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CLINICAL TRIAL

Sustained prognostic impact of circulating tumor cell status and kinetics upon further progression of metastatic breast cancer

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Abstract

Purpose Serial longitudinal enumeration of circulating tumor cells (CTCs) has shown its prognostic value on progression-free survival and overall survival (OS) in patients with stage IV breast cancer. This study prospectively evaluated the role of CTCs as a prognostic marker during further progression of metastatic breast cancer (MBC).

Methods Among 476 MBC patients recruited between 2010 and 2015, the 103 patients with a known CTC status at baseline (CTC_{BL}) and within 4 weeks of tumor progression (CTC_{PD}) were included. Progressive disease (PD) was defined according to the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1). Using the CellSearch method, <5 and ≥ 5 CTCs per 7.5 ml blood were determined as negative and positive, respectively. A shift in CTC status from baseline to progression ($CTC_{BL}{}^{+}$ to $CTC_{PD}{}^{-}$ and vice versa) was considered as alternating $Kinetics_{BL–PD}$.

Results Median follow-up was 29.9 [21.2, 40.0] months. CTC_{PD} positivity (37%, $n=38$) was associated with a significantly shorter OS than CTC_{PD} negativity (8.0 [5.1, 10.9] vs 22.6 [15.3, 39.8] months; $P<0.001$). Alternating $Kinetics_{BL–PD}$ was observed in 24% of the patients. This significantly changed the OS prediction of $CTC_{BL}{}^{+}$ patients ($CTC_{BL}{}^{+}CTC_{PD}{}^{-}$ vs $CTC_{BL}{}^{+}CTC_{PD}{}^{+}$, 11.4 [9.7, not available (NA)] vs. 7.6 [4.4, 11.5] months; $P=0.044$) and $CTC_{BL}{}^{-}$ patients ($CTC_{BL}{}^{-}CTC_{PD}{}^{+}$ vs. $CTC_{BL}{}^{-}CTC_{PD}{}^{-}$, 8.4 [4.0, NA] vs. 22.6 [18.9, NA] months, respectively; $P<0.001$).

Prediction of survival was significantly improved ($P=0.002$) by adding CTC_{PD} status to clinicopathological characteristics and CTC_{BL} status.

Conclusions CTC status upon further disease progression is a prognostic factor that could significantly improve well-established models. Thus, it represents a potential additional instrument supporting treatment decision.

Keywords CTCs · Circulating tumor cells · Metastatic breast cancer · Progressive disease · Survival · Prognostic marker

Abbreviations

MBC	Metastatic breast cancer
CTCs	Circulating tumor cells
PFS	Progression-free survival
OS	Overall survival
CEA	Carcinoembryonic antigen
CA15-3	Cancer antigen 15-3
iCTCs	Intact circulating tumor cells
aCTCs	Apoptotic circulating tumor cells
fCTCs	Enucleated fragments of circulating tumor cells
NCT	National Center for Tumor Diseases
REMARK	Reporting recommendations for tumor marker prognostic studies
RECIST	Response Evaluation Criteria in Solid Tumors
SD	Stable disease
PR	Partial response
CR	Complete response
PD	Progressive disease

Markus Wallwiener and Andreas Schneeweiss contributed equally to the study.

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BL	Baseline/study inclusion
CTC_{BL}	CTC status at baseline
CTC_{PD}	CTC status within 4 weeks of tumor progression
FDA	US Food and Drug Administration
anti-EpCAM	Anti-epithelial cellular adhesion molecule antibody
DAPI	4,2-Diamidino-2-phenylindole
$CTC_{BL}^{-}/CTC_{BL}^{+}$	Negative/positive CTC status at baseline
$CTC_{PD}^{-}/CTC_{PD}^{+}$	Negative/positive CTC status within 4 weeks of tumor progression
$iKinetics_{BL–PD}$	Course of intact CTCs from BL to PD
$aKinetics_{BL–PD}$	Course of apoptotic CTCs from BL to PD
$fKinetics_{BL–PD}$	Course of enucleated fragments of CTCs from BL to PD
$Kinetics_{BL\text{-}PD}^{-/+}$	CTC_{BL}^{-} to CTC_{PD}^{+}
$Kinetics_{BL\text{-}PD}^{+/-}$	CTC_{BL}^{+} to CTC_{PD}^{-}
$Kinetics_{BL\text{-}PD}^{+/+}$	CTC_{BL}^{+} to CTC_{PD}^{+}
$Kinetics_{BL\text{-}PD}^{-/-}$	CTC_{BL}^{-} to CTC_{PD}^{-}
r_s	Spearman correlation coefficient
CI	Confidence interval
BIC	Bayesian information criterion
HR	Hormone receptor or hazard ratio
HER2	Human epidermal growth factor receptor 2
ER	Estrogen receptor
PR	Progesterone receptor
TNBC	Triple-negative breast cancer
ctDNA	Circulating tumor DNA

