Clinical Cancer Research

Pooled Analysis of the Prognostic Relevance of Circulating Tumor Cells in Primary Breast Cancer

Wolfgang J. Janni[1], Brigitte Rack[2], Leon W.M.M. Terstappen[3], Jean-Yves Pierga[4], Florin-Andrei Taran[5], Tanja Fehm[6], Carolyn Hall[7], Marco R. de Groot[8], François-Clement Bidard[4], Thomas W.P. Friedl[1], Peter A. Fasching[9], Sara Y. Brucker[5], Klaus Pantel[10], and Anthony Lucci[7]

Abstract

Purpose: Although unequivocal evidence has shown the prognostic relevance of circulating tumor cells (CTC) in the peripheral blood of patients with metastatic breast cancer, less evidence is available for the prognostic relevance of CTCs at the time of primary diagnosis.

Experimental Design: We conducted a pooled analysis of individual data from 3,173 patients with nonmetastatic (stage I–III) breast cancer from five breast cancer institutions. The prevalence and numbers of CTCs were assessed at the time of primary diagnosis with the FDA-cleared CellSearch System (Janssen Diagnostics, LLC). Patient outcomes were analyzed using meta-analytic procedures, univariate log-rank tests, and multivariate Cox proportional hazard regression analyses. The median follow-up duration was 62.8 months.

Results: One or more CTCs were detected in 20.2% of the patients. CTC-positive patients had larger tumors, increased lymph node involvement, and a higher histologic tumor grade than did CTC-negative patients (all $P < 0.002$). Multivariate Cox regressions, which included tumor size, nodal status, histologic tumor grade, and hormone receptor and HER2 status, confirmed that the presence of CTCs was an independent prognostic factor for disease-free survival [HR, 1.82; 95% confidence interval (CI), 1.47–2.26], distant disease-free survival (HR, 1.89; 95% CI, 1.49–2.40), breast cancer–specific survival (HR, 2.04; 95% CI, 1.52–2.75), and overall survival (HR, 1.97; 95% CI, 1.51–2.59).

Conclusions: In patients with primary breast cancer, the presence of CTCs was an independent predictor of poor disease-free, overall, breast cancer–specific, and distant disease-free survival.
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Introduction

Dissemination of tumor cells from the primary tumor into the bloodstream is a critical step in tumorigenesis, and is considered a precursor of distant metastases. High-resolution imaging technologies often cannot detect the spread of early tumor cells and occult micrometastases (circulating tumor cells, CTCs, in blood and disseminated tumor cells, DTCs, in bone marrow) because these tumor cells are rare and, at least during the initial stages of the disease, are unaccompanied by clinical symptoms (1).

Recent advances in isolation and enrichment methods make it possible to detect and enumerate extremely rare CTCs in the peripheral blood (as few as 1 CTC per 10^6–10^8 leukocytes), facilitating their use as a surrogate marker of minimal residual disease (MRD) and enabling clinical researchers to perform real-time monitoring of disease progression and treatment responses through repeated blood sampling. Numerous published studies demonstrate that CTC prevalence predicts disease recurrence and survival in patients with metastatic breast cancer (MBC; refs. 2–6), and the persistence of CTCs after treatment has been shown to predict lack of responses to therapy in metastatic settings (7–9).

The use of CTCs as a prognostic and predictive marker might be more important for patients with early breast cancer than for patients with MBC, for whom treatment is palliative in nature, and preliminary prospective clinical trials suggest that the presence of CTCs at the time of primary diagnosis could predict early disease recurrence and reduced survival (10–15).

Herein, using CTC results obtained using the FDA-cleared CellSearch System, we present the first large pooled analysis of the prognostic value of the presence of CTCs at the time of primary diagnosis in patients with non-MBC. The primary aim of this study was to evaluate whether CTCs could serve as an independent prognostic factor of disease recurrence and survival, using individual patient data. In addition, the large number of individual patient data available allowed us to conduct subgroup analyses

[1]Department of Gynecology and Obstetrics, University Hospital Ulm, Ulm, Germany. [2]Department of Gynecology and Obstetrics, Ludwig-Maximilians University of Munich, Munich, Germany. [3]Medical Cell BioPhysics Group, University of Twente, Enschede, the Netherlands. [4]Department of Medical Oncology, Institut Curie, Université Paris Descartes, Paris, France. [5]Department of Obstetrics and Gynecology, University of Tuebingen, Tuebingen, Germany. [6]Department of Gynecology and Obstetrics, Heinrich-Heine-University of Düsseldorf, Düsseldorf, Germany. [7]Department of Surgical Oncology, University of Texas MD Anderson Cancer Center, Houston, Texas. [8]Medisch Spectrum Twente, Enschede, the Netherlands. [9]Department of Gynecology and Obstetrics, University Hospital Erlangen, Erlangen, Germany. [10]Department of Tumor Biology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Wolfgang J. Janni, Department of Gynecology and Obstetrics, University Hospital Ulm, Prittwitzstr. 43, D-89075 Ulm, Germany. Phone: +49-731-500-58500; Fax: +49-731-500-58502; E-mail: wolfgang.janni@uniklinik-ulm.de

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Translational Relevance

Circulating tumor cells (CTC) that are shed from the primary tumor into the bloodstream are thought to be responsible for tumor progression through initiation of metastatic growth in distant organs. Thus, CTCs detected in the peripheral blood of cancer patients have the potential to function as an easily accessible marker with high prognostic and predictive value. Our study shows the strong independent prognostic effect of CTCs on disease-free survival and overall survival in primary breast cancer patients. To our knowledge, it is both the first pooled analysis and largest study on the prognostic relevance of CTCs in primary breast cancer with CTC assessments being based on the only FDA-approved CTC detection method. Our data complement the results of a recently published pooled analysis showing an independent prognostic effect of CTCs in metastatic breast cancer, suggesting that CTCs can serve as valuable prognostic markers in all stages of breast cancer.

with sample sizes yielding statistically robust results to determine whether the prognostic value of CTCs varied among subgroups, and to identify patients who might be particularly responsive to additional adjuvant therapies.

Materials and Methods

Data collection

Breast cancer centers known to have conducted studies involving the determination of CTCs in the peripheral blood of primary breast cancer patients using the CellSearch System were personally contacted by the first author (W. Janni) and asked if they would be willing to provide individual patient data for a pooled analysis. Fully anonymized individual patient data for the pooled analysis were provided by five academic breast cancer units: Enschede (the Netherlands), Houston (TX), Munich (Germany), Paris (France), and Tübingen (Germany); hereafter, all studies will be referred to by their corresponding cities. Some of the patient cohort data were published previously [Enschede (10), Houston (12), Munich (14), and Paris (13)], and additional information regarding the data collection, patients, and treatments is available in these original publications. Because of differences in the inclusion and/or exclusion criteria, inclusion of additional patients, or updated follow-up information, the patient numbers and survival results reported herein might differ from those presented in the original publications.

Patients

Potentially eligible patients were diagnosed with histologically confirmed, operable, stage I–III invasive breast cancer without the evidence of metastatic disease (tumor size stages T1–T4, nodal stages N0–N3, and metastasis stage M0). Ineligible patients were those with no valid data on CTC presence at the time of primary diagnosis as assessed using the FDA-cleared CellSearch System (see Supplementary Fig. S1 in the Supplementary Appendix). Informed consent was obtained from all patients prior to blood collection for CTC analyses. All studies were approved by the responsible ethical boards and conducted in accordance with the Declaration of Helsinki on Ethical Principles for Medical Research.

