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# Circulating tumor cells in immunohistochemical subtypes of metastatic breast cancer: lack of prediction in HER2-positive disease treated with targeted therapy

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**Background:** Circulating tumor cells (CTCs) are associated with inferior prognosis in metastatic breast cancer (MBC). We hypothesized that the relationship between CTCs and disease subtype would provide a better understanding of the clinical and biologic behavior of MBC.
**Patients and methods:** We retrospectively analyzed 517 MBC patients treated at a single institution. Subtypes of primary tumors were analyzed by immunohistochemical (IHC) or fluorescent *in situ* hybridization analyses and CTCs were enumerated by CellSearch® at starting a new therapy. Overall survival (OS) and progression-free survival durations for each IHC subtype were determined.
**Results:** At a median follow-up of 24.6 months, 276 of 517 (53%) patients had died. The median OS for patients with <5 and ≥5 CTCs were 32.4 and 18.3 months, respectively ($P < 0.001$). Except in HER2+ patients, the prognostic value of CTCs was independent of disease subtype and disease site.
**Conclusions:** In this large retrospective study, CTCs were strongly predictive of survival in all MBC subtypes except HER2+ patients who had been treated with targeted therapy. Our results clearly demonstrate the value of enumerating CTCs in MBC and strongly suggest an interesting biological implication in the HER2+ subset of patients that need to be further explored.
**Key words:** circulating tumor cells, HER2, immunohistochemical subtypes, metastatic breast cancer, tumor markers

## introduction

Breast cancer is the most common cancer among women in the United States, with 194 280 new cases of invasive breast cancer and 40 610 confirmed breast cancer deaths during 2009 [1]. Only 5.6% of patients with newly diagnosed disease will present with advanced or metastatic breast cancer (MBC) [1]. However, ∼40% of patients initially presenting with localized disease eventually experience progression to MBC and die of their disease [2]. Recent evidence suggests that MBC is not a uniform disease and that breast cancer subtypes are associated with significant differences in distant spread patterns,

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independent of conventional clinical–pathologic variables [3]. Metastatic spread models demonstrate complex interactions of 'seed and soil' factors involving tumor intravasation, traversing the peripheral circulation, extravasation from the periphery, invasion, proliferation, and angiogenesis [4]. Detecting circulating tumor cells (CTCs) may provide a better understanding of the biological behavior of tumor changes during the metastatic process because they may represent the seed from primary tumor to metastatic lesion.

The detection of CTCs, as carried out using the US Food and Drug Administration-cleared CellSearch® system (Veridex, LLC, Warren, NJ) before the initiation of new systemic treatment, is a strong independent predictor of overall survival (OS) and progression-free survival (PFS) in MBC [5–18]. Moreover, the CTC enumeration is strongly correlated with radiographic determination of disease progression in patients undergoing chemotherapy or endocrine therapy [19]. Recent data suggest that the CTC phenotype and immunopathologic characteristics may be discordant among the primary tumor, metastatic deposits, and CTCs [20–23]. These data suggest the existence of an independent CTC phenotype that is associated with adverse prognosis and treatment effectiveness. Therefore, we determined the clinical value of the CTC count according to immunohistochemically defined subtypes, specific metastatic disease sites, and defined standard therapies to validate the prognostic information of CTCs within defined subset of disease.

Our study objectives were to confirm the differences in clinical behavior among subtypes of breast cancer and define the prognostic role of CTCs in relation to those factors. An independent association would suggest that these cells play a critical role in the metastatic process, that additional molecular characterization is needed, and that CTC-targeted therapies would be effective. To our knowledge, this is the largest retrospective study of CTCs in MBC patients.

## patients and methods

### patient population

We searched our prospectively maintained laboratory database to identify patients with MBC who had undergone standard baseline CTC evaluation at The University of Texas M. D. Anderson Cancer Center (Houston, TX) and had been treated between September 2002 and November 2009. For all patients, a baseline CTC evaluation had been carried out using the CellSearch® within 30 days before starting a new line of therapy. Moreover, to be included in our study, patients were required to have clinical and radiologic evidence of MBC, with measurable or evaluable disease, before initiating new therapy. All patients had undergone imaging studies, laboratory evaluations, and treatment planning at our institution. The institutional review board at the University of Texas M. D. Anderson Cancer Center approved the study (DR10-0227) and granted a waiver of informed consent, considering the retrospective nature of the study.

