NEOPLASIA
www.neoplasia.com

Volume 18 Number 11 November 2016 pp. 647–653 647

Prognostic Impact of HER2 and ER Status of Circulating Tumor Cells in Metastatic Breast Cancer Patients with a HER2-Negative Primary Tumor[1]

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Abstract

BACKGROUND: Preclinical and clinical studies have reported that human epidermal growth factor receptor 2 (HER2) overexpression yields resistance to endocrine therapies. Here the prevalence and prognostic impact of HER2-positive circulating tumor cells (CTCs) were investigated retrospectively in metastatic breast cancer (MBC) patients with a HER2-negative primary tumor receiving endocrine therapy. Additionally, the prevalence and prognostic significance of HER2-positive CTCs were explored in a chemotherapy cohort, as well as the prognostic impact of the estrogen receptor (ER) CTC status in both cohorts. *METHODS:* Included were MBC patients with a HER2-negative primary tumor, with ≥1 detectable CTC, starting a new line of treatment. CTCs were enumerated using the CellSearch system, characterized for HER2 with the CellSearch anti-HER2 phenotyping reagent, and characterized for ER mRNA expression. Primary end point was progression-free rate after 6 months (PFR6months) of endocrine treatment in HER2-positive versus HER2-negative CTC patients. *RESULTS:* HER2-positive CTCs were present in 29% of all patients. In the endocrine cohort ($n = 72$), the PFR6months was 53% for HER2-positive versus 68% for HER2-negative CTC patients ($P = .23$). In the chemotherapy cohort ($n = 82$), no prognostic value of HER2-positive CTCs on PFR6months was observed either. Discordances in ER status between the primary tumor and CTCs occurred in 25% of all patients but had no prognostic value in exploratory survival analyses. *CONCLUSION:* Discordances regarding HER2 status and ER status between CTCs and the primary tumor occurred frequently but had no prognostic impact in our MBC patient cohorts.

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Abbreviations: CTC, circulating tumor cell; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; MBC, metastatic breast cancer; PFR6months, progression-free-rate after 6 months; PT, primary tumor

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[1] This work was supported by KWF (Netherlands Cancer Foundation; grant number EMCR 2012-5390) and by Janssen Diagnostics. Janssen Diagnostics did not play any role in the design, analysis, and/or interpretation of the presented data.
Received 4 August 2016; Revised 24 August 2016; Accepted 29 August 2016

1476-5586
http://dx.doi.org/10.1016/j.neo.2016.08.007

Introduction

Treatment of metastatic breast cancer (MBC) is currently mainly driven by the characteristics of the primary tumor (PT). However, clinically relevant discordances between the PT and the metastases with respect to the estrogen receptor (ER) status and the human epidermal growth factor receptor 2 (HER2) status occur and influence systemic therapy choices and patient management [1]. As a result, both European and American guidelines [2,3] recommend to perform biopsies of metastatic lesions to assess their receptor status and decide on subsequent systemic therapy. Biopsies of metastases are, however, often not performed because this is regarded as a cumbersome procedure for patients, and it is sometimes even impossible due to inaccessibility of the metastases. Another limitation of taking single metastatic biopsies is that intratumor and intermetastatic heterogeneity is missed. To assess the characteristics of metastatic tumor cells in a minimally invasive way, it is attractive to obtain circulating tumor cells (CTCs) as a liquid biopsy from peripheral blood. Discrepancies between the ER status and the HER2 status of the PT and the CTCs in MBC have been demonstrated by numerous groups [4–18]. Some of these groups have also reported on the prognostic value of receptor discrepancies between PTs and CTCs, but such reports have been scarce [17,18]. Further, previous studies have been performed in rather small and heterogeneous groups of patients, making it difficult to draw firm conclusions on the exact clinical relevance of ER and HER2 expression in CTCs.