The primary tumor stages at diagnosis were classified according to the criteria defined by the revised American Joint Committee on Cancer and International Union against Cancer TNM classification system (16). Histologic grading was classified according to Black's nuclear grading system (ref. 17; Houston) or the Elston–Ellis modification of the Scarff–Bloom–Richardson grading system (ref. 18; all others). Tumors were defined as hormone receptor positive if the percentage of cells with immunohistochemical nuclear staining for estrogen, progesterone, or both was 10% or higher. Tumors with strong (3+) immunohistochemical membranous staining were defined as HER2-positive; tumors with moderate (2+) membranous staining were classified as HER2-positive only if an additional FISH analysis yielded a positive result.

Detection of CTCs

Presence and number of CTCs were analyzed using the standardized, semiautomatic CellSearch system, which has been described in detail previously (19, 20). Briefly, blood samples were collected in CellSave tubes (Janssen Diagnostics) and were centrifuged to separate the solid blood components from the plasma. After the immunomagnetic capture and enrichment of epithelial cell adhesion molecule (EpCAM)-positive cells via antibody-coated ferrofluid nanoparticles, the EpCAM-enriched cells were stained with phycoerythrin (PE)-conjugated antibodies C11 and A53-B/A2 specific for cytokeratins 8, 18, and 19 (epithelial cell markers), an allophycocyanin-conjugated mAb (HI30) specific for CD45 (leukocyte marker), and the fluorescent nucleic acid dye 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). CTCs were identified and counted using a semiautomated fluorescence-based microscope system that generated images of the stained cells; the CTCs were defined as cytokeratin-positive and CD45-negative nucleated cells >4 μm in size.

Blood samples for CTC status evaluations were collected prior to neoadjuvant chemotherapy (Paris) or at the time of primary surgery (Enschede, Houston, Munich, and Tübingen). One 7.5-mL blood sample per patient was used for CTC evaluations in Houston, Paris, and Tübingen, whereas four 7.5-mL blood samples per patient were analyzed separately in Enschede. In Munich, 30 mL of peripheral blood was collected per patient and was pooled and concentrated to a final volume of 7.5 mL prior to the CTC analysis (14). Patients were assessed as CTC-positive if at least 1 CTC was detected, regardless of the initial blood volume used for analysis. For statistical analyses involving the CTC numbers, the results for the four 7.5-mL blood samples per patient that had been collected in Enschede were pooled to obtain an average number of CTCs per 7.5 mL of blood.

Statistical analysis

Associations between the presence of CTCs and both baseline patient characteristics and established prognostic factors were evaluated using the t test for continuous variables, the Cochran–Armitage test for trends in the ordered categorical variables of tumor stage, nodal stage, and grading, and the χ^2 test for all other categorical variables.

We performed pooled analyses separately for four different survival endpoints defined according to the Standardized Definitions for Efficacy End Points (STEEP) criteria (21). Overall survival (OS) included death from any cause as an event. To calculate breast cancer–specific survival (BCSS), only death due to breast cancer–related causes (e.g., metastasis-dependent organ failure or

breast cancer progression) was considered an event. Disease-free survival (DFS) included invasive disease recurrence, second primary tumors, and death from any cause as events; all noninvasive *in situ* cancer events were excluded. To calculate distant disease-free survival (DDFS), only distant recurrence (metastasis and second primary tumors) and death from any cause were regarded as events. Ipsilateral or regional disease recurrences and contralateral breast cancers were excluded from analysis. All time-to-event intervals were measured from time of primary diagnosis to date of the event. If no endpoint was reached, data were censored at the date of the last follow-up. All median follow-up times were calculated using the reverse Kaplan–Meier method (22).

For all four survival endpoints (OS, BCSS, DFS, DDFS), the univariate HR and 95% confidence intervals (CI) for disease recurrence or death were initially calculated for each of the five centers on the basis of the individual patient data, using CTC positivity (yes/no) as the sole variable. Next, a summary estimate of the HRs and 95% CIs for each of the survival endpoints was obtained through a meta-analytic approach on the basis of random-effects models. If the HR for a survival endpoint could not be calculated for a single study because of a lack of events in CTC-negative or CTC-positive patients, the study was subsequently excluded from the meta-analytic HR calculation for the survival endpoint. This was the case for the OS and BCSS endpoint calculations, in which the Tübingen data had to be excluded because of missing events among the CTC-positive patients. Interstudy heterogeneity was assessed with the Q-test, and sensitivity analyses were conducted by computing meta-analytic HRs and CIs that were calculated following the omission of one study at a time.

Univariate significance values of the study variables were determined according to Kaplan–Meier estimates and log-rank tests. The simultaneous effects of multiple covariates on survival endpoints were calculated using Cox proportional hazards regression models stratified by study center. Because of the missing values with regard to clinicopathologic variables included in the multivariate models, 96 patients had to be excluded from all multivariate analyses. Our initial model included tumor grade (G1, G2, G3), histologic type (ductal, lobular, other), tumor stage (T1, T2, T3, T4), nodal stage (N0, N1, N2, N3), hormone receptor status (positive, negative), HER2 status (positive, negative), menopausal status (premenopausal, postmenopausal), neoadjuvant chemotherapy (yes, no), and adjuvant chemotherapy (yes, no). Following a stepwise backward selection procedure to exclude variables that did not contribute significantly to the model (significance level cutoff for exclusion, 0.05; likelihood ratio test), CTC presence (yes/no) was added to the model to determine whether inclusion of this variable significantly improved the model, that is, whether the presence of CTCs was a significant independent prognostic factor. Finally, the two-way interactions between CTC presence and each of the other factors that remained in the model after the stepwise backward selection procedure were added to the model already including CTC presence (one at a time) to test whether the addition of the interaction term further improved the model significantly, that is, to test whether the prognostic value of CTCs was significantly affected by one of the other factors in the model. The assumptions for the proportional hazards regression models were met for all four survival endpoints (no significant CTC presence by time-interaction term; calculated using a multivariate Cox regression model with a time-dependent covariate). Statistical analyses were performed with the IBM SPSS Statistics software package, version 21 (SPSS Inc.). All statistical tests were two-sided and P values less than 0.05 were considered statistically significant.

This article has been written in accordance to guidelines and recommendations for tumor marker prognostic studies (REMARK; ref. 23).

Results

Prevalence of CTCs and associations with clinical parameters

A total of 3,173 patients with stage I–III invasive breast cancer and known CTC statuses were included in our pooled analysis (Supplementary Fig. S1 in the Supplementary Appendix). Median age was 54 years (range, 21–91 years), and median follow-up duration was 62.8 months. At least one CTC was detected in 640 (20.2%) of the patients; the numbers of detected CTCs ranged from 1 to 827. Data regarding patient number, age, and enrollment period as well as presence and number of CTCs from the five centers are presented in Supplementary Table S1 of the Supplementary Appendix.

The presence of CTCs was associated with a large tumor size, increased lymph node involvement, unfavorable histologic grade, and lobular tumor type, whereas no significant association was identified between CTC presence and menopausal status, hormone receptor status, or HER2 status (Table 1). Patients with CTCs more often received neoadjuvant and/or adjuvant chemotherapy than did patients without CTCs, whereas no association was identified between the presence of CTCs and endocrine therapy, HER2-targeted therapy, or radiotherapy (Table 1).

Meta-analysis

Data on the number of patients and follow-up duration as well as on the number of events for the four study endpoints OS, BCSS, DFS, and DDFS by center are shown in Supplementary Table S1 of the Supplementary Appendix. The meta-analytic summary estimate for the OS HR according to the presence of CTCs was 2.444 (95% CI, 1.811–3.298; $P < 0.001$). The HRs calculated for the single studies ranged from 1.538 (Houston) to 4.212 (Paris), and were significant for all studies, except Houston. No significant interstudy heterogeneity was observed (Q-test, $P = 0.344$). Sensitivity analyses confirmed that the exclusion of any one study did not markedly affect the HR or confidence limit summary estimates (HR estimate range, 2.273–2.623; all $P < 0.003$). Regarding BCSS, the HR summary estimate was 2.540 (95% CI, 1.910–3.378; $P < 0.001$). The single-study HRs ranged from 1.734 (Houston) to 4.212 (Paris) and, again, were significant for all studies, except Houston. The Q-test revealed no significant heterogeneity among the studies ($P = 0.561$), and the sensitivity analyses revealed that the HR and corresponding CI summary estimate, which were calculated after the removal of any one study at a time, remained virtually unchanged (HR estimate range, 2.435–2.693; all $P < 0.001$).