### immunohistopathologic findings and staging definition

Histological type and grade of invasive disease were coded according to the World Health Organization classification system [24] and modified Black nuclear grading system, respectively [25]. Consistent with institutional standard, all specimens from within and without the institution were analyzed by a pathologist at this institution. The method used to determine hormone receptor (HR) status depended on the year of primary diagnosis. For specimens obtained before 1993 ($n = 13$), estrogen and progesterone receptors (ER and PR) status were determined using the dextran-coated charcoal ligand-binding method. For specimens obtained after 1993, immunohistochemical (IHC) staining with monoclonal antibodies 6F11 and 1A6 (Novacastra Laboratories, Ltd., Burlingame, CA) were used to determine ER and PR status, respectively, on 4-μm paraffin-embedded tissue. Patients with at least one positive HR (ER or PR ≥ 1%) were considered HR+. HER2 status was determined using IHC (AB8 Neo Markers) and FISH using the PathVysion HER-2/neu DNA Probe Kit (LSI HER-2/neu SpectrumOrange/CEP17 SpectrumGreen). Specimens scored as IHC 0, 1+, 2+ and no gene amplification by FISH (HER2/CEP17 signal ratio <2) were considered HER2 constitutive or negative. Specimens scored as IHC 3+ or demonstrating gene amplification by FISH were considered HER2+ or amplified. Triple-receptor negative (TN) status was assigned to patients whose tumors were negative for ER, PR, and HER2. In our study, we refer at IHC breast cancer subtypes as follows: HR+/HER2−, HR+/HER2+, HR−/HER2−, and TN breast cancer.

Metastatic sites were evaluated at the time of phlebotomy and characterized on the basis of radiologic imaging findings and patients' cancer history. Visceral and non-visceral metastases were defined in a previous paper [6].

### CTC count

CTCs were isolated and counted using the USA Food and Drug Administration-cleared CellSearch® technology (Veridex, LLC) as previously reported [26]. Briefly, 7.5 ml of peripheral blood were collected in CellSave™ tubes and incubated with anti-EpCAM-coated ferrous particles to enrich for epithelial cells. The EpCAM-enriched cell fraction was labeled with fluorescent nucleic acid dye 4,2-diamidino-2-phenylindole dihydrochloride (DAPI), stained with antibodies to identify cytoplasmic cytokeratins (CKs)-8, CK18 and CK19 as well as with anti-CD45 to identify contaminating leukocytes. CTCs were identified and counted using the CellSpotter®, a semi-automated fluorescence-based microscopy system that permits computer-generated reconstruction of cellular digitized images. CTCs were identified as cells with the appropriate morphologic characteristics: CK positive, DAPI positive, and CD45 negative. Technical details of the CellSearch® and CellSpotter® systems, including accuracy, precision, linearity, and reproducibility, have been described previously [26]. All CTC assessments were carried out in a central laboratory (M. D. Anderson Cancer Center, Houston, TX) by an experienced operator and the digitized images of CTCs were reviewed and validated by a board-certified pathologist. A cut-off of five CTCs per 7.5 ml of blood was chosen to distinguish patients with an unfavorable prognosis from patients with a favorable prognosis [5].

### statistical analysis

Differences among patient characteristics between CTC groups (<5 or ≥5) were tested using Fisher's exact test or Pearson chi-square test. OS duration was defined as the time of basal blood draw for CTCs to the date of death. All living patients were censored at the last follow-up date. PFS duration, defined as the time of basal blood draw to documentation of disease progression (according to RECIST), and all clinical data available in M. D. Anderson's electronic medical records (ClinicStation) were independently verified by two physicians (AG and MG). All data, such as survival and treatments, were collected from patients' records. Patients without progressive disease were censored at the last follow-up date. Kaplan–Meier plots were compared using the log-rank test. To evaluate the interaction between IHC subtypes of disease and CTC count, we quantified the heterogeneity between subgroups (CTCs <5 and ≥5) with the Higgins' I2 index [27]. A backward stepwise Cox regression test was used to model and assess the relationship among PFS, OS, and CTC value. After adjusting for clinical variables, we removed bone metastasis and performance status from the analysis because they were not

statistically significant. In the final regression model, we considered CTC number (<5 versus ≥5), HR (positive versus negative), HER2 (positive versus negative), visceral metastasis (yes versus no), and number of metastatic sites (1 versus 2 versus ≥3). All statistical test analyses were two-sided, and P values <0.05 were considered statistically significant. Analyses were carried out using SPSS 17 statistical software (SPSS, Inc. Somers, NY).

