Clinical Cancer Research

Prognostic Impact of Circulating Tumor Cells for Breast Cancer Patients Treated in the Neoadjuvant "Geparquattro" Trial

Sabine Riethdorf[1], Volkmar Müller[2], Sibylle Loibl[3], Valentina Nekljudova[3], Karsten Weber[3], Jens Huober[4], Tanja Fehm[5], Iris Schrader[6], Jörn Hilfrich[7], Frank Holms[8], Hans Tesch[9], Christian Schem[10], Gunter von Minckwitz[3], Michael Untch[11], and Klaus Pantel[1]

Abstract

Purpose: This study aimed to evaluate the prognostic impact of circulating tumor cells (CTC) detected in patients with operable or locally advanced breast cancer before and after neoadjuvant therapy (NT) within the clinical trial GeparQuattro.

Experimental Design: Data on CTCs enumerated with the CellSearch system were available for 213 and 207 patients before and after NT, respectively. Associations of CTCs with disease-free survival (DFS) and overall survival (OS) were analyzed by nonparametric Kaplan–Meier estimates and parametric Cox regression.

Results: After a median follow-up of 67.1 months, the detection of $\geq$1 CTC/7.5 mL and $\geq$2 CTCs/7.5 mL before NT was associated with reduced DFS ($P = 0.031$ and $P < 0.0001$, respectively) and OS ($P = 0.0057$ and $P < 0.0001$, respectively), whereas CTCs detected after NT did not correlate with DFS or OS. In parametric univariate and multivariate Cox models, $\geq$1 CTC/7.5 mL, $\geq$2 CTCs/7.5 mL, and absolute CTC numbers before NT revealed to be independent prognostic parameters of DFS and OS. CTC-negative patients with pathologic complete response (pCR) exhibited the best prognosis, whereas those with CTCs and less tumor response were at high risk of tumor relapse. In HER2 (ERBB2)-positive and triple-negative patients, $\geq$2 CTCs/7.5 mL detected before NT also were significantly associated with worse DFS and OS.

Conclusions: Detection of CTCs before NT is an independent prognostic factor of impaired clinical outcome, and combined with pCR, it could be helpful to stratify breast cancer patients for therapeutic interventions. *Clin Cancer Res; 23(18); 5384–93. ©2017 AACR.*

Introduction

Therapeutic efficacy of neoadjuvant chemotherapy or targeted treatment for patients with breast cancer can be assessed rapidly without long follow-up periods (1, 2). The assumed better long-term benefit for patients that achieve a pathologic complete response (pCR) has led to the approval of new therapeutic approaches especially for this setting (1). However, the correlation between pCR and long-term outcome was not robust in all studies, and formal surrogacy of pCR has not been unambiguously demonstrated yet (3). Thus, a meta-analysis involving data from 6,377 primary breast cancer patients treated with neoadjuvant therapy (NT) in seven randomized trials revealed that pCR is a suitable surrogate end point marker for patients with luminal B/HER2 (ERBB2)-negative, HER2-positive (nonluminal), and triple-negative disease but to a lower extend for those with luminal B/HER2-positive or luminal A tumors (4). Therefore, the availability of additional factors allowing the assessment of prognosis is of high clinical relevance.

Over the last years, the German Breast Group has conducted numerous successful clinical NT trials (5, 6) among them the phase III Geparquattro study that incorporated epirubicin/cyclophosphamide prior to randomization to either docetaxel alone, docetaxel in combination with capecitabine or docetaxel followed by capecitabine and additionally trastuzumab for patients with HER2-positive tumors (6). Several accompanying translational research projects to this study that examined biomarkers prognostic for therapy response were already performed (7–13).

In this context, also the detection of circulating tumor cells (CTCs) as liquid biopsy has gained importance, and we previously could demonstrate that both the CTC incidence and the number of CTCs per patient were significantly reduced during neoadjuvant interventions within the GeparQuattro study (14). However, we did not find any correlations between CTCs, standard clinicopathologic parameters, and primary tumor response

[1]Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. [2]Department of Gynecology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. [3]German Breast Group, Neu Isenburg, Germany. [4]Department of Gynecology and Obstetrics, University Medical Center, Ulm, Germany. [5]Department of Obstetrics and Gynecology, University Medical Center, Düsseldorf, Germany. [6]Gynecologic/Oncological Office, Hannover, Germany. [7]Eilenriede-Hospital, Hannover, Germany. [8]Department of Obstetrics and Gynecology, St. Barbara-Klinik, Hamm-Heessen, Germany. [9]Oncology Bethanien, Frankfurt, Germany. [10]Department of Gynecology and Obstetrics, University Medical Center, Kiel, Germany. [11]Department of Obstetrics and Gynecology, Heliosklinik Buch, Berlin, Germany.

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S. Riethdorf and V. Müller contributed equally to this article.

Corresponding Author: Sabine Riethdorf, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. Phone: 0049-40-7410-51891; Fax: 0049-40-7410-56546; E-mail: sriethdorf@uke.de

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Translational Relevance

Neoadjuvant systemic therapy has become an important approach for evaluation of new therapeutic options in patients with breast cancer since a correlation of tumor response and long-term outcome was observed. In this study, the detection of circulating tumor cells (CTCs) in the peripheral blood before neoadjuvant therapy was not associated with the primary tumor response but significantly correlated to an unfavorable outcome. Thus, enumeration of CTCs delivers independent prognostic information on the success or failure of neoadjuvant systemic therapy in breast cancer. This could be of relevance for the selection of patients for postneoadjuvant trials that should help to optimize the outcome.