It has been suggested that the HER2 status of the tumor impacts response to endocrine therapy. Several preclinical studies demonstrated that the introduction of the HER2 transcript in ER-positive breast cancer cell lines confers endocrine resistance [19–21]. In addition, a meta-analysis in 2379 MBC patients demonstrated that patients with a HER2-positive PT were less sensitive to endocrine treatment than patients with a HER2-negative PT [22]. In the current study, we investigated whether or not MBC patients with a HER2-negative PT, but HER2-positive CTCs, have a worse outcome to endocrine treatment compared with patients with HER2-negative CTCs. In addition, a separate control group of MBC patients with a HER2-negative PT receiving first-line chemotherapy was included in which the prognostic impact of HER2-positive CTCs was explored. Also explored in both cohorts were the clinical impact of switches in ER expression between the PT and CTCs.

Methods

Patients and Treatment

MBC patients with a HER2-negative PT and the presence of at least one detectable CTC by the CellSearch system (see below), who started a new line of systemic treatment for MBC, were eligible for this study. We used data from our CTC studies with patients starting first-line chemotherapy (study 06-248 [9,17,23]) or starting a new line of endocrine therapy (study 09-405 [24]) for MBC. All patients had been included between February 2008 and March 2015 in six centers in the Netherlands and Belgium. From all patients, 2 × 7.5 mL of blood was drawn for CTC enumeration and CTC characterization. The local institutional review board of each participating center approved the study protocols (Erasmus MC ID MEC-06-248 and MEC-09-405). All patients provided written informed consent.

Enumeration of CTCs and HER2 Staining

Before the start of a new line of systemic treatment for MBC, 7.5 ml of blood was drawn in CellSave tubes (Janssen Diagnostics, Raritan, NJ). CTC enumerations were performed within 96 hours of the blood draw using the CellSearch system (Janssen Diagnostics). CTCs were characterized for HER2 expression directly in the CellSearch system using the anti-HER2 antibody as described by the manufacturer (CellSearch tumor phenotyping reagent HER2, Janssen Diagnostics). We used the cutoff for HER2 positivity in CTCs as described by Riethdorf and colleagues [25]; a gallery of representative images for scoring is presented in Supplementary Figure 1. When at least one CTC immunofluorescently stained 2+ or 3+ for HER2, the patient was considered as having HER2-positive CTCs. The results of the enumeration were always checked by a second certified reviewer. The results of the HER2 staining were reviewed in a HER2 consensus meeting involving two investigators (N.B. and J.K.).

CTC ER Assay

Simultaneously with the blood draw for CTC enumeration, 7.5 ml of blood was drawn in EDTA tubes and enriched for CTCs using the CellSearch profile kit (Janssen Diagnostics). Samples were processed within 24 hours and subsequently frozen at −80°C for RNA isolation and analysis. Larger than 200 bp RNA was isolated using the AllPrep DNA/RNA Micro Kit (Qiagen, Germantown, MD). Generation of cDNA, preamplification, and reverse transcription quantitative polymerase chain reaction to quantify the expression of *ESR1* were performed as described in detail before using a validated *ESR1* Taqman assay (Hs00174860 m1; Applied Biosystems, San Francisco, CA) [9]. ER positivity in CTCs was defined as an *ESR1* mRNA ΔCq level higher than −3.89 corrected for background healthy donor blood signal, which we previously demonstrated to be a reliable cutoff for *ESR1* with excellent sensitivity and specificity [17].

Statistical Considerations

The primary end point of this study was the progression-free rate after 6 months of treatment (PFR6months) in patients receiving endocrine therapy. A small survey among medical oncologists revealed that a PFR6months of 20% for endocrine therapy alone in MBC patients with HER2-positive CTCs would be convincing enough for medical oncologist not to treat an MBC patient with an ER-positive PT with endocrine therapy alone. Given that the expected PFR6months for endocrine therapy in unselected MBC patients is around 70% (and certainly not lower than 50%) and the prevalence of HER2-positive CTCs was expected to be around 25%, we calculated that 60 patients would render 15 patients with HER2-positive CTCs to detect a PFR6months of 20% with a 95% confidence interval (CI) not higher than 50% (4%-48%), with a type I error probability (α) of .05 and a type II error probability (β) of .2.