## results

### patient characteristics

This study was restricted to a cohort of 517 MBC patients. Table 1 shows patients' pathological and clinical characteristics according to CTC count. Two hundred and six (40%) patients had ≥5 CTCs at baseline blood draw, and 311 (60%) had <5 CTCs. The distribution of tumor by subgroup classification was as follows: (56.4%) had HR+/HER2−, 9.7% had HR+/HER2+, 9.9% had HR−/HER2+, and 24% had HR−/HER2−. A larger proportion of HR+/HER2− patients had ≥5 CTCs than did patients with other subtypes of tumor ($P = 0.024$). No significant differences in CTC counts were found within the other subtypes of tumor. CTCs in patients with visceral metastasis (62%) were equally distributed between patients with <5 CTCs and those with ≥5 CTCs. However, 80% of patients with ≥5 CTCs presented with bone involvement versus 56% of patients with fewer than 5 CTCs ($P < 0.001$). The number of metastatic sites was associated with a high CTCs count ($P = 0.02$); this difference was not more significant after adjusting for bone metastasis (data not shown).

**Table 1.** Patient characteristics by CTC count

| Variable | Overall | No. of CTCs | | | | *P* value |
|---|---|---|---|---|---|---|
| | | <5 | | ≥5 | | |
| All patients, *n*, % | 517 | 100% | 311 | 60.2% | 206 | 30.8% | |
| Median age (years) | 49 | | 49 | | 49 | | |
| Grade, *N* (%) | | | | | | | |
| 1 | 18 | 3.5 | 10 | 3.2 | 8 | 3.9 | |
| 2 | 182 | 35.2 | 100 | 32.2 | 82 | 39.8 | |
| 3 | 277 | 53.6 | 182 | 58.5 | 95 | 46.1 | |
| Unknown | 40 | 7.7 | 19 | 6.1 | 21 | 10.2 | 0.061 |
| HR+/HER2−, *N* (%) | 292 | 56.4 | 163 | 52.4 | 129 | 62.6 | 0.024 |
| HR+/HER2+, *N* (%) | 50 | 9.7 | 33 | 10.6 | 17 | 8.3 | 0.448 |
| HR−/HER2+, *N* (%) | 51 | 9.9 | 33 | 10.6 | 18 | 8.7 | 0.548 |
| Triple negative, *N* (%) | 124 | 24 | 82 | 26.4 | 42 | 20.4 | 0.141 |
| Visceral metastasis, *N* (%) | 319 | 61.7 | 188 | 60.5 | 131 | 63.6 | 0.518 |
| Bone metastasis, *N* (%) | 339 | 65.6 | 175 | 56.3 | 164 | 79.6 | <0.001 |
| Metastatic sites, *N* (%) | | | | | | | |
| 1 | 153 | 29.6 | 104 | 33.5 | 49 | 23.8 | |
| 2 | 153 | 29.6 | 94 | 30.2 | 59 | 28.6 | |
| ≥3 | 211 | 40.8 | 113 | 36.3 | 98 | 47.6 | 0.02 |
| Line of therapy, *N* (%) | | | | | | | |
| 1 | 237 | 45.8 | 142 | 45.7 | 95 | 46.1 | |
| 2 | 122 | 23.6 | 80 | 25.7 | 42 | 20.4 | |
| ≥3 | 158 | 30.6 | 89 | 28.6 | 69 | 33.5 | 0.294 |

CTC, circulating tumor cell.

### administered treatments

Approximately 46% of patients had undergone first-line treatment of newly diagnosed MBC. Chemotherapy alone, chemotherapy plus bevacizumab, anti-HER2 combination treatment, hormonal treatment, or other investigational treatments were administered in 48%, 13%, 15%, and 19% or 5% of cases, respectively (supplemental Table 1, available at *Annals of Oncology* online). Among the 292 ER+/HER2− patients, 92 (32%) had undergone hormonal treatment. Of the 101 HER2+ patients, 84 (83%) had received trastuzumab or lapatinib. Seventy-six percent of ER+/HER2+ patients and 90% of ER−/HER2+ patients had received anti-HER2 agents.