The summary estimate for the DFS HR in association with the presence of CTCs was 2.080 (95% CI, 1.688–2.563; $P < 0.001$). The single-study HRs ranged from 1.896 (Tübingen) to 2.706 (Paris) and were significant for all studies, except Tübingen. There was no significant heterogeneity among the studies (Q-test, $P = 0.899$), and sensitivity analyses showed that exclusion of any one study at a time had no marked effect on

Table 1. Presence of CTCs according to clinical variables in a pooled analysis of early breast cancer patients

Variable	All patients ($n = 3,173$)	Patients without CTCs ($n = 2,533$)	Patients with CTCs ($n = 640$)	*P*
Age (years)				0.320[a]
Mean ± SD	54.1 ± 11.2	54.2 ± 11.3	53.7 ± 11.0	
Range	21–91	21–91	26–90	
Menopausal status, *n* (%)				0.697[b]
Premenopausal	1,236 (39.0)	982 (38.8)	254 (39.7)	
Postmenopausal	1,922 (60.6)	1,538 (60.7)	384 (60.0)	
Unknown	15 (0.5)	13 (0.5)	2 (0.3)	
Tumor stage, *n* (%)				<0.001[c]
T1	1,403 (44.2)	1,159 (45.8)	244 (38.1)	
T2	1,451 (45.7)	1,137 (44.9)	314 (49.1)	
T3	203 (6.4)	148 (5.8)	55 (8.6)	
T4	99 (3.1)	75 (3.0)	24 (3.8)	
Unknown	17 (0.5)	14 (0.6)	3 (0.5)	
Nodal stage, *n* (%)				<0.001[c]
N0	1,385 (43.6)	1,136 (44.8)	249 (38.9)	
N1	1,230 (38.8)	997 (39.4)	233 (36.4)	
N2	357 (11.3)	268 (10.6)	89 (13.9)	
N3	190 (6.0)	122 (4.8)	68 (10.6)	
Unknown	11 (0.3)	10 (0.4)	1 (0.2)	
Histologic grading, *n* (%)				0.002[c]
G1	291 (9.2)	252 (9.9)	39 (6.1)	
G2	1,506 (47.5)	1,206 (47.6)	300 (46.9)	
G3	1,364 (43.0)	1,064 (42.0)	300 (46.9)	
Unknown	12 (0.4)	11 (0.4)	1 (0.2)	
Histologic type, *n* (%)				0.022[b]
Ductal	2,569 (81.0)	2,063 (81.4)	506 (79.1)	
Lobular	370 (11.7)	276 (10.9)	94 (14.7)	
Other	228 (7.2)	188 (7.4)	40 (6.3)	
Unknown	6 (0.2)	6 (0.2)	0 (0.0)	
Hormone receptor status, *n* (%)				0.317[b]
Negative	778 (24.5)	611 (24.1)	167 (26.1)	
Positive	2,388 (75.3)	1,915 (75.6)	473 (73.9)	
Unknown	7 (0.2)	7 (0.3)	0 (0.0)	
HER2 status, *n* (%)				0.532[b]
Negative	2,441 (76.9)	1,953 (77.1)	488 (76.3)	
Positive	688 (21.7)	543 (21.4)	145 (22.7)	
Unknown	44 (1.4)	37 (1.5)	7 (1.1)	
Endocrine therapy				0.512[b]
No	956 (30.1)	756 (29.8)	200 (31.3)	
Yes	2,196 (69.2)	1,759 (69.4)	437 (68.3)	
Unknown	21 (0.7)	18 (0.7)	3 (0.5)	
HER2-targeted therapy				0.933[b]
No	2,234 (70.4)	1,779 (70.2)	455 (71.1)	
Yes	526 (16.6)	418 (16.5)	108 (16.9)	
Unknown	413 (13.0)	336 (13.3)	77 (12.0)	
Radiotherapy				0.469[b]
No	560 (17.6)	453 (17.9)	107 (16.7)	
Yes	2,600 (81.9)	2,068 (81.6)	532 (83.1)	
Unknown	13 (0.4)	12 (0.5)	1 (0.2)	
Neoadjuvant chemotherapy				0.095[b]
No	2,912 (91.8)	2,335 (92.2)	577 (90.2)	
Yes	261 (8.2)	198 (7.8)	63 (9.8)	
Adjuvant chemotherapy				0.014[b]
No	629 (19.8)	524 (20.7)	105 (16.4)	
Yes	2,534 (79.9)	2,000 (79.0)	534 (83.4)	
Unknown	10 (0.3)	9 (0.4)	1 (0.2)	

[a] *t* test.
[b] χ^2 test (without unknowns).
[c] Cochran–Armitage test for trend (without unknowns).

the HR or confidence limit summary estimates, which were calculated from the remaining studies (HR estimate range, 2.018–2.321; all $P < 0.001$). The results of the DDFS meta-analysis were similar to those obtained for the other three survival endpoints. The HR summary estimate associated with the presence of CTCs was 2.196 (95% CI, 1.737–2.776; $P < 0.001$), with single-study HRs ranging from 1.916 (Munich) to 2.920 (Houston). Again, there was no significant heterogeneity among the studies (Q-test, $P = 0.664$), and the HR summary estimates determined from the sensitivity analyses, in which single studies were excluded from the analysis one at a time, ranged from 2.099 to 2.801 (all $P < 0.001$).

Survival, disease recurrence, and CTC status

Overall, 238 of 3,173 patients (7.5%) died during follow-up, and 93 (39.1%) of these patients presented with CTCs at the time of primary diagnosis. The presence of CTCs significantly predicted shorter OS in both the univariate analysis (HR, 2.413; 95% CI, 1.859–3.131; log-rank test: $P < 0.001$; Fig. 1A), and in the multivariate Cox proportional hazard models after controlling for tumor grade and stage, nodal stage, hormone receptor, and HER2 receptor expression (HR, 1.974; 95% CI, 1.505–2.590; $P < 0.001$; Table 2). The prognostic value of CTCs for OS was evident not only when comparing patients with and without CTCs, but also when using CTC cut-off values ranging from 1 (0–1 CTC vs. > 1 CTC) to 20 (0–20 CTCs vs. > 20 CTCs), as revealed in univariate analyses using log-rank tests (all $P < 0.04$; Fig. 2A).