Secondary objectives were 1) to explore the association between the HER2 status of CTCs and the PFR6months on chemotherapy (as a control cohort), 2) to establish the incidence of ER-positive CTCs in the endocrine and chemotherapy cohort, 3) to establish discrepancy rates of the ER and HER2 status in CTCs compared with PT characteristics in both cohorts, and 4) to explore whether an ER-status switch between the primary breast tumor and the CTCs was associated with outcome in both cohorts. The date of progression was established by the treating physician and was defined as radiological progression according to Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1 [26]. In case of poorly evaluable disease, the treating physician was allowed to use other techniques considered to be appropriate (e.g., bone scan, serum biomarkers, CTC counts) to assess progressive disease. Data on the

HER2 and ER status [including HercepTest (Dako, Glostrup, Denmark) scoring and the percentage of ER-positive tumor cells] of the PT were collected from the pathology report. A HER2-negative PT was defined as having 0 or 1+ scoring according to the HercepTest (scored according to the manufacturer's instructions) on the PT, or 2+ scoring in combination with negative HER2 *in situ* hybridization. An ER-negative PT was defined as having <10% of the primary tumor cells staining for ER using immunohistochemistry.

Differences in the PFR6months between patients with HER2-positive versus HER2-negative CTCs were analyzed using the χ^2 test. Univariate Cox regression was used to evaluate whether the presence of at least one HER2-positive CTC (as a dichotomous variable) was associated with progression-free survival (PFS) or overall survival (OS). The HER2/CTC ratio was calculated by dividing the number of HER2-positive CTCs by the total number of CTCs. No statistics were performed in the ER-CTC–related analyses, as this study was not appropriately powered to evaluate the prognostic power of the ER-CTC status. Instead, Kaplan-Meier curves were constructed to explore the potential prognostic power of the ER-CTC status. Associations between the HercepTest score and the HER2-CTC status were investigated with the χ^2 test, whereas associations between the percentage of ER-positive tumor cells in the PT and ER switches were compared with the Kruskal-Wallis test. Reported *P* values are two-sided, and a significance level $\alpha = .05$ was used. REMARK criteria [27] were taken into account for this report. Analyses were done using Stata/SE version 12 (StataCorp LP, College Station, TX).

Results

Patient Characteristics

A total of 154 MBC patients were included in the current analysis (Table 1); 72 patients were treated with endocrine therapy and 82 patients with first-line chemotherapy for MBC. Patients treated with endocrine therapy mainly started this as first-line therapy (69%) for MBC and mostly received an aromatase inhibitor (64%). The patients in the chemotherapy cohort predominantly received taxane-based (48%) or anthracycline-based chemotherapy (35%). No patients in either cohort received targeted therapies such as trastuzumab or everolimus. Median follow-up of all patients was 15 months.

Table 1. Baseline Characteristics

	Endocrine Therapy (*n* = 72)	Chemotherapy (*n* = 82)
Age at inclusion, median (range)	67 (37-88)	61 (33-85)
Primary tumor ER positive	72 (100%)	57 (70%)
Previous chemotherapy lines for MBC		
0	68 (94%)	82 (100%)
1	4 (6%)	
Previous endocrine therapy lines for MBC		
0	50 (69%)	78 (96%)
1	17 (24%)	2 (2%)
2	5 (7%)	2 (2%)
Chemotherapy regimen received after inclusion		
Taxane based		39 (48%)
Anthracycline based		29 (35%)
Other		14 (17%)
Endocrine therapy regimen received after inclusion		
AI based	46 (64%)	
Tamoxifen based	17 (24%)	
Other	9 (12%)	
CTC count at baseline		
1-4 CTCs/7.5 ml	30 (42%)	24 (29%)
≥ 5 CTCs/7.5 ml	42 (58%)	58 (71%)
Follow-up, median days (range)	511 (30-1436)	406 (8-1430)

Incidence and Prognostic Value of HER2-Positive CTCs

Results regarding the prevalence of HER2-positive CTCs (2+ or 3+ staining) and their relation to the PFR6months are presented in Table 2. HER2-positive CTCs were present in 19 patients receiving endocrine treatment (26%) and in 26 patients receiving chemotherapy (32%). The PFR6months in MBC patients treated with endocrine therapy with at least one HER2-positive CTC was not different from the PFR6months in patients without HER2-positive CTCs (53% vs 68%, $P = .23$). When the analysis was restricted to patients receiving first-line endocrine therapy, PFR6months did not differ between patients with HER2-positive CTCs ($n = 15$, 60%) versus patients without HER2-positive CTCs ($n = 35$, 71%; $P = .43$). In the chemotherapy cohort, no difference in PFR6months with regard to the HER2-CTC status was observed either (65% vs 57%, $P = .48$). In patients in whom HER2-positive CTCs were present, the median HER2 to CTC ratio (total number of HER2-positive CTCs divided by total number of CTCs) was 0.08 (Q1 0.03-Q3 0.22).