### overall outcome and CTCs

At a median follow-up period of 24.6 months, 456 (88%) of 517 patients had showed progression of disease and 276 (53%) patients had died. In the largest group of patients (HR+/HER2−, $n = 292$), the median OS duration was 27.3 months [95% confidence interval (CI) 23.6–30.9 months] and PFS was 6.4 months (95% CI 5.6–7.3 months). In HR+/HER2+ patients ($n = 50$), the median OS duration was not yet reached and PFS was 7.6 months (95% CI 5.3–9.8); in HR−/HER2+ patients ($n = 51$), the median OS was 26 months (95% CI 18.3–33.6 months) and PFS was 7.5 months (95% CI 5.4–9.7 months). In TN patients ($n = 124$), the median OS duration was 15.8 months (95% CI 13.2–18.4 months) and PFS was 4.9 months (95% CI 4.2–5.5 months) (Figure 1A and B). One hundred and forty-one (68%) of the 206 patients with ≥5 CTCs had died by the time of this analysis, compared with 135 (43%) of 311 with <5 CTCs. As shown in Figure 1C and D, shorter median OS and PFS durations were observed in patients with ≥5 CTCs than in those with <5 CTCs (OS, 18.3 versus 32.4 months, $P < 0.001$; and PFS, 5.8 versus 6.3, $P = 0.006$).

### IHC subtype and CTCs

The median OS and PFS were significantly different in HR+/HER2− patients ($n = 292$) with ≥5 CTCs than in patients with <5 CTCs (OS, 18.8 versus 48.7 months, $P < 0.001$; and PFS, 5.9 versus 7.1, $P = 0.004$) (Figures 2A and 3A).

In HER2+/HR+ patients with ≥5 CTCs, the median OS was 29.5 months versus not yet reached in patients with <5 CTCs ($P = 0.084$) (Figure 2B). In HER2+/HR− patients with ≥5 CTCs, the median OS was 27.2 versus 21.4 months in patients with <5 CTCs ($P = 0.991$) (Figure 2C). In brief, the hazard ratio of death in patients with ≥5 CTCs who had undergone anti-HER2-targeted therapy did not significantly differ from that of patients with <5 CTCs (Table 2). Also the PFS among HER2+ groups was similar according the CTC count (HER2+/HR+ patients PFS, 7.6 versus 8.6, $P = 0.458$; HER2+/HR− patients PFS, 6.9 versus 7.5, respectively, $P = 0.719$) (Figure 3B and C).

Finally, among TN breast cancers ($n = 124$), patients with ≥5 CTCs had a median OS significantly shorter than patients with <5 CTCs (10.4 versus 17.8 respectively, $P = 0.001$) (Figure 2D). Median PFS was similar for TN breast cancer patients with ≥5 CTCs and patients with <5 CTCs (PFS, 5.1 versus 4.8, respectively, $P = 0.274$) (Figure 3D).

The interaction test between the clinical outcomes and subtypes was not significant for both PFS ($P = 0.56$) and OS ($P = 0.17$).