Breast cancer–specific deaths were reported for 198 patients (83.2% of all recorded deaths). Patients with CTCs were more likely to die from breast cancer than were patients without CTCs (HR, 2.606; 95% CI, 1.962–3.460; log-rank test: $P < 0.001$; Fig. 1B), and the independent prognostic value of CTCs was confirmed in a multivariate Cox regression analysis (HR, 2.042; 95% CI, 1.519–2.746; $P < 0.001$; Table 2). Similar to OS, the prognostic value of CTCs for BCSS was significant for all CTC

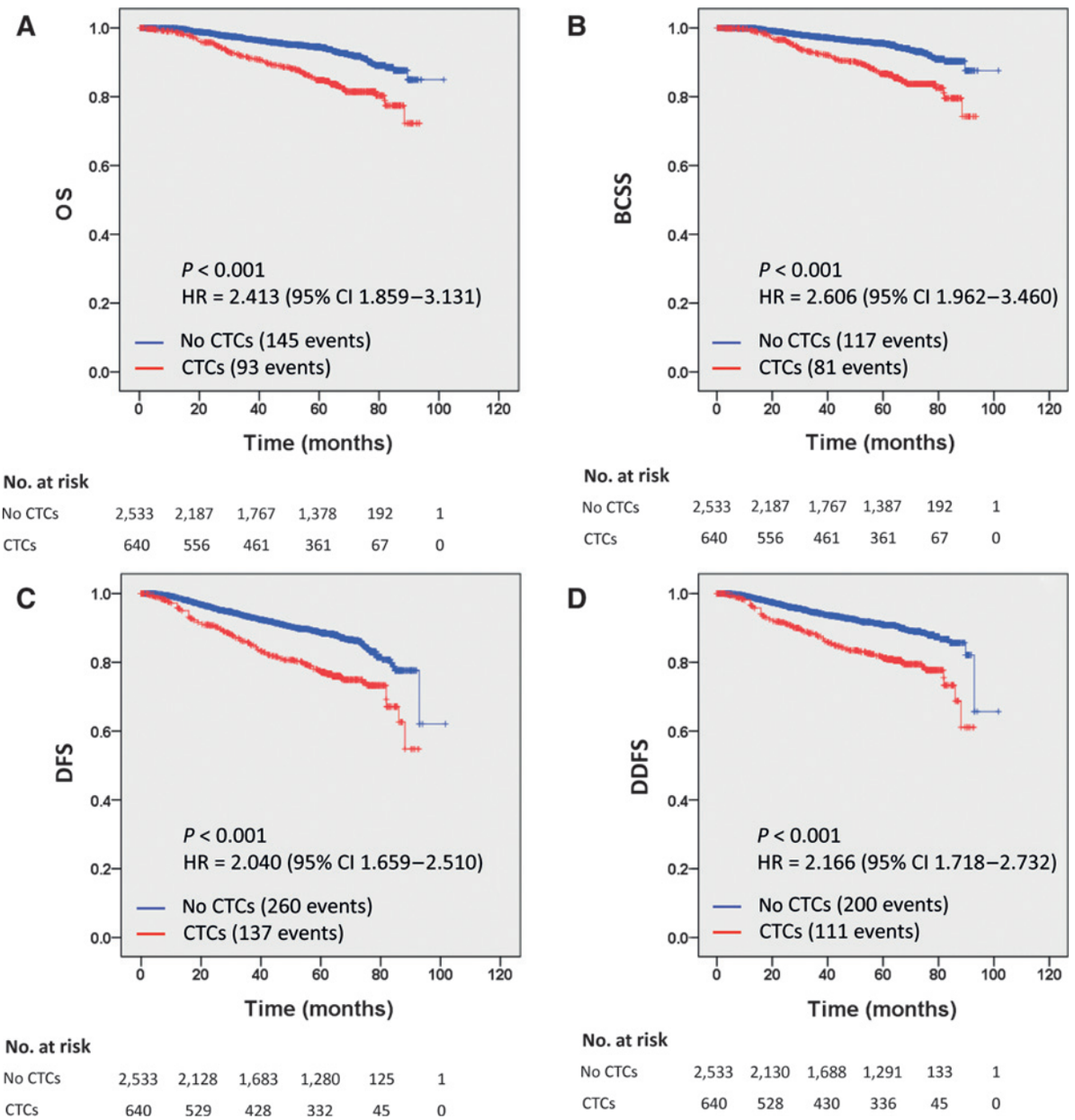

Figure 1.

Kaplan–Meier plots of survival according to presence of CTCs at the time of primary diagnosis. OS (A), BCSS (B), DFS (C), and DDFS (D). HR denotes the HR, and *P* values refer to log-rank tests.

Table 2. Multivariate HRs (cox proportional hazards regression model, stratified for study center) for OS, BCSS, DFS, and DDFS in a pooled analysis of early breast cancer patients

Survival endpoint	HR (95% CI)	*P*
OS ($n = 233$ events)		
CTCs		
Positive vs. negative	1.974 (1.505–2.590)	<0.001
Tumor grade		<0.001
G2 vs. G1	1.137 (0.540–2.391)	0.735
G3 vs. G1	2.440 (1.164–5.112)	0.018
Tumor stage		<0.001
T2 vs. T1	1.847 (1.323–2.580)	<0.001
T3 vs. T1	3.882 (2.429–6.204)	<0.001
T4 vs. T1	3.165 (1.730–5.788)	<0.001
Nodal stage		<0.001
N1 vs. N0	2.549 (1.771–3.667)	<0.001
N2 vs. N0	3.838 (2.529–5.822)	<0.001
N3 vs. N0	6.738 (4.275–10.621)	<0.001
Hormone receptor status		
Positive vs. negative	0.381 (0.282–0.514)	<0.001
HER2 status		
Positive vs. negative	0.593 (0.426–0.825)	0.002
BCSS ($n = 193$ events)		
CTCs		
Positive vs. negative	2.042 (1.519–2.746)	<0.001
Tumor grade		<0.001
G2 vs. G1	1.347 (0.532–3.407)	0.530
G3 vs. G1	3.291 (1.310–8.265)	0.011
Tumor stage		<0.001
T2 vs. T1	2.233 (1.516–3.289)	<0.001
T3 vs. T1	5.090 (3.025–8.565)	<0.001
T4 vs. T1	3.217 (1.613–6.416)	0.001
Nodal stage		<0.001
N1 vs. N0	2.759 (1.844–4.128)	<0.001
N2 vs. N0	3.894 (2.451–6.185)	<0.001
N3 vs. N0	7.526 (4.575–12.383)	<0.001
Hormone receptor status		
Positive vs. negative	0.353 (0.255–0.488)	<0.001
HER2 status		
Positive vs. negative	0.633 (0.444–0.902)	0.011
DFS ($n = 387$ events)		
CTCs		
Positive vs. negative	1.822 (1.470–2.258)	<0.001
Tumor grade		<0.001
G2 vs. G1	1.584 (0.869–2.887)	0.133
G3 vs. G1	3.076 (1.686–5.614)	<0.001
Tumor stage		<0.001
T2 vs. T1	1.429 (1.126–1.814)	0.003
T3 vs. T1	2.246 (1.542–3.273)	<0.001
T4 vs. T1	2.915 (1.825–4.657)	<0.001
Nodal stage		<0.001
N1 vs. N0	1.935 (1.480–2.529)	<0.001
N2 vs. N0	2.660 (1.929–3.670)	<0.001
N3 vs. N0	5.359 (3.793–7.573)	<0.001
Hormone receptor status		
Positive vs. negative	0.551 (0.435–0.698)	<0.001
HER2 status		
Positive vs. negative	0.701 (0.546–0.901)	0.005
DDFS ($n = 304$ events)		
CTCs		
Positive vs. negative	1.888 (1.485–2.401)	<0.001
Tumor grade		<0.001
G2 vs. G1	1.610 (0.775–3.345)	0.202
G3 vs. G1	3.604 (1.738–7.475)	0.001
Tumor stage		<0.001
T2 vs. T1	1.657 (1.254–2.191)	<0.001
T3 vs. T1	2.585 (1.683–3.971)	<0.001
T4 vs. T1	3.276 (1.951–5.499)	<0.001
Nodal stage		<0.001
N1 vs. N0	2.240 (1.646–3.047)	<0.001
N2 vs. N0	3.139 (2.183–4.514)	<0.001
N3 vs. N0	6.207 (4.192–9.191)	<0.001
Hormone receptor status		
Positive vs. negative	0.508 (0.391–0.661)	<0.001
HER2 status		
Positive vs. negative	0.722 (0.547–0.951)	0.021

(Continued on the following column)

cut-off values ranging from 1 to 20 (all $P < 0.02$; see Supplementary Fig. S2A in the Supplementary Appendix).