(14). A lack of association between CTCs detected by the CellSearch system and pCR was also described by Onstenk and colleagues for HER2-negative patients recruited in the NEOZOTAC trial (15) and by Pierga and colleagues for the REGAMUS 02 trial (16). However, from these and several other studies that investigated the presence of CTCs by RT-PCR and reported a decline of CTC detection rates in the course of NT, it could be concluded that CTCs as biomarker can support therapy monitoring (17–20). In contrast, in a small study including 51 HER2-positive breast cancer patients within the NeoALTTO phase III trial, no significant decrease of the CTC incidence during NT could be observed (21). Interestingly, subsets of both EMT-like CTCs and/or stem cell–like CTCs seem to be resistant to neoadjuvant therapies (17, 22).

Prognostic relevance of CTCs for overall, disease-free, and progression-free survival (OS, DFS, PFS) has been defined during the last decade not only for large cohorts of breast cancer patients with metastatic disease (23–25), but also for patients with primary nonmetastatic breast cancer. Hence, in the thus far largest prospective trial on a cohort of 2,026 primary breast cancer patients, the independent prognostic relevance of CTCs detected both before and after adjuvant chemotherapy for DFS and OS could be verified (26). Conflicting results, however, were published regarding CTC detection before and after NT and its prognostic value for OS and relapse-free survival of breast cancer patients. Although Bidard and colleagues described prognostic relevance of CTCs detected prior to NT for distant metastasis-free survival and OS mainly during the first 3 to 4 years of follow-up in a cohort of 115 patients (27, 28), only the persistent detection of CTCs before and after NT identified a subpopulation of patients with increased risk of recurrence in a small study on breast cancer patients ($n = 24$; ref. 29). The presence of CTCs after NT correlated with reduced relapse-free survival and OS in a study enrolling 57 triple-negative breast cancer (TNBC) patients (30). In contrast, no significant correlations were found for CTCs detected by RT-PCR before and after therapy to PFS and OS in a recent study enrolling 115 breast cancer patients (17).

In our previous thus far largest study in the context of NT, CTCs were prospectively enumerated in a cohort of 287 patients with analyses before therapy in 213 patients and after therapy in 207 patients (14). Now, long-term follow-up data of these patients over 9 years with a median follow-up time of 67.1 months are available. Hence, in the present study, we evaluated the prognostic value of CTCs detected before and after NT. In addition to the analysis of the whole patient cohort, subgroup analyses for patients with luminal, HER2-positive, and TNBC were performed.

Materials and Methods

GeparQuattro study

For the Geparquattro study, patients with either large operable or locally advanced tumors, tumors with negative hormone receptor status, or receptor-positive tumors but clinically node-positive disease were recruited to receive preoperatively 4 cycles of epirubicin/cyclophosphamide (EC; 90 mg/m^2/600 mg/m^2) and to be then randomized to either 4 cycles of docetaxel (T; 100 mg/m^2) or 4 cycles of T + capecitabine (X; 75 mg/m^2/1,800 mg/m^2; TX) or 4 cycles of T (75 mg/m^2) followed by 4 cycles of X (1,800 mg/m^2; T→X). Patients with HER2-positive tumors received trastuzumab (6 mg/kg i.v. every 3 weeks) concomitantly to cytotoxic treatment, starting with a loading dose of 8 mg/kg i.v. on day 1 of the first EC cycle. Primary objectives were to assess the effect of X by comparing EC→T versus EC→TX + EC→T→X and to assess the effect of duration (24 vs. 36 weeks) by comparing EC→T + EC→TX versus EC→T→X (6). The study was performed as joined trial of the GBG and AGO-B study groups (http://www.gbg.de/de/studien/geparquattro.php). HER2 positivity of the tumors was defined as 3+ (strong) scored by immunohistochemistry or as positive for *HER2* gene amplification as assessed by FISH. For immunohistochemistry, the standardized HercepTest by Dako-Cytomation was mandatory, and all immunohistochemistry 2+ cases had to be centrally analyzed by FISH in one of five German reference centers.

As previously reported, patients for the CTC substudy within the GeparQuattro trial were recruited from 14 participating centers (14). At the time of present follow-up data analysis, median follow-up of the patients was 67.1 months. Sixty-eight of 287 patients had relapsed, and 40 of 287 patients had died.

For the here presented analysis, pCR was defined as the absence of invasive tumor in the breast and axillary lymph nodes.

Ethical considerations

All patients gave informed consent to provide a prespecified amount of extra blood before entering the Geparquattro study with the informed consent form. Participation on the clinical trial was still possible if a patient did not agree to provide extra blood samples. Patients were not informed about the laboratory results due to their experimental character. The clinical treatment study as well as the translational research project described here was approved by the central ethics committee at the University of Frankfurt as well as in all ethics committees of the participating centers.

Enumeration of CTCs

Peripheral blood samples of 7.5 mL each were collected into CellSave tubes (Janssen) (1) prior to treatment and (2) after NT, before surgery. Samples were shipped to the Department of Tumor Biology and processed with a maximum interval of 96 hours after the blood draw as necessitated by the vendor for CTC detection. Sample processing and enumeration of CTC counts were performed at the Department of Tumor Biology, University Medical Center Hamburg-Eppendorf.

For the detection of CTCs, the Cell Search Epithelial Cell Test (Janssen) was applied. Here, CTCs were enriched from

peripheral blood by anti–EpCAM-antibody-coated ferrofluid and subsequently visually identified by keratin-positivity, negativity for the leukocyte common antigen CD45, and DAPI staining to ensure integrity of the nucleus.

In our previous analysis that formed the basis for the present study, we detected ≥1 CTC/7.5 mL (≥1 CTC) in 46 of 213 patients (21.6%) before NT. Applying the cut-off of ≥2 CTCs/7.5 mL (≥2 CTCs), 19 of 213 patients (8.9%) revealed to be CTC-positive before NT. We could not find any significant association of the presence of CTCs to pCR (14). Detection of CTCs associated with patient and tumor characteristics for both time points is listed in Supplementary Table S1. There was no significant correlation of ≥1 CTC or ≥2 CTCs with any of these parameters.