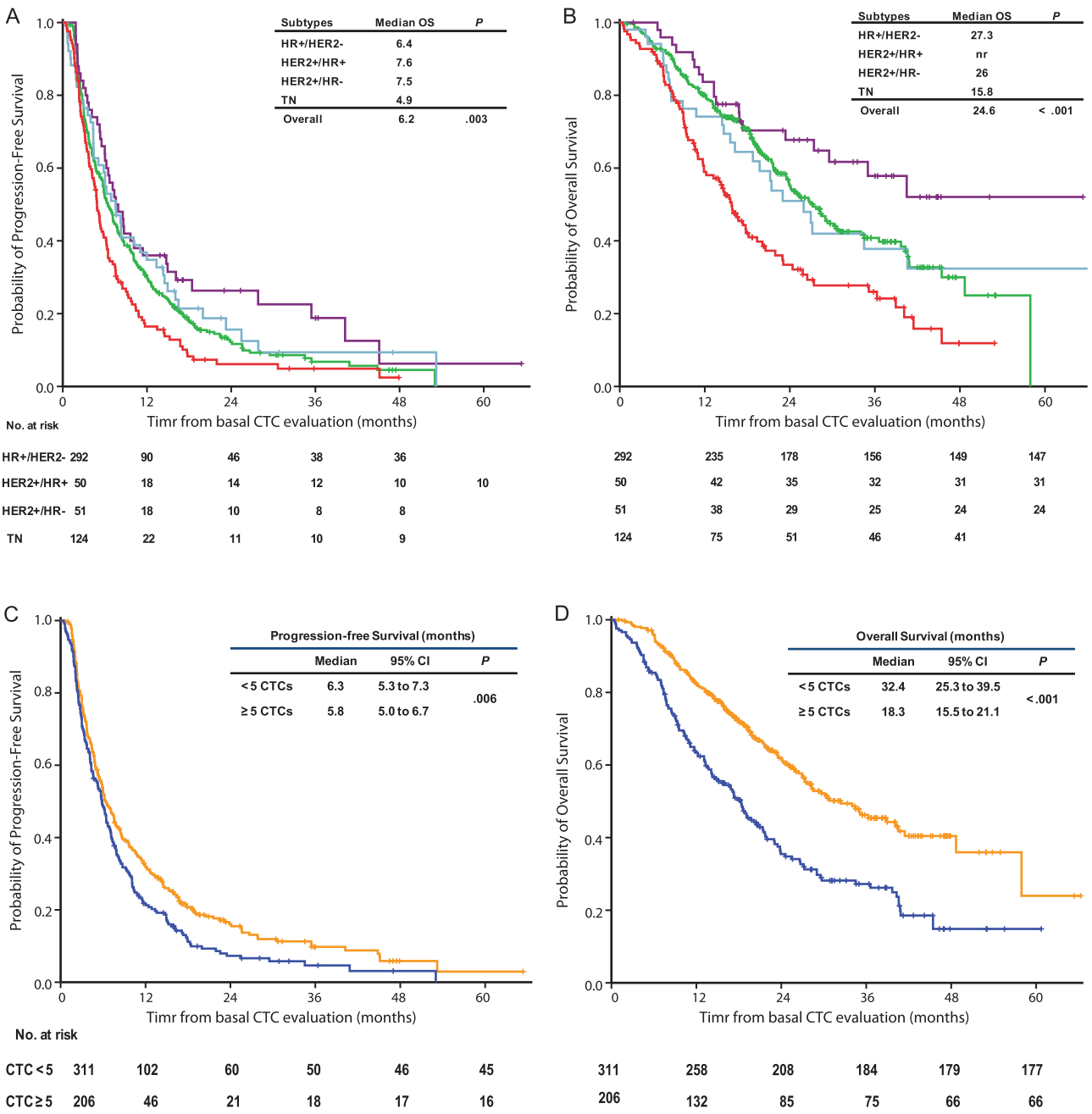


**Figure 1.** (A) Overall survival (OS) and (B) progression-free survival (PFS) for all 517 patients, according to immunohistochemical subtype. HR+/HER2− in green ($n = 292$); HR+/HER2+ in purple ($n = 50$); HR−/HER2+ in light blue ($n = 51$); triple-receptor negative (TN) in red ($n = 124$). OS (C) and PFS (D) for all 517 patients with <5 (blue) versus ≥5 (orange) circulating tumor cells (CTCs). Time was measured from basal blood draw to death for OS and to radiologic evidence of disease progression (PFS).

### multivariate analysis of predictors of PFS and OS

A multivariate Cox proportional hazards regression analysis was carried out to determine the association between factors of interest, PFS, and OS. In the backward stepwise Cox regressions test, CTCs were predictors of both PFS and OS, considering HR, HER2, visceral metastasis involvement, and number of metastatic disease sites (Table 3). Patients with ≥5 CTCs had a hazard of death of 2.08 (95% CI, 1.64–2.66; $P < 0.001$) compared with those with <5 CTCs.