Because we had ample follow-up time for the included patients, we also explored whether the presence of HER2-positive CTCs was associated with PFS or OS in a univariate Cox regression model. The presence of at least one HER2-positive CTC in the endocrine therapy cohort was not associated with a difference in PFS [hazard ratio (HR) 1.17, 95% CI 0.65-2.09] or OS (1.72, 95% CI 0.73-4.03) (Figure 1, *A* and *C*). Similarly, in the chemotherapy cohort, no association of HER2-positive CTCs with change in PFS (HR 1.09, 95% CI 0.67-1.78) or OS (HR 0.93, 95% CI 0.53-1.63) was observed (Figure 1, *B* and *D*).

When the cutoff for HER2 positivity was shifted to only the CTCs that had a 3+ immunofluorescent staining, HER2-positive 3+ CTCs were observed in 6 patients (8%) in the endocrine therapy cohort and in 9 patients (11%) in the chemotherapy cohort. As the number of patients with HER2-positive 3+ CTCs was very limited, we did not perform formal statistics on differences in PFR6months.

Incidence and Prognostic Value of Switches in ER Status Between PT and CTCs

The ER status of CTCs was assessed on the mRNA level and determined using our predefined cutoff for *ESR1* positivity as described before [17]. We compared the ER status of the CTCs with the ER status of the PT as reported by the pathologist. The ER-CTC status could not be determined in 38 patients (25%): in 9 patients, no sample for mRNA analysis was available; in 29 patients, the mRNA was of poor quality or the epithelial mRNA signal was too low, the latter being indicative of a CTC count too low for a reliable mRNA

Table 2. Prevalence and PRF6 Months in Relation to the HER2-CTC Status

	Endocrine Therapy (*n* = 72)	Chemotherapy (*n* = 82)
HER2-positive CTCs (2+ or 3+ HER2 staining) present	19 (26%)	26 (32%)
HER2-positive CTCs (3+ HER2 staining) present	6 (8%)	9 (11%)
PFR 6 months		
Absent HER2-positive CTCs (2+ or 3+ HER2 staining)	68%	57%
≥ 1 HER2-positive CTCs (2+ or 3+ HER2 staining) present	53%	65%
χ^2 *PFR 6 months P value (absent vs present)*	.23	.48