Disease recurrence during the follow-up period was observed in 397 of 3,173 patients (12.5%), and 311 of these patients (9.8%) had distant metastases (alone or in combination with local recurrences). The presence of CTCs was significantly associated with shorter DFS and DDFS (Fig. 1C and D), with multivariate HRs of 1.822 (95% CI, 1.470–2.258; $P < 0.001$) for DFS and 1.888 (95% CI, 1.485–2.401; $P < 0.001$) for DDFS (Table 2). The prognostic value of CTCs was significant for all CTC cut-off values ranging from 1 to 20 both for DFS (all $P < 0.01$; Fig. 2B) and for DDFS (all $P < 0.01$; see Supplementary Fig. S2B in the Supplementary Appendix).

The multivariate analyses revealed that for all four survival endpoints alike, grading, tumor stage, nodal stage, hormone receptor status, and HER2 status were additional significant independent prognostic factors, while histologic type, menopausal status, neoadjuvant chemotherapy, and adjuvant chemotherapy were not significantly associated with disease recurrence or survival (Table 2). The addition of the interaction terms between CTC presence and tumor characteristics never led to a significant model improvement (OS: all $P > 0.14$; BCSS: all $P > 0.07$; DFS: all $P > 0.20$; DDFS: all $P > 0.27$).

Subgroup analyses

The univariate HRs for OS according to the presence of CTCs in various subgroups (plus both the number of patients and the number of events observed within each subgroup) are shown in Fig. 3. The HRs were 2.801 (95% CI, 1.989–3.944; $P < 0.001$) for patients with hormone receptor–positive tumors and 1.894 (95% CI, 1.261–2.845; $P = 0.002$) for patients with hormone receptor–negative tumors. The prognostic relevance of CTC status was similar in patients with HER2-negative tumors (HR, 2.388; 95% CI, 1.783–3.199; $P < 0.001$) and those with HER2-positive tumors (HR, 2.521; 95% CI, 1.394–4.559; $P = 0.002$).

CTC status had significant prognostic relevance in triple-negative breast cancer patients (HR, 1.973; 95% CI, 1.254–3.104; $P = 0.003$), in patients with hormone receptor–positive/HER2-negative tumors (HR, 2.621; 95% CI, 1.784–3.850; $P < 0.001$) and in those with hormone receptor–positive/HER2-positive tumors (HR, 3.616; 95% CI, 1.649–7.928; $P = 0.001$); however, the CTC status was not significantly associated with prognosis in patients with hormone receptor–negative/HER2-positive tumors (HR, 1.594; 95% CI, 0.618–4.111; $P = 0.331$).

Presence of CTCs did not significantly predict OS in the subgroup of patients with nodal stage N0 disease (HR, 1.322; 95% CI, 0.705–2.478; $P = 0.383$), while it was significantly associated with OS in patients with nodal stage N1 (HR, 2.523; 95% CI, 1.623–3.923; $P < 0.001$), N2 (HR, 2.375; 95% CI, 1.340–4.211;

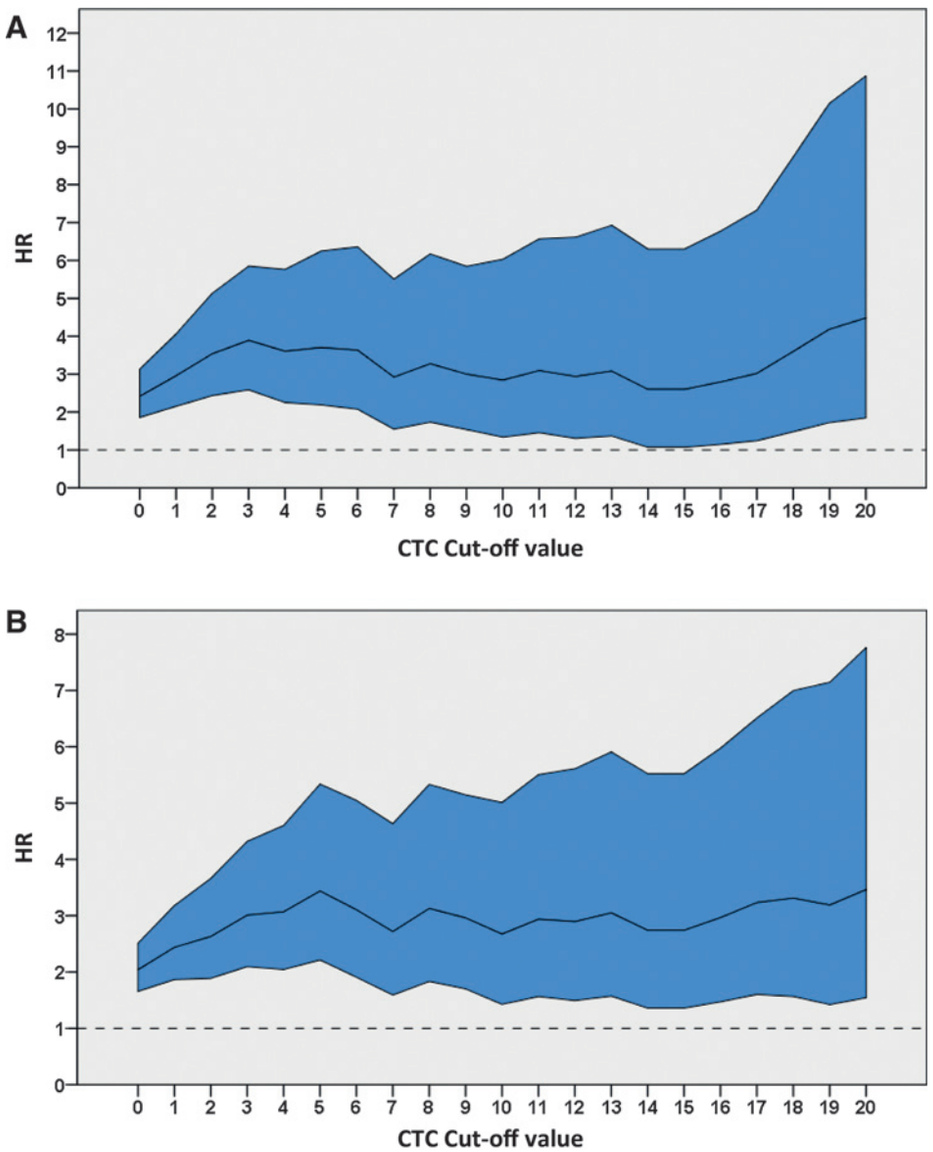

Figure 2.
HRs (black line) and 95% CIs (blue area) from the univariate survival analyses according to the presence of CTCs categorized using different cut-off values (e.g., cutoff 0: 0 CTCs vs. >0; cutoff 1: 0–1 CTCs vs. >1; cutoff 2: 0–2 CTCs vs. >2). OS (A) and DFS (B).

$P = 0.002$), or N3 disease (HR, 2.186; 95% CI, 1.274–3.750; $P = 0.004$). In addition, the presence of CTCs did not affect OS in low-risk patients, defined here as those with stage T1N0 primary tumors (HR, 1.030; 95% CI, 0.294–3.618; $P = 0.963$). In contrast, the presence of CTCs had a highly significant effect on OS in high-risk patients, defined as those with primary tumors larger than 2 cm (T2–T4) and lymph node involvement (HR, 2.460; 95% CI, 1.784–3.390; $P = 0.001$; see Supplementary Fig. S3 in the Supplementary Appendix).