## discussion

In this large retrospective study, we confirm the prognostic value of assessing CTCs in MBC and provide a further classification of prognostic groups. In the HR+/HER2− subgroup, patients had more frequently ≥5 CTCs ($P = 0.024$). However, this finding is not concordant with results from previously published reports using the CellSearch® for CTC enumeration in similar but smaller population of MBC patients [10, 14, 16]. We showed that baseline CTCs enumeration had

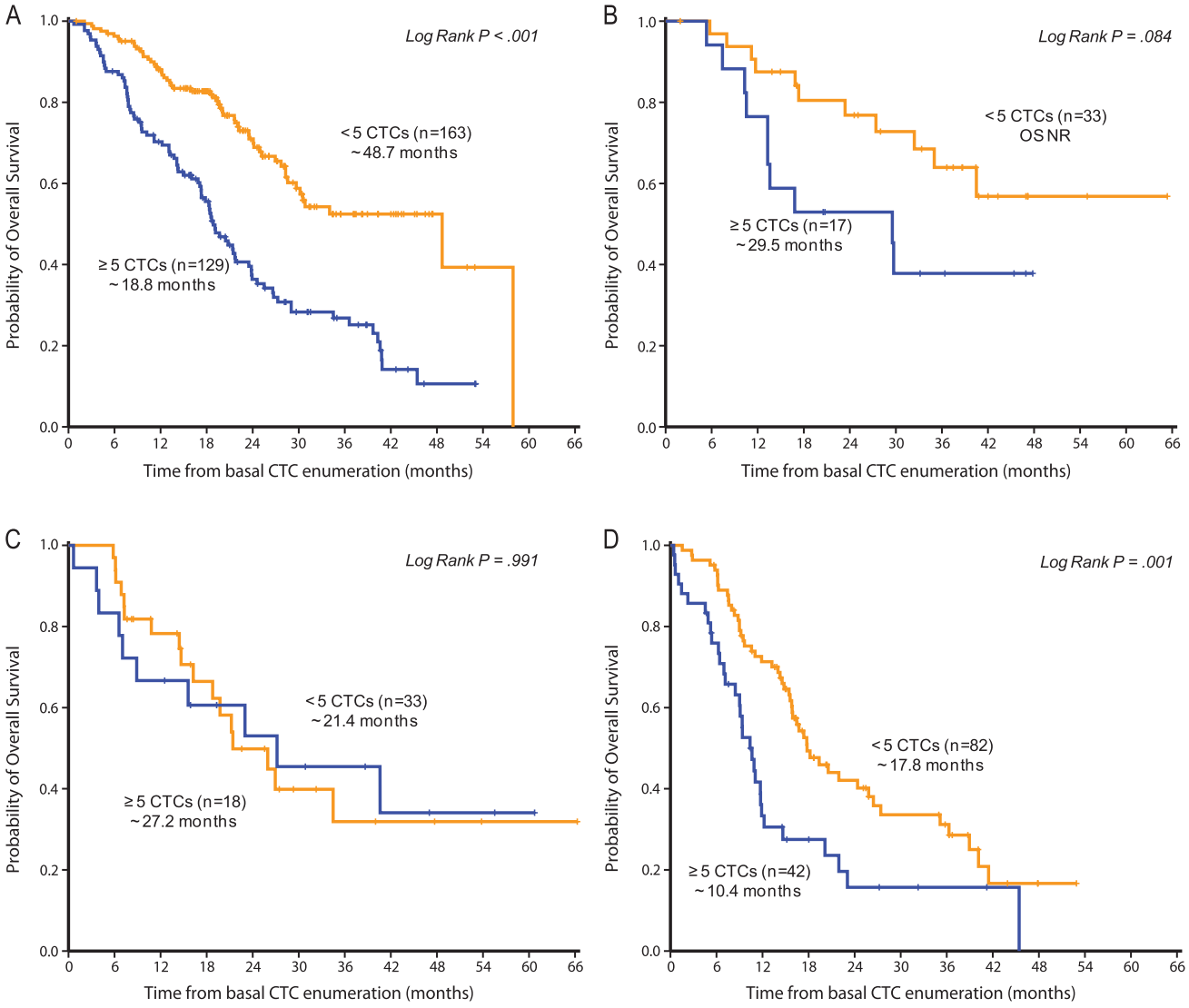


**Figure 2.** Overall survival (OS) in months according to immunohistochemical subtype and circulating tumor cell (CTC) value (patients with <5 CTCs in blue versus ≥5 CTCs in orange). (A) HR+/HER2− ($n = 292$); (B) HER2+/HR+ ($n = 50$); (C) HER2+/HR− ($n = 51$); (D) Triple-receptor negative ($n = 124$).

prognostic value in all breast cancer subtypes but appeared to be most valuable in HR+ and TN breast cancers and least valuable in HER2+ cancer treated with targeted therapy, suggesting an interaction between CTCs and such therapies. Therefore, we confirmed that in HR+/HER2− and TN breast cancers subgroups, CTCs were a strong prognostic factor irrespective of type and number of metastatic disease site.