Statistical analysis

Numbers of CTCs were categorized with two cut-off values: The variable ≥1 CTC is binary with the two levels ≥1 CTC/7.5 mL and 0 CTC/7.5 mL; variable ≥2 CTCs uses the cut-off value 2 and has the levels ≥2 CTCs/7.5 mL and <2 CTCs/7.5 mL. The

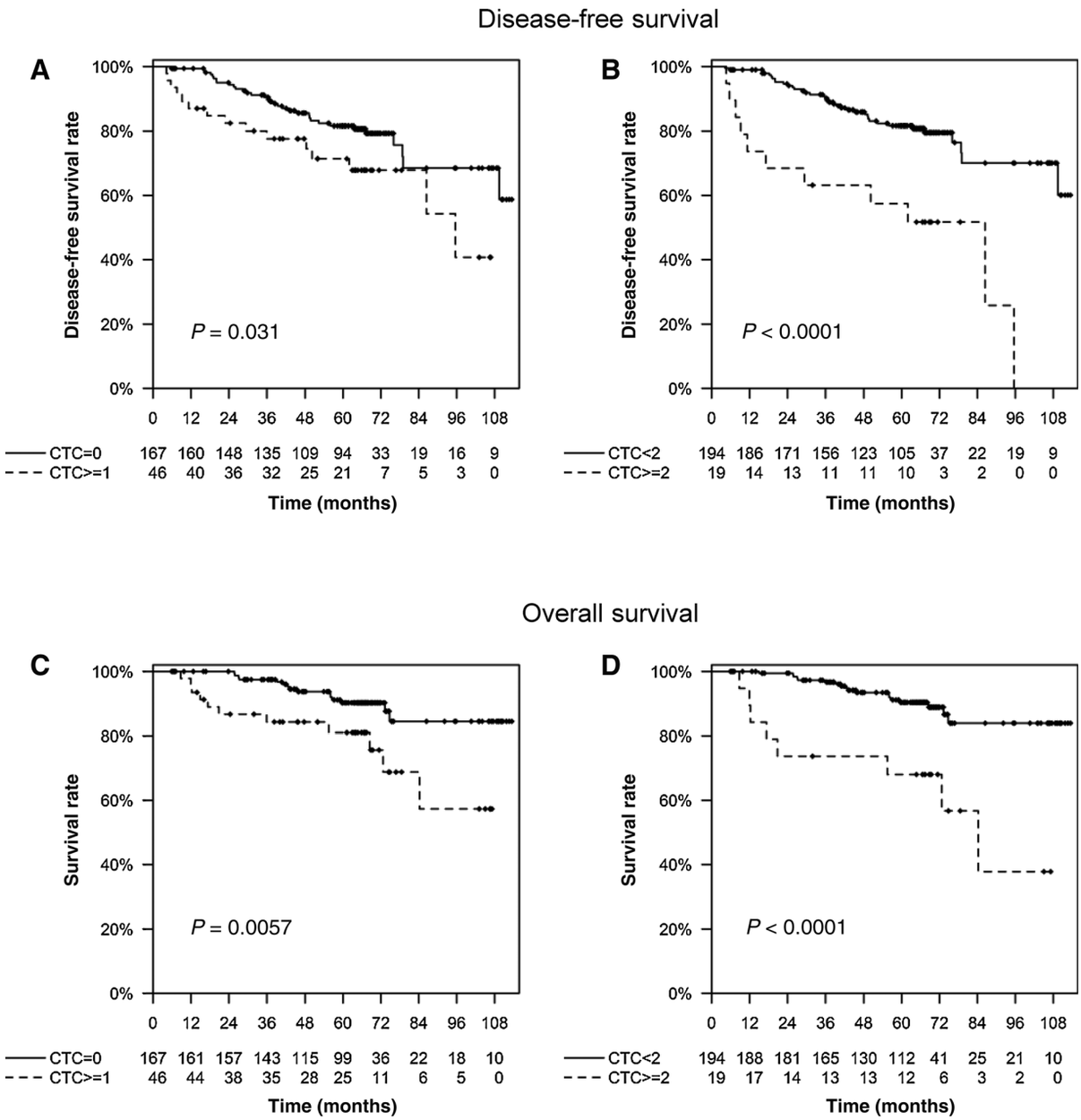

Figure 1.

Kaplan–Meier plots for DFS and OS according to baseline CTC detection. **A** and **B,** DFS, analysis of the whole patient cohort at CTC cut-offs ≥1 (**A**) and ≥2 (**B**) CTCs/7.5 mL blood. **C** and **D,** OS, analysis of the whole patient cohort at CTC cut-offs ≥1 (**C**) and ≥2 (**D**) CTCs/7.5 mL blood.

Table 1. HRs and CIs for DFS using the two CTC variables and absolute CTC numbers before therapy (univariate and multivariate analyses in different patient subgroups)