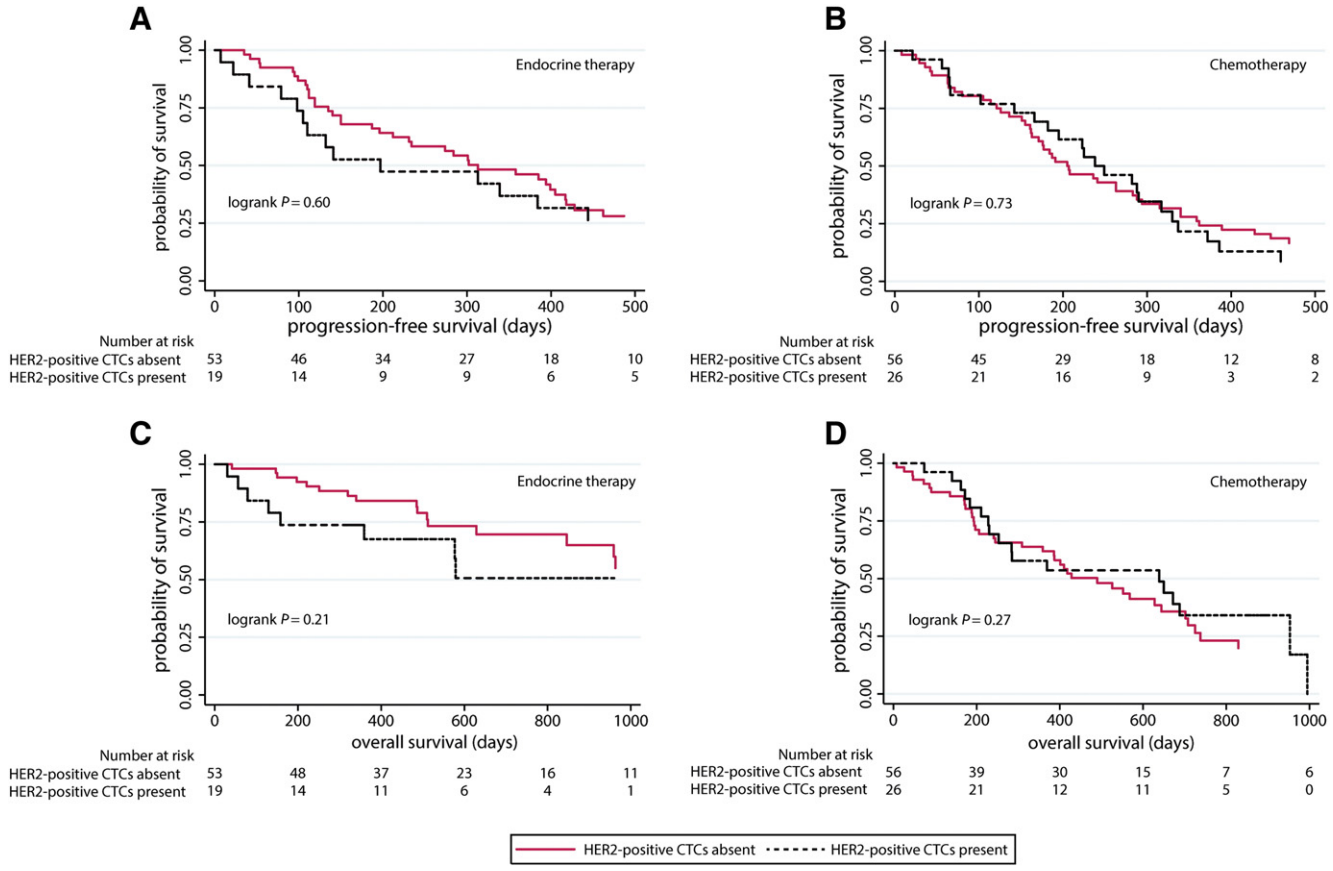

Figure 1. PFS and OS according to HER2-CTC status. (A and C) PFS and OS, respectively, for the endocrine therapy cohort. (B and D) PFS and OS, respectively, for chemotherapy cohort.

analysis. We were thus able to determine the ER status on CTCs in 116 patients (75%) (Table 3). In the endocrine therapy cohort, consisting solely of patients with ER-positive PTs, 10 patients (14%) had ER-negative CTCs. In the chemotherapy cohort, 31% of the patients had a discordant ER status between the PT and the CTCs. Interestingly, out of 19 patients who had an ER-negative PT, 13 patients (68%) had ER-positive CTCs. In addition, in 7 out of 46 patients (15%) with an ER-positive PT, the CTCs were negative for ER.

Exploratory Kaplan-Meier curves for the prognostic impact of ER switches between the PT and the CTCs were constructed as planned. As depicted in Figure 2, no clear prognostic impact of ER switch appeared to be present in either the endocrine therapy cohort or the chemotherapy cohort.

Table 3. Discordances between Primary Tumor and CTC Regarding the ER Status

	ESR1 Status CTCs Negative	*ESR1* Status CTCs Positive	*Total*
Endocrine therapy			
ER status primary tumor			
Positive	10	41	*51*
Chemotherapy			
ER status primary tumor			
Negative	6	13	*19*
Positive	7	39	*46*
Total	*13*	*52*	*65*

Associations Between HER2 and ER Status of CTCs and the PT

We explored whether HER2-positive CTCs were related to the HER2 HercepTest score as assessed on the PT by the pathologist in the context of standard clinical care. For 42 patients, no data on the PT HercepTest score were available. In the remaining 112 patients, the HercepTest score (0, 1+, or 2+) was not found to be associated with the presence or absence of HER2-positive CTCs (P = .24, Supplementary Table 1). We also explored whether switches in patients with an ER-positive PT to ER-negative CTCs were associated with the percentage of ER-positive tumor cells in the PT. Patients who had an ER-positive PT but ER-negative CTCs had lower percentages of ER-positive tumor cells in the PT than patients with an ER-positive PT in whom the CTCs remained ER positive (P = .03; Supplementary Figure 2). No data on the percentage of ER-positive tumor cells were available for patients with an ER-negative PT.