HER2-targeted therapy combined with chemotherapy was highly effective, regardless of CTC value, in patients with HER2+ primary tumors. We previously showed that the effect of chemotherapy plus HER2-targeting drugs in patients with a high baseline CTC count was considerable, with the number of CTCs reduced to below the threshold of 5 in 16 of 17 (94%) subjects [18]. Accordingly, other groups have shown that biological therapies markedly decrease the number of CTCs at follow-up CTC assays [14, 16]. In our study, HER2+ MBC patients with ≥5 CTCs showed a PFS and OS similar to patients with <5 CTCs. Since 84 of 101 HER2+ breast cancer patients received an anti-HER2 treatment, we speculate that the high effectiveness of trastuzumab and lapatinib may eliminate a predominant population of circulating epithelial cells with HER2 amplification or overexpression thereby reducing the prognostic value of CTCs enumeration. However, we should state that the HER2+ group of patients is the smallest in number among subtypes and that the interaction test between subtypes and CTC count was negative. Moreover, tumor specimens obtained before 1993 and the absence of primary tumor gene expression profiling may lead to a misclassification of breast cancer subtypes [28].

Our data confirm differences in overall prognosis among different IHC subtypes. HER2+ breast cancer patients who had received trastuzumab or lapatinib had the best overall outcome, supporting the superior value of targeted therapy in breast cancer. Several studies have shown that women with luminal A, luminal B, and HER2 breast cancer subtypes [29] have superior prognostic outcomes in the trastuzumab era to those of women with TN tumors [30, 31]. The difference in prognostic value tends to support the hypothesis that various