		Univariate		Multivariate	
Patients	**CTCs/7.5 mL**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
All patients	0 vs. ≥1	1.941 (1.051–3.585)	***0.034***	2.204 (1.182–4.109)	***0.013***
	<2 vs. ≥2	3.723 (1.893–7.323)	<***0.001***	4.087 (2.046–8.162)	<***0.001***
	Absolute number	1.018 (1.008–1.029)	***0.001***	1.018 (1.007–1.029)	***0.001***
Luminal	0 vs. ≥1	1.661 (0.582–4.740)	0.343	2.202 (0.724–6.696)	0.164
	<2 vs. ≥2	2.686 (0.872–8.269)	0.085	5.395 (1.510–19.274)	***0.009***
	Absolute number	1.038 (0.900–1.196)	0.609	1.110 (0.949–1.298)	0.191
HER2-positive	0 vs. ≥1	1.780 (0.566–5.601)	0.324	1.596 (0.496–5.133)	0.433
	<2 vs. ≥2	4.996 (1.390–17.964)	***0.014***	5.877 (1.148–30.100)	***0.034***
	Absolute number	1.259 (1.024–1.548)	***0.029***	1.197 (0.956–1.499)	0.117
Triple-negative	0 vs. ≥1	1.993 (0.709–5.605)	0.191	3.342 (1.033–10.817)	***0.044***
	<2 vs. ≥2	4.707 (1.481–14.958)	***0.009***	7.227 (1.754–29.778)	***0.006***
	Absolute number	1.089 (0.877–1.352)	0.441	1.178 (0.932–1.489)	0.170

collective comprises 287 patients. In addition to the analysis of the whole patient cohort, subgroup analyses for patients with luminal (ER- and/or PR-positive, HER2-negative), HER2-positive, and TNBC were performed. As endpoints DFS and OS were analyzed. DFS was defined as locoregional recurrence, distant recurrence, or death by any cause. OS was defined as death by any cause. Nonparametric Kaplan–Meier estimates of the survival function and parametric Cox models were used to compare CTC-positive and CTC-negative samples. In addition to the univariate analyses, the two CTC variables ≥1 CTC and ≥2 CTCs were separately used in multivariate Cox models with the following clinical parameters: age (≥50 years vs. <50 years), clinical tumor stage (T1–3 vs. T4), lymph node status (lymph node metastasis positive vs. lymph node metastasis negative), and grade of differentiation (G1–2 vs. G3). Analyses of this report were conducted using the statistical programming language R version 3.2.2 (R Foundation for Statistical Computing). For reporting of results, the REMARK criteria (31) were followed. Our primary endpoint was the impact of CTC detection on DFS and OS in the entire patient cohort. Secondary endpoints were subgroup analyses stratified on ER and HER2 status as well as the role of dynamics of CTC before/after therapy and correlation with pCR.

Results

CTC detection and DFS

The detection of both ≥1 CTC ($n = 46$) and ≥2 CTCs ($n = 19$) in blood samples taken before chemotherapy from 213 patients was significantly associated with worse DFS ($P = 0.031$ and $P < 0.0001$, respectively) in univariate nonparametric Kaplan–Meier analysis (Fig. 1A and B). Table 1 provides the results of a parametric Cox model for the two CTC variables and absolute CTC counts/7.5 mL. In addition to the univariate analysis, the CTC variables were separately used in a multivariate Cox model with the following clinical parameters: age, tumor size, nodal status, and grading. In the total cohort of patients, ≥1 CTC, ≥2 CTCs, and the absolute CTC number were strong independent parameters for a reduced DFS ($P = 0.013$, $P < 0.001$, and $P = 0.001$).

CTCs with both used cut-off values and absolute CTC counts detected after completion of chemotherapy were not significantly associated with DFS in the total patient cohort (≥1 CTC, $P = 0.43$; ≥2 CTCs, $P = 0.69$; Supplementary Fig. S1A and S1B).

CTC detection and OS

Among the 213 patients enrolled in CTC measurements before chemotherapy, positivity for ≥1 CTC as well as for ≥2 CTCs was significantly associated with worse OS ($P = 0.0057$ and $P < 0.0001$; Fig. 1C and D). For the total cohort, HRs and confidence intervals (CI) presented in Table 2 demonstrate that both CTC variables (≥1 CTC and ≥2 CTCs) and absolute numbers of CTCs were independent prognostic factors for OS ($P = 0.008$, $P = 0.001$, and $P < 0.001$, respectively).

In contrast, applying parametric univariate and multivariate Cox models for the total cohort of patients, CTC detection at both chosen cut-offs after NT (Supplementary Table S1) was not significantly correlated to OS (Supplementary Fig. S1C and S1D).

Table 2. HRs and CIs for OS using the two CTC variables and absolute CTC numbers before therapy (univariate and multivariate analyses in different patient subgroups)

		Univariate		Multivariate	
Patients	**CTCs/7.5 mL**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
All patients	0 vs. ≥1	2.855 (1.310–6.220)	***0.008***	2.904 (1.321–6.387)	***0.008***
	<2 vs. ≥2	4.544 (1.968–10.491)	<***0.001***	4.109 (1.738–9.716)	***0.001***
	Absolute number	1.019 (1.008–1.030)	<***0.001***	1.021 (1.010–1.033)	<***0.001***
Luminal	0 vs. ≥1	1.338 (0.138–12.933)	0.801	6.559 (0.372–115.560)	0.199
	<2 vs. ≥2	2.919 (0.300–28.406)	0.356	48.007 (0.868–2654.784)	0.059
	Absolute number	1.038 (0.766–1.406)	0.811	1.075 (0.822–1.407)	0.597
HER2-positive	0 vs. ≥1	3.021 (0.850–10.740)	0.088	2.145 (0.544–8.462)	0.276
	<2 vs. ≥2	4.627 (1.153–18.563)	***0.031***	3.498 (0.520–23.533)	0.198
	Absolute number	1.094 (0.902–1.326)	0.361	1.047 (0.931–1.177)	0.447
Triple-negative	0 vs. ≥1	2.819 (0.908–8.757)	0.073	6.611 (1.608–27.178)	***0.009***
	<2 vs. ≥2	5.188 (1.548–17.381)	***0.008***	7.397 (1.916–28.554)	***0.004***
	Absolute number	1.107 (0.894–1.370)	0.352	1.206 (0.953–1.525)	0.118

Association of combined results for pCR and CTC detection with DFS and OS

Bivariate Cox regression models were applied for a survival analysis by CTC detection (≥1 CTC and ≥2 CTCs) at baseline and pCR (yes or no). Significant contribution of pCR and CTC when using both CTC cut-offs before NT to DFS could be demonstrated (Table 3; Fig. 2A and B). Patients with pCR, but without CTCs, exhibited the best prognosis (5-year DFS of 96.0%, 95%-CI 88.6%-100.0%, while those with CTCs and less tumor response were at high risk of tumor recurrence or metastasis (≥1 CTC: 5-year DFS of 68.6%, 95% CI, 53.7%–87.5%; ≥2 CTCs: 5-year DFS of 59.3%, 95% CI 38.7%–90.7%). CTCs detected after NT did not have any significant additional impact on DFS (Table 3; Supplementary Fig. S2A and S2B).