Discussion

Our study is the first pooled analysis to demonstrate that CTC positivity at the time of primary early breast cancer diagnosis is an independent and highly significant prognostic marker of OS, DFS, BCSS, and DDFS. This study, based on a large sample size with long follow-up duration (median, 62.8 months) at five breast cancer centers, confirmed the independent prognostic relevance of CTCs for OS, BCSS, DFS, and DDFS in non-MBC with the highest level of evidence. Our study also adds important new data not reported in previous studies on the prognostic value of CTCs in early breast cancer in terms of a comprehensive analysis of the prognostic role of CTCs in various breast cancer subgroups and a detailed evaluation of the prognostic impact of CTCs using a broad range of different CTC cut-off values. Importantly, there was no significant heterogeneity among the five centers, and sensitivity analyses showed that the large patient cohort from Munich did not bias the results, as the presence of CTCs remained a significant prognostic factor with respect to all four survival endpoints even after excluding the Munich data from the analysis.

As shown by the subgroup analyses, the prognostic value of CTCs varied among breast cancer subtypes. CTC positivity was not a significant prognostic factor of outcome in low-risk patients with T1N0 tumors, suggesting that early-stage breast cancers detected

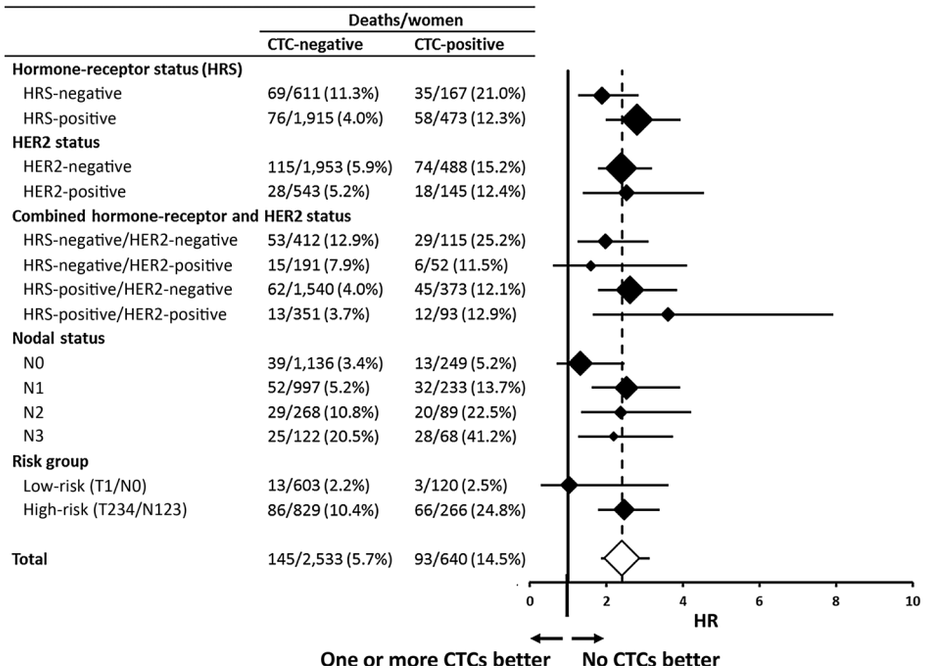

Figure 3.
Forest plot of OS and comparison of patients with and without CTCs in various subgroups. The black diamonds indicate the HRs (CTC positive vs. CTC negative) for the subgroup analyses, and the white diamond represents the overall HR for the pooled analysis including all 3,173 patients (for better comparison also indicated by the dashed vertical line). The size of the diamonds is proportional to the sample size (number of patients) in the groups, and the horizontal lines indicate the corresponding 95% CIs for the HRs. The solid vertical line represents an HR of 1.0 (i.e., no difference in survival between CTC-positive and CTC-negative patients).

before axillary spreading could be treated successfully and independently of CTC status. In contrast, the significant prognostic value of CTCs in high-risk patients suggests a potential clinical relevance for CTCs in this group, and CTC presence might direct decisions regarding the appropriateness of dose-intensified chemotherapy, new investigational drugs, or other therapeutic regimens.

The biologic subtype of tumors (definitions following the recommendations of the 12th St Gallen International Breast Cancer Conference Expert Panel; ref. 24) also affected the prognostic value of the CTCs. Presence of CTCs significantly predicted outcome in patients with hormone receptor–positive tumors (i.e., subtypes luminal A and B, including luminal B HER2-positive subtype) and with triple-negative tumors (basal-like subtype), but not in patients with HER2-positive, hormone receptor–negative tumors (nonluminal HER2-overexpressing subtype), although the lack of statistical significance in the latter group might also be related to reduced statistical power due to small sample sizes. Our data revealed a highly significant prognostic value of CTCs when all HER2-positive breast cancer patients were combined irrespectively of hormone receptor status, and this finding remained true when the analysis included only patients who were receiving HER2-targeted therapy (HR for OS, 2.439; CI, 1.139–5.208; $P = 0.018$). Most of the patients with HER2-positive/hormone receptor–positive tumors received endocrine therapy in addition to HER2-targeted therapy; this regimen appeared to enhance the prognostic relevance of CTCs relative to that associated with HER2-positive but hormone receptor–negative tumors. It is also plausible that HER2-positive tumors respond differently to cytotoxic treatments depending on the hormone receptor status, as HER2-positive tumors comprise two different biologic subtypes, that is, the nonluminal HER2-overexpressing subtype (HER2-positive/hormone receptor–negative tumors) or the luminal B HER2-positive subtype (HER2-positive/hormone receptor–positive tumors; ref. 24).

It is of considerable interest to assess whether there is an optimal cut-off point with regard to the prognostic impact of CTCs. In accordance with previously reported studies (3), a cut-off value of five CTCs has been used to evaluate the prognostic role of CTCs in metastatic settings. However, this fixed cut-off value has been criticized on the basis of biologic, technical, and statistical reasons (25–28), and recent data indicate that there is a direct relationship between CTC number and outcome (2, 29, 30). Given that CTCs are detected at much lower frequencies in nonmetastatic compared with MBC, most studies to date have used a cut-off point of $\geq$1 CTC to assess the prognostic role of CTCs.

Because of the limited number of non-MBC patients with multiple CTCs, no information regarding the prognostic value of CTCs with respect to different cut-off points was previously available. Our large pooled analysis revealed significant prognostic relevance for CTCs independent of the selected cut-off points (cut-off values ranged from 1 to 20 CTCs; corresponding HRs ranged from 2.413 to 4.480 for OS and from 2.040 to 3.463 for DFS), indicating that the presence of at least one CTC yields relevant prognostic information.

Limitations of this study include the difference in blood volumes used for the CTC analyses among the contributing centers. However, CTC positivity rates did not differ significantly among four of the five participating centers (χ^2 test, degrees of freedom = 3, $P = 0.143$). The only exception was the Tübingen study, which reported a considerably lower CTC positivity rate than the other centers (Supplementary Table S1). This discrepancy was probably due to the fact that the patient cohort from Tübingen had higher frequencies of N0 (72.8%) and T1 tumors (66.8%) compared with the other centers. A more general

limitation is the fact that the CellSearch system is based on detection of the epithelial-specific marker EpCAM. One potential problem with the use of epithelial-specific markers is that more aggressive CTC types are likely to undergo phenotypic changes associated with the epithelial-to-mesenchymal transition (EMT). In cancer cells, EMT is considered a crucial event for metastatic spreading, because it facilitates primary tumor dissemination into the blood stream as well as migration and invasion into secondary metastatic sites (1, 31, 32). During EMT, cells exhibit reduced levels of cell–cell adhesion and increased mesenchymal protein expression, which is accompanied by a corresponding loss of epithelial marker expression (33). Therefore, EpCAM-based CTC detection methods such as the CellSearch system might miss circulating cancer cells that are particularly likely to initiate distant organ metastasis (34, 35). EpCAM-negative CTCs have been detected in the blood of patients with different subtypes of MBC patients (36), but it has been argued that the problem of detecting only EpCAM-positive CTCs and missing EpCAM-negative circulating cancer cells might apply particularly to some basal-like tumors, as these are characterized by low or even nonexistent EpCAM expression (37). However, EpCAM-positive CTCs are commonly found also in triple-negative (basal-like) breast cancer, and the presence of EpCAM-positive CTCs as detected using the CellSearch system has been identified as a prognostic marker for disease-free and overall survival in patients with both early (this study) and metastatic triple-negative breast cancer (2, 6, 38).