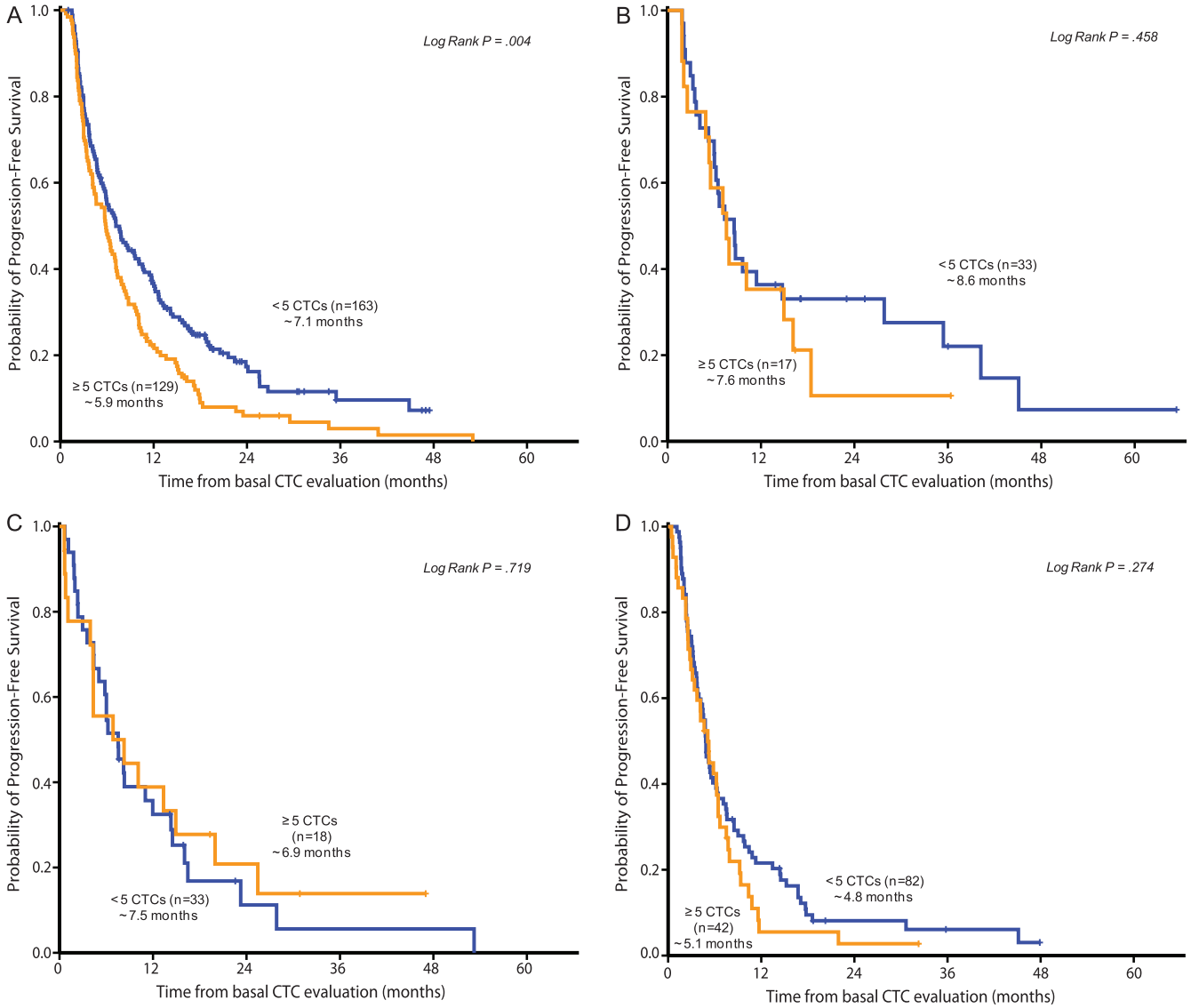


**Figure 3.** Progression-free survival (PFS) in months according to immunohistochemical subtype and circulating tumor cell (CTC) value (patients with <5 CTCs in blue versus ≥5 CTCs in orange). (A) HR+/HER2− ($n = 292$); (B) HER2+/HR+ ($n = 50$); (C) HER2+/HR− ($n = 51$); (D) Triple-receptor negative ($n = 124$).

**Table 2.** Median OS duration (months) and hazard ratio of death (in favor of ≥5 CTCs) in all 101 HER2+ breast cancer patients, according to HR status and treatment

| Subtype | *N* | Median OS CTC < 5 | Median OS CTC ≥ 5 | Hazard ratio | 95% CI | *P* |
|---|---|---|---|---|---|---|
| HER2+/HR+ | 50 | N/A | 29.5 | 2.18 | 0.88–5.37 | 0.092 |
| HER2+/HR− | 51 | 21.4 | 27.2 | 1.01 | 0.46–2.21 | 0.991 |
| HER2+ treated with anti-HER2 agents | 84 | 40.5 | 29.5 | 1.4 | 0.73–2.7 | 0.315 |

N/A: median OS not yet reached. Anti-HER2 agents: trastuzumab or lapatinib. OS, overall survival; CTC, circulating tumor cell; HR, hormone receptor; CI, confidence interval.

initiating pathways of tumor progression underlie the clinical heterogeneity and survival outcome of MBC subtypes and that CTC detection and characterization will help us better understand the biological behavior of tumor changes during the metastatic process.

In conclusion, we provided for the first time, strong evidence of a relationship between the IHC disease subtypes of breast cancer, HER2-targeted therapy and CTC prognostic value in MBC. These data suggest that breast cancer subtypes are associated with strong differences in patterns of metastatic

**Table 3.** Multivariate Cox regression analysis for prediction of PFS and OS

| Parameter | PFS | 95% CI | *P* value | OS | 95% CI | *P* value |
|---|---|---|---|---|---|---|
| ≥5 versus <5 CTCs | 1.23 | 1.01–1.48 | 0.036 | 2.1 | 1.65–2.67 | <0.001 |
| HR (positive versus negative) | 0.76 | 0.62–0.92 | 0.006 | 0.49 | 0.38–0.63 | <0.001 |
| HER2 (positive versus negative) | 0.72 | 0.56–0.92 | 0.009 | 0.57 | 0.41–0.79 | 0.001 |
| Visceral metastasis (yes versus no) | 1.31 | 1.04–1.66 | 0.023 | 1.67 | 1.22–2.29 | 0.002 |
| No. of metastatic sites (1 versus 2 versus ≥3) | 1.29 | 1.12–1.48 | <0.001 | 1.38 | 1.15–1.66 | 0.001 |

PFS, progression-free survival; OS, overall survival; CI, confidence interval; CTC, circulating tumor cell.

spread. Moreover, we believe future therapeutic trials in MBC should include CTC count to better stratify patients among different prognostic groups.

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## disclosure

The authors have declared no conflicts of interest.

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