Applying this analysis for OS, we did not observe a significant influence of pCR. However, CTCs detected at baseline (≥1 CTC and ≥2 CTCs) contributed significantly to OS (Table 3; Fig. 2C and D). No significant additional prognostic impact of CTCs detected after NT to pCR for OS was apparent (Table 3; Supplementary Fig. S2C and S2D).

Detection of CTCs before NT and survival for subgroups of patients

In addition to the analysis of the whole patient cohort, subgroup analyses were performed. Considering patients with luminal tumors analyzed before NT separately ($n = 102$), there was no statistically significant difference in DFS for patients with ≥1 CTC ($n = 20$, $P = 0.34$) or ≥2 CTCs ($n = 9$, $P = 0.073$) compared with patients with 0 or 0 to 1 CTC, respectively. Results of a parametric Cox model for patients with luminal tumors, however, revealed a significant prognostic impact of ≥2 CTCs, but not of ≥1 CTC detected before NT on DFS in multivariate analysis ($P = 0.009$; Table 1). No impact of CTC detection before NT on OS could be observed for patients with luminal tumors (Table 2).

Applying the cut-off ≥2 CTCs detected before NT for the subgroup of patients with HER2-positive tumors ($n = 59$), the presence of CTCs was significantly correlated with reduced DFS ($P = 0.0062$, Fig. 3A), whereas no impact of ≥1 CTC on DFS ($n = 11$) was observed ($P = 0.32$). For this patient subgroup, the significant prognostic impact of ≥2 CTCs but not of ≥1 CTC detected before NT on DFS holds true in univariate and multivariate Cox regression analysis ($P = 0.014$ and $P = 0.034$, respectively, Table 1). The presence of ≥2 CTCs detected before NT had a significant influence on OS for patients with HER2-positive tumors ($P = 0.018$, Fig. 3B). For these patients, ≥2 CTCs were associated with OS in univariate ($P = 0.031$), but not in multivariate analysis ($P = 0.198$, Table 2).

Also for triple-negative patients ($n = 52$), detection of ≥2 CTCs, but not ≥1 CTC ($n = 15$), before NT was significantly associated with a shorter DFS compared with patients with <2 CTCs, respectively ($P = 0.0038$, Fig. 3C). In a parametric Cox model, both ≥1 CTC and ≥2 CTCs as cut-offs revealed to be independent prognostic factors of DFS ($P = 0.044$ and $P = 0.006$, Table 1). Detection of ≥2 CTCs, but not ≥1 CTC ($n = 15$), before NT was significantly associated with a shorter OS compared with patients with <2 CTCs, respectively ($P = 0.0029$, Fig. 3D). Both CTC variables were independently associated with OS using the parametric Cox model in multivariate analysis ($P = 0.009$ and $P = 0.004$ for ≥1 CTC and ≥2 CTCs, respectively, Table 2).

Table 3. Bivariate Cox regression models with pCR and CTCs for DFS and OS

Cox regression model	Variable	HR (95% CI)	*P*
	Before neoadjuvant therapy		
DFS	pCR	0.28 (0.09–0.90)	***0.0322***
	≥1 CTC	2.09 (1.13–3.87)	***0.0186***
DFS	pCR	0.30 (0.09–0.96)	***0.0431***
	≥2 CTCs	3.75 (1.90–7.37)	***0.0001***
OS	pCR	0.50 (0.15–1.68)	0.2633
	≥1 CTC	3.03 (1.38–6.63)	***0.0055***
OS	pCR	0.54 (0.16–1.81)	0.3214
	≥2 CTCs	4.67 (2.02–10.79)	***0.0003***
	After neoadjuvant therapy		
DFS	pCR	0.37 (0.13–1.03)	0.0572
	≥1 CTC	0.62 (0.22–1.73)	0.3655
DFS	pCR	0.38 (0.14–1.06)	0.0649
	≥2 CTCs	1.16 (0.42–3.21)	0.7816
OS	pCR	0.35 (0.08–1.46)	0.1489
	≥1 CTC	0.30 (0.04–2.22)	0.2398
OS	pCR	0.36 (0.08–1.50)	0.1590
	≥2 CTCs	0.56 (0.08–4.12)	0.5681

Detection of CTCs after NT and survival for subgroups of patients

CTCs with any used cut-off values and absolute CTC counts detected after completion of NT were not significantly associated with DFS by the Kaplan–Meier analysis in patients with luminal (≥1 CTC $P = 0.45$; ≥2 CTCs $P = 0.053$), HER2-positive (≥1 CTC $P = 0.13$; ≥2 CTCs $P = 0.23$; Supplementary Fig. S3A), and triple-negative tumors (≥1 CTC $P = 0.50$; ≥2 CTCs $P = 0.98$, Supplementary Fig. S3B). Only in patients with luminal tumors, ≥2 CTCs displayed statistical relevance for DFS in multivariate Cox analysis ($P = 0.041$; HR = 3.999, 95% CI, 1.055–15.163).

