2+ phenotype (age >50 years) as a subgroup with extremely poor prognosis and the highest mortality rate (100%) deserves further consideration regarding the efficacy of the therapeutic interventions.

We consider this finding important as a small contribution to the better understanding of MBC. Treatment options indeed affect MBC survival, but a better subgrouping of patients based on parameters that we know at the time of primary diagnosis even before metastatic occurrence could be helpful to determine the course of disease and refine treatment choices.

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