CTC detection holds great potential as a prognostic and predictive tool for early breast cancer, and is likely superior to serum tumor markers such as CEA and CA15-3 (2). An important avenue of future research involves the phenotypic characterization and molecular profiling of CTCs; such research could considerably enhance our understanding of tumor biology, particularly metastatic spread, and facilitate the development of more personalized treatment interventions (39). Currently, therapeutic decisions are mainly based on primary tumor markers. However, several studies have shown a high degree of discordance between the primary tumor and metastatic sites or CTCs with respect to hormone receptor and HER2 expression (40–43). Given the estimated short life span of CTCs in the circulation (44), the presence of CTCs in the peripheral blood of breast cancer patients after primary tumor resection suggests that the pool of CTCs is replenished by occult micrometastases. Thus, CTC analyses may be considered as liquid biopsy for cancer patients, providing unique and important information about the molecular characteristics of occult MRD in the absence of clinically detectable overt metastases (45, 46).

Advancing the field of CTC research is an important requirement for developing and conducting interventional trials to assess the potential of implementing CTCs in clinical praxis. The pivotal SWOG0500 trial (NCT00382018) was the first prospectively randomized clinical trial that evaluated the clinical utility of CTCs (47). In this trial, 123 patients with MBC showing no decrease in CTCs after one cycle of first-line chemotherapy were randomized to continue the first-line therapy or to change to second-line chemotherapy. While early switching to an alternative chemotherapy did not result in a significant improvement in progression-free or overall survival, the trial confirmed the prognostic significance of persistently increased CTCs in patients with MBC receiving first-line chemotherapy (47). The lack of improved outcome in early-switching patients does not question the potential clinical utility of CTCs—rather it suggests that in MBC patients the failure of a first-line chemotherapeutic regimen to reduce CTCs after one cycle might indicate a general chemotherapy resistance.

There are several ongoing large prospective interventional studies in which therapeutic decisions are based on CTC presence and/or CTC phenotypes in both non-MBC and MBC (48). For example, the German randomized two-arm DETECT III trial (NCT01619111) is evaluating the efficacy (assessed on the basis of CTC clearance rate after treatment) of a HER2-targeted treatment with lapatinib in patients with HER2-negative MBC but at least one HER2-positive CTC. On the basis of findings that suggest a treatment benefit of trastuzumab in patients with non-HER2–overexpressing tumors (49), the European Organization for Research and Treatment of Cancer TREAT-CTC trial (NCT01548677), a multicenter randomized phase II study, aims to assess the use of trastuzumab in patients with nonmetastatic HER2-negative disease and at least one CTC in 15 mL of peripheral blood. In the French STIC CTC trial (NCT01710605), patients with hormone receptor–positive MBC are randomized between a standard clinician choice arm and a CTC count driven arm, in which patients with a baseline CTC count equal to or higher than 5 CTC/7.5 mL presumably at a higher risk receive chemotherapy, while patients with a baseline CTC count below 5 CTC/7.5 mL receive endocrine therapy. The aim of the French CirCé01 trial (Circulating Tumor Cells to Guide Chemotherapy for Metastatic Breast Cancer; NCT01349842) is to evaluate repeatedly over several lines of chemotherapy whether patients whose CTC count does not decrease after the first treatment cycle benefit from a switch of chemotherapeutic regimens. COMETI P2 (NCT 01701050) is an American observational trial on ER-positive, HER2-negative MBC patients that aims to determine a CTC-Endocrine Therapy Index (CTC-ETI) to predict whether patients either will respond favorably to a new endocrine therapy or will be resistant to endocrine treatment and thus need a chemotherapeutic treatment. The results of these trials will be crucial for evaluating the potential for CTC implementation in clinical breast cancer management.

In conclusion, this large pooled analysis provides strong evidence for the independent prognostic relevance of CTCs in primary breast cancer patients, and can thus be regarded as proof-of-principle for the use of CTCs as prognostic markers in early breast cancer. The next step toward clinical application of CTCs is incorporating CTC phenotypes or changes in CTC numbers during treatment to facilitate the selection of appropriately targeted and individualized therapy that will hopefully improve survival and quality of life in patients with non-MBC. Future technologies for CTC detection with enhanced sensitivity that also incorporate stem cell and EMT markers might further improve the value of CTCs as prognostic and predictive tool in early breast cancer.

Disclosure of Potential Conflicts of Interest

W.J. Janni reports receiving commercial research grants from AstraZeneca, Chugai, GlaxoSmithKline, Janssen Diagnostics, Novartis, Pfizer, Roche, and Sanofi-Aventis. B. Rack reports receiving commercial research grants from Janssen Diagnostics, Lilly, Novartis, and Sanofi Aventis; and is a consultant/advisory board member for Novartis. L.W.M.M. Terstappen reports receiving commercial research grants from Janssen Diagnostics. J.-Y. Pierga reports receiving commercial research grants from Janssen Diagnostics, and Roche. P.A. Fasching reports receiving commercial research grants from Amgen, Celgene, and Novartis; speakers bureau honoraria from Amgen, Genomic Health, GlaxoSmithKline, Nanostring, Novartis, Pfizer, Roche, and Teva; and is a

consultant/advisory board member for Celgene, Novartis, Pfizer, and Roche. A. Lucci reports receiving speakers bureau honoraria from Genomic Health, Inc. No potential conflicts of interest were disclosed by the other authors.

Disclaimer

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Authors' Contributions

Conception and design: W.J. Janni, B. Rack, L.W.M.M. Terstappen, K. Pantel, A. Lucci
Development of methodology: B. Rack, L.W.M.M. Terstappen
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W.J. Janni, B. Rack, L.W.M.M. Terstappen, J.-Y. Pierga, F.-A. Taran, T. Fehm, C. Hall, M.R. de Groot, F.-C. Bidard, P.A. Fasching, S.Y. Brucker, K. Pantel, A. Lucci
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W.J. Janni, B. Rack, T. Fehm, T.W.P. Friedl, K. Pantel, A. Lucci
Writing, review, and/or revision of the manuscript: W.J. Janni, B. Rack, L.W.M.M. Terstappen, J.-Y. Pierga, F.-A. Taran, T. Fehm, C. Hall, M.R. de Groot, F.-C. Bidard, T.W.P. Friedl, P.A. Fasching, S.Y. Brucker, K. Pantel, A. Lucci
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.Y. Brucker
Study supervision: W.J. Janni, B. Rack, A. Lucci