Applying nonparametric Kaplan–Meier (Supplementary Fig. S3C and S3D) and parametric univariate or multivariate Cox models in these subtypes, CTC detection at both chosen cut-offs after NT (Supplementary Table S1) was not significantly correlated to OS.

Changes of CTC counts during NT related to OS and DFS

Results for measurements of CTCs before and after NT are available for 133 patients (14). The dynamic of changes in absolute CTC counts/7.5 mL for all patients is illustrated in Supplementary Fig. S4. Both the total number of patients and the number of patients with two measurements in each subgroup in relation to clinical parameters are displayed in Supplementary Table S2. However, using univariate and multivariate Cox models, we did not observe that changes of CTC numbers in the total study cohort or in any of the different subgroups were related to DFS or OS (Supplementary Tables S3 and S4).

Discussion

The aim of our study was to explore whether CTC detection can provide additional prognostic impact to tumor response and help predicting clinical outcome of breast cancer patients treated in the GeparQuattro study. Overall, our results demonstrate a prognostic relevance of CTCs detected prior to NT for DFS and OS, which was independent from the primary tumor response and other established risk factors. In contrast, CTCs detected after NT before surgery did not have any prognostic influence on DFS and OS. Whether CTCs detected after NT represent different tumor cell

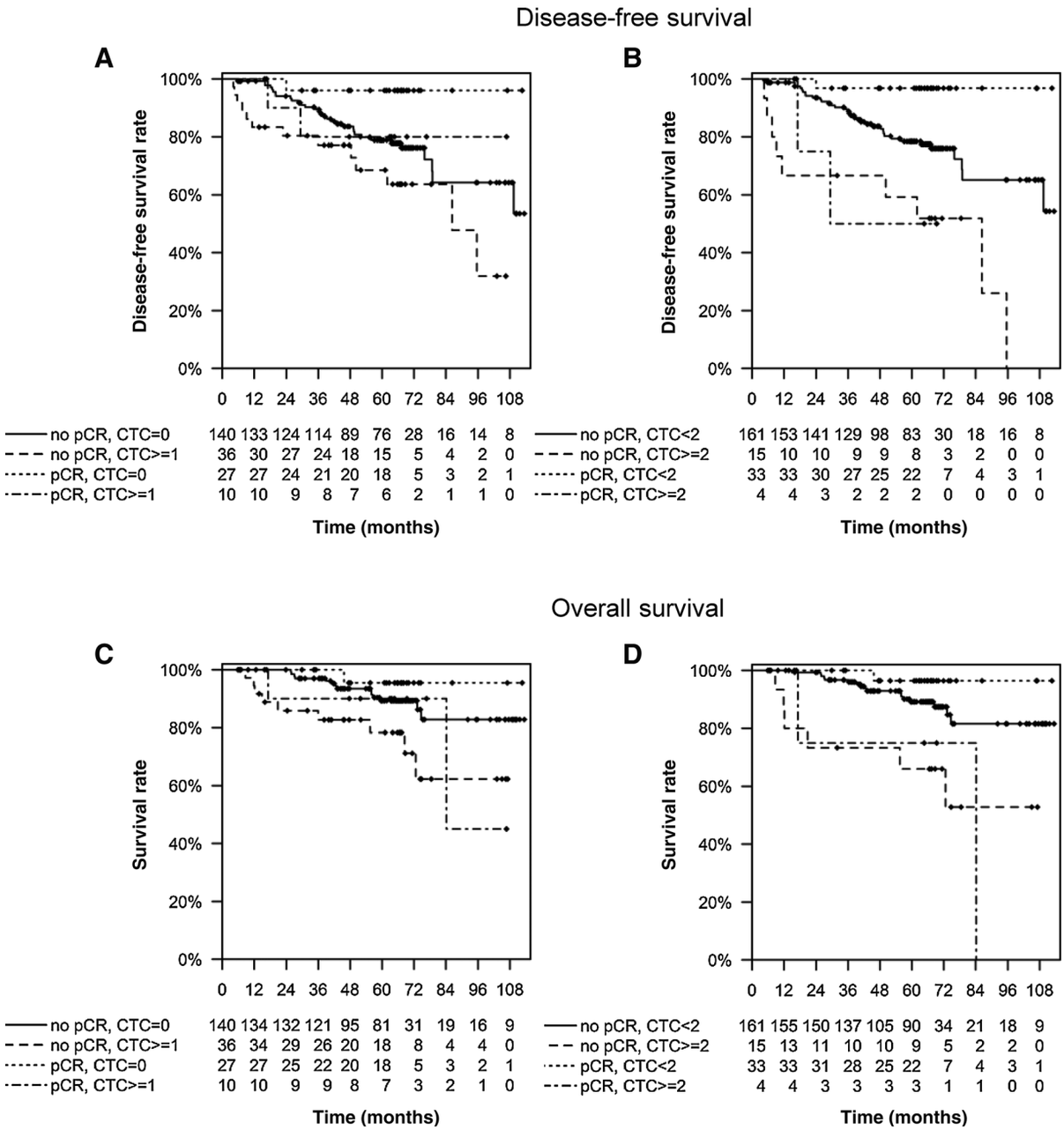

Figure 2.

Kaplan–Meier plots by pCR and CTCs detected at baseline. **A** and **B,** DFS, analysis of the whole patient cohort at cut-off $\geq$1 (**A**) CTC/7.5 mL and $\geq$2 (**B**) CTCs/7.5 mL blood. **C** and **D,** OS, analysis of the whole patient cohort at cut-off $\geq$1 (**C**) CTC/7.5 mL and $\geq$2 (**D**) CTCs/7.5 mL blood.

populations probably due to selection and/or mobilization by chemotherapy or bone marrow–stimulating agents (26, 32) or by surgical manipulation (33) has to be investigated in future trials.