Discussion

CTCs are an attractive alternative to solid biopsies and may give insight into intratumor and intermetastatic heterogeneity [28,29]. Previous studies demonstrated discrepancies in the HER2 and ER status between the PT and the metastases, as well as discordances of these markers between the PT and the CTCs. However, appropriately powered studies evaluating the prognostic impact of HER2-positive CTCs have been scarce. We set out to evaluate the prevalence and

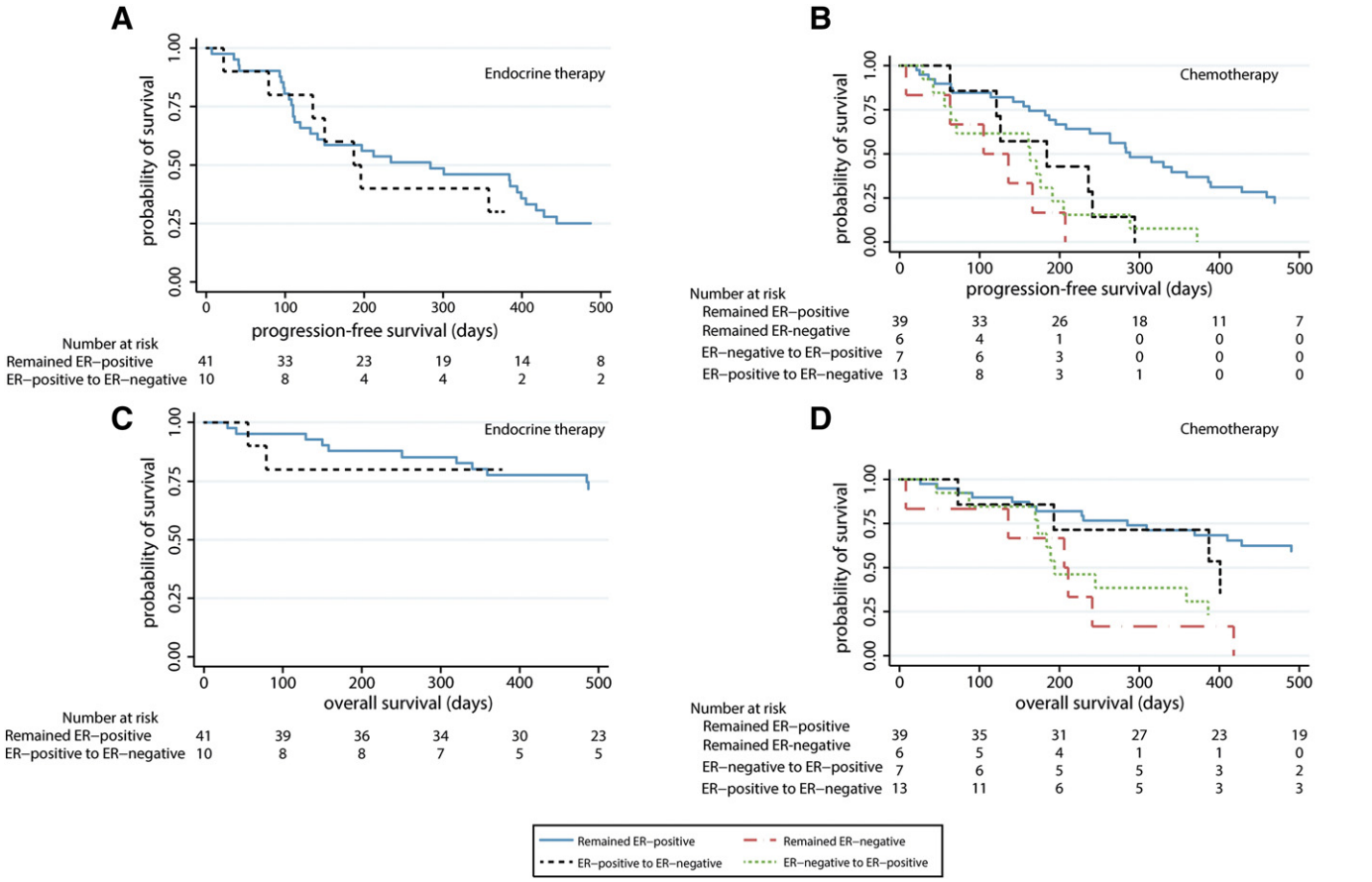

Figure 2. PFS and OS according to ER-CTC status. (A and C) PFS and OS, respectively, for the endocrine therapy cohort. (B and D) PFS and OS, respectively, for chemotherapy cohort.

prognostic value of HER2-positive CTCs in MBC patients with HER2-negative PTs. We hypothesized that MBC patients with HER2-positive CTCs would have a lower PFS after 6 months of endocrine therapy compared with patients with HER2-negative CTCs. Although discordance in the HER2 status between the PT and CTCs was frequently present, no prognostic value of HER2-positive CTCs was observed in MBC patients treated with endocrine therapy.

Our findings that HER2-positive CTCs occur in a relatively large subset of HER2-negative MBC patients (45/154 patients, 29%) are in accordance with previous reports. Wallwiener and colleagues [18] used the same method as we did to score HER2-positive CTCs and found HER2-positive CTCs in 30% of their 107 MBC patients with ≥5 CTCs and HER2-negative PTs. In contrast to our results, this study observed that patients with ≥5 CTCs and HER2-positive CTCs had a longer PFS than patients with ≥5 CTCs without HER2-positive CTCs. However, because also patients with HER2-positive PTs who were treated with HER2-targeted treatments were included in that study, the results cannot directly be compared with our results. Slightly higher numbers of HER2-positive CTCs than in our study were observed by Fehm et al. [30], who used an immunofluorescent staining on