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References

1. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. Nat Rev Clin Oncol 2009;6:339–51.
2. Bidard F-C, Peeters DJ, Fehm T, Nolé F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014;15:406–14.
3. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
4. Munzone E, Botteri E, Sandri MT, Esposito A, Adamoli L, Zorzino L, et al. Prognostic value of circulating tumor cells according to immunohistochemically defined molecular subtypes in advanced breast cancer. Clin Breast Cancer 2012;12:340–6.
5. Pierga J-Y, Hajage D, Bachelot T, Delaloge S, Brain E, Campone M, et al. High independent prognostic and predictive value of circulating tumor cells compared with serum tumor markers in a large prospective trial in first-line chemotherapy for metastatic breast cancer patients. Ann Oncol 2012;23:618–24.
6. Wallwiener M, Hartkopf AD, Baccelli I, Riethdorf S, Schott S, Pantel K, et al. The prognostic impact of circulating tumor cells in subtypes of metastatic breast cancer. Breast Cancer Res Treat 2013;137:503–10.
7. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006;12:4218–24.
8. Liu MC, Shields PG, Warren RD, Cohen P, Wilkinson M, Ottaviano YL, et al. Circulating tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer. J Clin Oncol 2009;27:5153–9.
9. Nakamura S, Yagata H, Ohno S, Yamaguchi H, Iwata H, Tsunoda N, et al. Multi-center study evaluating circulating tumor cells as a surrogate for response to treatment and overall survival in metastatic breast cancer. Breast Cancer 2010;17:199–204.
10. Franken B, de Groot MR, Mastboom WJ, Vermes I, van der Palen J, Tibbe AG, et al. Circulating tumor cells, disease recurrence and survival in newly diagnosed breast cancer. Breast Cancer Res 2012;14:R133.
11. Ignatiadis M, Kallergi G, Ntoulia M, Perraki M, Apostolaki S, Kafousi M, et al. Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. Clin Cancer Res 2008;14:2593–600.
12. Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, et al. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012;13:688–95.
13. Pierga J-Y, Bidard F-C, Mathiot C, Brain E, Delaloge S, Giachetti S, et al. Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial. Clin Cancer Res 2008;14:7004–10.
14. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 2014;106: pii: dju066.
15. Serrano MJ, Rovira P, Martinez-Zubiaurre I, Rodriguez M, Fernandez M, Lorente JA. Dynamics of circulating tumor cells in early breast cancer under neoadjuvant therapy. Exp Ther Med 2012;4:43–8.
16. Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editor. AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer Berlin Heidelberg; 2010.
17. Black MM, Speer FD. Nuclear structure in cancer tissues. Surg Gynecol Obstet 1957;105:97–102.
18. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403–10.
19. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004;10:6897–904.
20. Riethdorf S, Fritsche H, Müller V, Rau T, Schindlbeck C, Rack B, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res 2007;13:920–8.
21. Hudis CA, Barlow WE, Costantino JP, Gray RJ, Pritchard KI, Chapman JAW, et al. Proposal for standardized definitions for efficacy end points in adjuvant breast cancer trials: The STEEP system. J Clin Oncol 2007;25:2127–32.
22. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. Control Clin Trials 1996;17:343–6.
23. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst 2005;97:1180–4.
24. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ. Strategies for subtypes-dealing with the diversity of breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 2011;22:1736–47.
25. Allan AL, Keeney M. Circulating tumor cell analysis: technical and statistical considerations for application to the clinic. J Oncol 2010;2010:426218.
26. Botteri E, Sandri MT, Bagnardi V, Munzone E, Zorzino L, Rotmensz N, et al. Modeling the relationship between circulating tumour cells number and prognosis of metastatic breast cancer. Breast Cancer Res Treat 2010;122:211–7.
27. Fehm T, Sauerbrei W. Information from CTC measurements for metastatic breast cancer prognosis-we should do more than selecting an "optimal cut point." Breast Cancer Res Treat 2010;122:219–20.
28. Tibbe AGJ, Miller MC, Terstappen LWMM. Statistical considerations for enumeration of circulating tumor cells. Cytom Part A 2007;71A:154–62.
29. Coumans FA, Ligthart ST, Uhr JW, Terstappen LWMM. Challenges in the enumeration and phenotyping of CTC. Clin Cancer Res 2012;18:5711–8.
30. Coumans FA, Ligthart ST, Terstappen LWMM. Interpretation of changes in circulating tumor cell counts. Transl Oncol 2012;5:486–91.

31. Barrière G, Riouallon A, Renaudie J, Tartary M, Rigaud M, Barriere G. Mesenchymal and stemness circulating tumor cells in early breast cancer diagnosis. BMC Cancer 2012;12:114.
32. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2002;2:442–54.
33. May CD, Sphyris N, Evans KW, Werden SJ, Guo W, Mani SA. Epithelial-mesenchymal transition and cancer stem cells: a dangerously dynamic duo in breast cancer progression. Breast Cancer Res 2011;13:202.
34. Gorges TM, Tinhofer I, Drosch M, Röse L, Zollner TM, Krahn T, et al. Circulating tumour cells escape from EpCAM-based detection due to epithelial-to-mesenchymal transition. BMC Cancer 2012;12:178.
35. Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. Breast Cancer Res 2012;14:R15.
36. Lustberg MB, Balasubramanian P, Miller B, Garcia-Villa A, Deighan C, Wu Y, et al. Heterogeneous atypical cell populations are present in blood of metastatic breast cancer patients. Breast Cancer Res 2014;16:R23.
37. Blick T, Widodo E, Hugo H, Waltham M, Lenburg ME, Neve RM, et al. Epithelial mesenchymal transition traits in human breast cancer cell lines. Clin Exp Metastasis 2008;25:629–42.
38. Peeters DJE, van Dam PJ, Van den Eynden GGM, Rutten A, Wuyts H, Pouillon L, et al. Detection and prognostic significance of circulating tumour cells in patients with metastatic breast cancer according to immunohistochemical subtypes. Br J Cancer 2014;110:375–83.
39. Parkinson DR, Dracopoli N, Petty BG, Compton C, Cristofanilli M, Deisseroth A, et al. Considerations in the development of circulating tumor cell technology for clinical use. J Transl Med 2012;10:138.
40. Fehm T, Muller V, Aktas B, Janni W, Schneeweiss A, Stickeler E, et al. HER2 status of circulating tumor cells in patients with metastatic breast cancer: a prospective, multicenter trial. Breast Cancer Res Treat 2010; 124:403–12.
41. Nadal R, Fernandez A, Sanchez-Rovira P, Salido M, Rodriguez M, Garcia-Puche JL, et al. Biomarkers characterization of circulating tumour cells in breast cancer patients. Breast Cancer Res 2012;14:R71.
42. Pestrin M, Bessi S, Galardi F, Truglia M, Biggeri A, Biagioni C, et al. Correlation of HER2 status between primary tumors and corresponding circulating tumor cells in advanced breast cancer patients. Breast Cancer Res Treat 2009;118:523–30.
43. Somlo G, Lau SK, Frankel P, Hsieh HB, Liu X, Yang L, et al. Multiple biomarker expression on circulating tumor cells in comparison to tumor tissues from primary and metastatic sites in patients with locally advanced/inflammatory, and stage IV breast cancer, using a novel detection technology. Breast Cancer Res Treat 2011;128:155–63.
44. Meng S, Tripathy D, Frenkel EP, Shete S, Naftalis EZ, Huth JF, et al. Circulating tumor cells in patients with breast cancer dormancy. Clin Cancer Res 2004;10:8152–62.
45. Alix-Panabières C, Pantel K. Circulating tumor cells: liquid biopsy of cancer. Clin Chem 2013;59:110–8.
46. Alix-Panabières C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer 2014;14:623–31.
47. Smerage JB, Barlow WE, Hortobagyi GN, Winer EP, Leyland-Jones B, Srkalovic G, et al. Circulating tumor cells and response to chemotherapy in metastatic breast cancer: SWOG S0500. J Clin Oncol 2014;32: 3483–9.
48. Bidard F-CC, Fehm T, Ignatiadis M, Smerage JB, Alix-Panabieres C, Janni W, et al. Clinical application of circulating tumor cells in breast cancer: overview of the current interventional trials. Cancer Metastasis Rev 2013;32:179–88.
49. Georgoulias V, Bozionelou V, Agelaki S, Perraki M, Apostolaki S, Kallergi G, et al. Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CK-19mRNA-positive circulating tumor cells: results of a randomized phase II study. Ann Oncol 2012;23:1744–50.