Importantly, combining CTC detection and pCR results provides additional prognostic impact for DFS. Hence, in our study, CTC-negative patients who achieved pCR exhibited the best prognosis, whereas those with CTCs and less tumor response were at high risk of tumor recurrence or metastasis. In addition, patients with <2 CTCs but without achieving pCR turned out to be at intermediate risk for disease recurrence. Similar data were obtained by Pierga and colleagues in the BEVERLY-2 trial enrolling 52 patients with highly aggressive HER2-positive inflammatory breast cancer. Here, a 3-year follow-up identified CTC detection as additional prognostic marker to pCR for DFS (34).

In our study, we applied two different cut-off values ($\geq$1 CTC/7.5 mL blood and $\geq$2 CTCs/7.5 mL blood). The cut-off of $\geq$5 CTCs/7.5 mL blood has been proven yet for metastatic breast cancer patients in several large clinical trials to enable

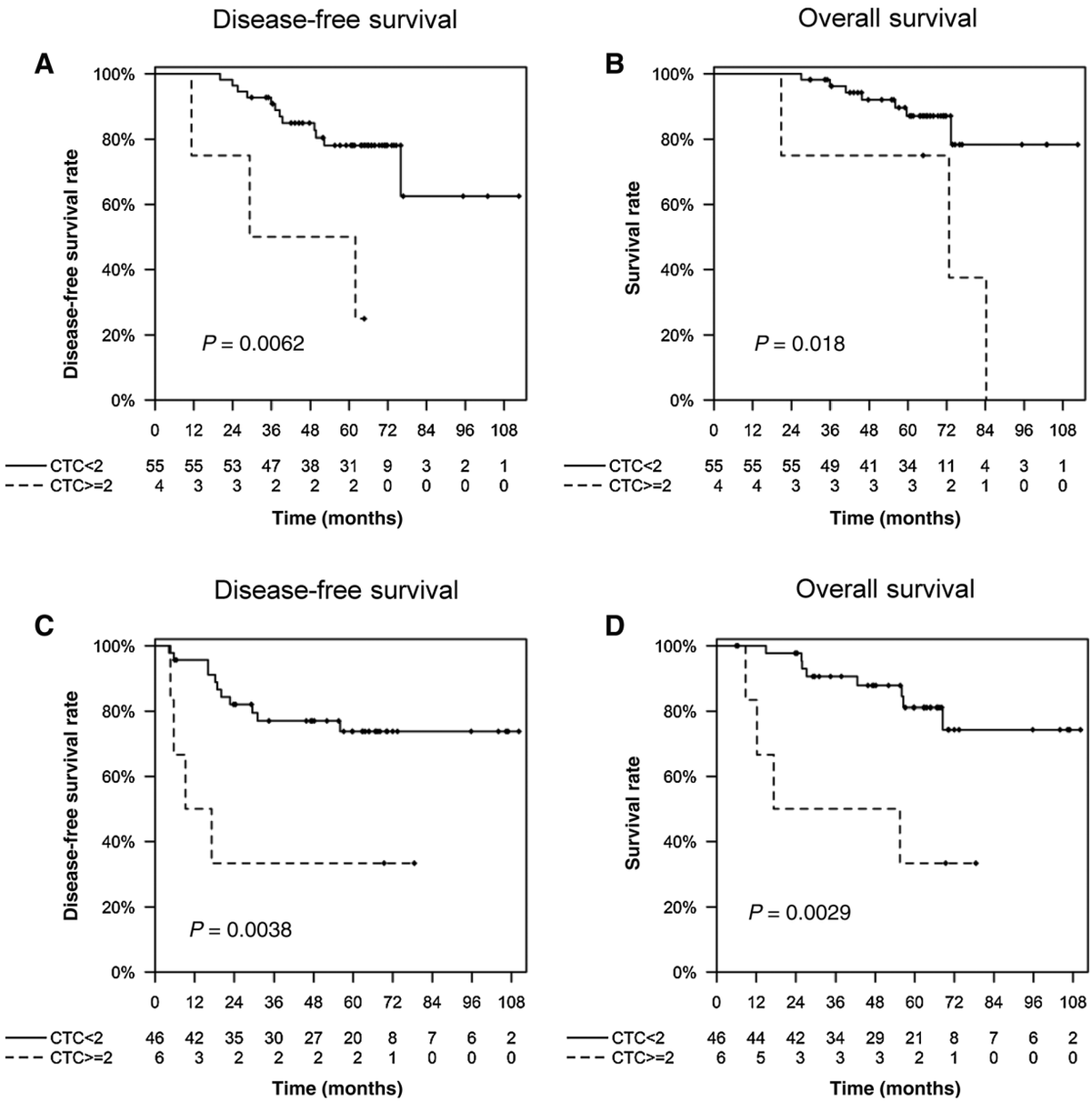

Figure 3.

Kaplan–Meier plots for DFS and OS according to baseline CTC detection at cut-off ≥2 CTCs/7.5 mL. **A,** DFS, analysis of patients with HER2-positive tumors. **B,** OS, analysis of patients with HER2-positive tumors. **C,** DFS, analysis of patients with triple-negative tumors. **D,** OS, analysis of patients with triple negative tumors.

discrimination between patients with favorable and those with unfavorable outcome (23, 35). Because of very low CTC numbers, however, its application for patients with nonmetastatic breast cancer is limited. Analyzing the thus far largest number of nonmetastatic breast cancer patients (n = 2,026), Rack and colleagues detected ≥1 CTC/30 mL blood in 435 of 2,026 (21.5%) breast cancer patients before taxane-based adjuvant chemotherapy significantly associated with poor DFS, OS, and breast cancer–specific survival. Increasing the cut-off, the HR also increased significantly (≥1 CTC/30 mL: 2.11 for DFS and 2.18 for OS to ≥5 CTCs/30 mL 4.51 for DFS and 3.60 for OS), demonstrating that the prognosis is getting worse with increasing CTC numbers (26). Also in our study, patient outcome as measured by DFS and OS was not only significantly associated with CTCs at arbitrary CTC cut-offs but also with the absolute CTC counts as evidenced by increased HRs applying the cut-off of ≥2 compared with ≥1 CTC/7.5 mL.