CellSearch for HER2 of 3+ and observed HER2-positive CTCs in 25 of 76 (33%) MBC patients with HER2-negative PTs. Although other studies have investigated the prognostic impact of HER2-CTC status in MBC [4,8,12], either these studies did not use an FDA-cleared method to enumerate CTCs followed by characterization for HER2 expression, or they did not sufficiently describe their technique to score HER2-positive CTCs. For these reasons, direct comparison of the results from these studies with our study is not possible.

Although HER2-positive CTCs were frequently present in our MBC patients, their presence was not of prognostic value. Our study was powered to detect a 20% PFR6months in patients with HER2-positive CTCs receiving endocrine therapy versus 70% PFR6months in patients with HER2-negative CTCs, which was a difference considered clinically relevant after a small survey among medical oncologists. We believe that it is justified to have powered this study for these sorts of large differences in PFR because only such large differences will have a clear clinical impact and affect clinical decision making. Our data at least suggest that the presence of HER2-positive CTCs in patients with HER2-negative PTs does not have a major impact on their prognosis. There are several explanations for the lack of prognostic value of HER2-positive CTCs. First, it is possible that there is indeed no association between HER2 overexpression in CTCs of MBC patients and outcome to endocrine treatment. Second, specificity issues may have occurred when using the CellSearch Tumor Phenotyping reagent HER2. We found a moderate but significant correlation between the number of CTCs and the number of HER2-positive CTCs (Spearman $r = 0.31$, $P < .001$). This suggests that when a higher number of CTCs would be present, there would be larger chance of finding at least one HER2-positive CTC. This may be in line with the observed heterogeneity for HER2 expression in CTCs as seen in the median