Generally, assessing the surrogate value of pCR in the GeparQuattro study was hindered by strong differences in tumor biology and patient management between HER2-positive and HER2-negative tumor diseases (36). For example, additionally

to neoadjuvant chemotherapy, in GeparQuattro trastuzumab was administered for patients with HER2-positive tumors. Based on these conditions, we separately analyzed the presence of CTCs in different prognostically relevant subgroups. A multivariate analysis including age, clinical tumor size, clinical nodal status, histological tumor type, tumor grade, hormone-receptor status, and HER2 status/trastuzumab treatment to adjust for variations in baseline characteristics showed that estrogen/progesterone receptor–positive patients presented with a significantly better DFS and OS independent on the different chemotherapy regimens (36). Although in our study Kaplan–Meier estimates for patients with luminal tumors did not reveal a significant association of CTCs detected before and after NT with DFS and OS, the detection of $\geq$2 CTCs/7.5 mL before NT appears to have independent prognostic value in multivariate analysis for DFS, but not for OS.

Although patients with trastuzumab-treated HER2-positive tumors showed similar DFS as HER2-negative patients, they presented with significantly better adjusted OS than HER2-negative patients (36). Moreover, 16.7% of HER2-positive patients without response to the first 4 chemotherapy cycles presented with pCR compared to only 3.3% of nonresponding HER2-negative patients (6). In our study, detection of $\geq$2 CTCs/7.5 mL defines HER2-positive patients with reduced DFS and OS, which holds true in multivariate analysis for DFS, suggesting that at least part of preoperatively detected CTCs is resistant against chemotherapy and/or trastuzumab as basis for later tumor relapse.

In GeparQuattro, patients with TNBC responded better to NT as non-TNBC patients; however, long-term prognosis of TNBC patients was worse compared with patients with other breast cancer subtypes (37). Overall, in our study, 21 of 97 (21.6%) and 10 of 97 (10.3%) TNBC samples were detected CTC-positive with $\geq$1 and $\geq$2 CTCs/7.5 mL blood, respectively (Supplementary Table S1). Positivity for CTCs before NT was independently associated with reduced DFS and OS in multivariate analysis. Possibly, these TNBC patients might profit from a more intense postoperative chemotherapy treatment, e.g., with additional carboplatin.

For CTC enumeration, we used the CellSearch system, the only standardized and FDA-approved approach currently available for CTC detection (35, 38–40). It is important to note that this system is only able to detect CTCs with Epithelial cell adhesion molecule (EpCAM) and keratin expression. Therefore, CTCs that have completely lost their epithelial features, e.g., in the course of epithelial–mesenchymal transition, will not be identified with the CellSearch system. Several new techniques have been developed to capture and detect also mesenchymal-like CTCs; however, comprehensive validation in large clinical trials is still needed (40). Thus, EPCAM-independent CTC detection methods could complement current application of CTCs within NT studies.

Besides CTCs, circulating tumor DNA (ctDNA) mainly released by apoptotic cells in primary or metastatic lesions has become a key component of "liquid biopsies" (40). Monitoring ctDNA is therefore currently investigated as additional blood-based biomarker to determine response to NT in breast cancer (41–43). A recent pilot study on 38 patients showed that measurable ctDNA levels after one cycle of NT were associated with reduced DFS and OS. However, detection of ctDNA was not associated with pCR at any time point during therapy (43). In another study, decreased levels of methylated ctDNA were correlated to clinical tumor response in a cohort of 87 patients treated with NT (44). Future studies can test whether CTC and ctDNA measurements might provide complementary or redundant information on response to NT (45).

In summary, detection of CTCs before NT is associated with an impaired DFS and OS of nonmetastatic breast cancer patients independent of tumor response. In combination with pCR, enumeration of CTCs might be helpful to stratify prognostic subgroups for specific treatment schedules. Future clinical trials can investigate if patients found to be CTC-positive prior to NT might benefit from more intense or novel forms of therapy (e.g., immune checkpoint inhibition). Besides enumeration, molecular characterization of CTCs might help to predict which therapy has the highest chance to improve the unfavorable outcome of patients with CTCs.

Disclosure of Potential Conflicts of Interest

J. Huober reports receiving commercial research grants from GlaxoSmithKline, speakers bureau honoraria from and is a consultant/advisory board member for Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Riethdorf, V. Müller, S. Loibl, J. Huober, J. Hilfrich, G. von Minckwitz, M. Untch, K. Pantel

Development of methodology: S. Riethdorf, M. Untch, K. Pantel

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Riethdorf, V. Müller, S. Loibl, J. Huober, T. Fehm, I. Schrader, J. Hilfrich, F. Holms, H. Tesch, C. Schem, G. von Minckwitz, M. Untch, K. Pantel

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Riethdorf, V. Müller, S. Loibl, K. Weber, T. Fehm, H. Tesch, C. Schem, G. von Minckwitz, M. Untch, K. Pantel

Writing, review, and/or revision of the manuscript: S. Riethdorf, V. Müller, S. Loibl, V. Nekljudova, K. Weber, J. Huober, T. Fehm, J. Hilfrich, C. Schem, G. von Minckwitz, M. Untch, K. Pantel

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Riethdorf, S. Loibl, V. Nekljudova, K. Weber, I. Schrader, M. Untch

Study supervision: S. Riethdorf, V. Müller, M. Untch, K. Pantel

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