HER2 to CTC ratio, or indicate specificity issues. Third, a limitation of our study is that we did not perform fluorescence *in situ* hybridization analysis on CTCs to confirm amplification of HER2, which may have further improved the specificity of the HER2-CTC assay. Fourth, a subset of patients in the endocrine therapy cohort had already received prior endocrine therapy for MBC, which may have impacted the analyses regarding PFS in this cohort. However, in a subgroup analysis of patients receiving first-line endocrine therapy which also met our power calculation, no prognostic value of HER2-positive CTCs was observed either. Fifth, the fact that HER2 is overexpressed does not necessarily mean that it is also an active driver of tumor growth in that particular patient. The determination of phosphorylated HER2 or markers downstream of HER2 in CTCs may provide better insight into the activity of the HER2 signaling pathway in CTCs [31]. Lastly, there is currently no consensus on the optimal cutoff for HER2 positivity. We chose CTCs immunofluorescently staining 2+ or 3+ as HER2 positive given that this was the cutoff used in the CellSearch/Veridex interreader variability study [32] and good agreement for this cutoff was demonstrated between academic readers and Veridex consensus. However, other cutoffs for HER2 positivity on CTCs might yield different results regarding the prognostic impact of HER2-positive CTCs. Consensus on the optimal cutoff for HER2-positive CTCs is needed and should be driven by the prognostic power and clinical utility of such a cutoff (for example, for response to anti-HER2 targeted agents).

With regard to ER status, we found discordance between the PT and CTCs in 26% of our patients, which is in line with our previous reports [9,17] and reports by others [6,13,33–35] describing heterogeneity of CTCs for ER and discordances in ER status in 24% to 45% of the MBC cases. Especially of interest is that ER-positive CTCs were observed in 68% of the patients with an ER-negative PT, which might indicate that a subset of patients with an ER-negative PT might benefit from endocrine therapy. Also worth noting is the finding that in patients in whom the PT was ER positive but the CTCs ER negative, the number of ER-positive tumor cells in the primary was significantly lower than in patients in whom the CTCs remained ER positive. This suggests that heterogeneity in ER expression in the PT may give a higher chance of clonal evolution of an ER-negative clone. Although heterogeneity and discordances between PTs and CTCs for ER expression have frequently been described, little is known about the prognostic impact of the ER-CTC status. We previously explored the prognostic value of the ER-CTC status and found patients with ER-negative PTs but ER-positive CTCs to have a longer time-to-treatment-switch than patients who remained ER negative [17]. However, in the present study (comprising 30 of the patients who were also included in our previous report), we were unable to confirm these findings. This could be due to the facts that our previous cohort was smaller (n = 62) and that included patients were treated with either endocrine therapy or chemotherapy. In addition, the applied cutoff for ER positivity of CTCs in our previous study was based on *ESR1* mRNA levels in the PTs. Although this cutoff was demonstrated to have excellent sensitivity and specificity [17], it was not feasible to validate that cutoff value for the current study because the PT tissue was not available for all patients. The exploratory analyses here indicating lack of prognostic value should however be interpreted with caution, as the number of patients who had a switch in ER status was limited. Larger studies are required to evaluate the prognostic value of ER discordances between the PT and CTCs, preferably also evaluating ER expression in CTCs at the single cell level to enable the evaluation of heterogeneity in ER expression between single CTCs. We have recently started a study in which we are evaluating heterogeneity of ER-positive CTCs and their prognostic impact using a proximity ligation assay technique (CareMore-Trastuzumab study and CareMore-AI study; NTR5121 [36,37]), whereas others have reported on immunofluorescent ER staining directly in the CellSearch machine, similar to HER2 in this study [35].

In conclusion, a lack of prognostic value was observed for HER2-positive CTCs and ER-positive CTCs with respect to outcome to endocrine therapy or chemotherapy in MBC patients. Future research should focus on ER characterization of CTCs in a larger patient cohort but may also focus on looking beyond classical predictive factors (such as HER2 and ER expression) that are related to endocrine resistance. This could for example be done by determining resistant mutations in *ESR1* [34] or measuring gene expression panels associated with resistance to antitumor therapy [23,24]. Such characterization of several prognostic and predictive markers on CTCs correlated with resistance to either endocrine therapy or chemotherapy may eventually lead to improved prognostication and prediction of therapeutic response in MBC patients.

Conflict of Interest

None.

Funding

This work was supported by KWF (Netherlands Cancer Foundation; grant number EMCR 2012-5390) and by Janssen Diagnostics. Janssen Diagnostics did not play any role in the design, analysis, and/or interpretation of the presented data.

Acknowledgements

The authors would like to thank M. van der Vlugt-Daane for her assistance with isolating and profiling CTCs.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.08.007.

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