Introduction

As a result of diagnostic and therapeutic achievements, the breast cancer death rate has declined by 39% in the last 2–3 decades [1, 2]. However, metastatic breast cancer (MBC) is still an incurable disease [3, 4] with a median survival of approximately 20 months [5–11] and a 5-year relative survival rate of only 27% [2]. Thereby, heterogeneity regarding treatment response and prognosis constitute important challenges [12]. Although it has been shown that tumor biology may alter during disease progression, primary tumor characteristics often guide treatment of stage IV breast cancer [13–16]. Hence, new, easily accessible prognostic and predictive markers are required to better stratify patients, simplify risk-directed treatment selection, reduce unnecessary treatment side effects, and improve the therapeutic efficacy and outcome of patients [17].

Since different metastatic sites within one patient could harbor distinct genetic aberrations, the blood seems to be a more representative reservoir of tumor cell diversity than biopsies of single metastases [18, 19]. Circulating tumor cells (CTCs), shed into the bloodstream by the primary tumor mass or metastatic lesions, are readily available via simple liquid biopsy. In addition to reflecting intratumor heterogeneity, CTCs are attributed to contain metastasis-initiating potential and cancer stem cell properties [20, 21]. Large multicenter studies have already demonstrated the prognostic capacity of CTCs on progression-free survival (PFS) and overall survival (OS) in disseminated breast cancer: CTC levels of at least 5 cells per 7.5 ml of blood were associated with significantly shorter OS and PFS [22–24]. Furthermore, CTCs seem to be superior to established markers such as carcinoembryonic antigen (CEA) and cancer antigen 15-3 (CA15-3). These established serum tumor markers are currently used to support treatment decision-making in MBC according to the valid 'ASCO Clinical Practice Guideline' [16, 20, 22].

In reaction to multiple stressors, CTCs undergo apoptosis and necrosis, leading to an individual equilibrium of intact CTCs (iCTCs), apoptotic CTCs (aCTCs), and enucleated fragments of CTCs (fCTCs). Regarding the prognostic effect of aCTCs, contradictory theories exist. Some studies suggest that high numbers of aCTCs are a negative prognostic factor due to insufficient therapy response, high tumor proliferation, and cell turnover [25, 26]. In contrast, Kallergi et al. [27] showed the relation of CTC viability and disease stage with lower aCTC detection rates in metastatic compared to early breast cancer patients. To date, there are no sufficient data investigating fCTCs.

Thus far, the role of CTC status has been evaluated periodically, corresponding to predefined timepoints, before and after introducing a new line of therapy [12, 22–24]. In this context, serial enumeration seems to be useful with regard to therapy monitoring [12, 22–24]. To our knowledge, this article represents a novel substantial analysis of the prognostic impact of CTCs upon further progression of MBC.

Patients and methods

Study design

This is a prospective, partially blinded, single-center study (S-295/2009) of the National Center for Tumor Diseases (NCT)/Department of Obstetrics and Gynecology, University Hospital Heidelberg, Germany [12, 15, 17, 21, 26, 28, 29]. It was conducted in agreement with the Declaration of Helsinki and the principles of good clinical practice. Approval of the Local Ethics Committee and written informed consent of patients were obtained before enrollment. Reporting recommendations for tumor marker prognostic studies (REMARK) were followed where applicable [30]. Inclusion criteria were distant metastases according to

the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1) [31] and start of a new line of systemic therapy. Patients and attending physicians as well as the scientists quantifying CTCs were blinded concerning CTC count or patient history and therapy regimen, respectively. Treatment response was categorized approximately every 3 months as stable disease (SD), partial response (PR), complete response (CR), and progressive disease (PD) in line with the RECIST guideline [31], whereby radiologists had no access to treatment plans. CTC_{BL} blood was collected at study inclusion/baseline (BL) before application of a new line of systemic therapy. CTC_{PD} samples drawn within 4 weeks of further progression of MBC and at least 6 weeks after study inclusion were considered.

CTC enumeration

As previously described [12, 15, 17, 26], CTCs were measured using the US Food and Drug Administration (FDA)-approved CellSearch system (Janssen Diagnostics, LLC, Raritan, NJ, USA) [23, 24, 32, 33]. Here, 7.5 ml of peripheral blood was drawn into a CellSave Preservative Tube and processed within 96 h at room temperature. The assay contained an anti-epithelial cellular adhesion molecule antibody (anti-EpCAM), conjugated to ferrofluid, and antibodies against epithelial keratins 8, 18, and 19 as well as against the leukocyte-specific antigen CD45, magnetically enriching CTCs against the background of other cellular components of the blood. For nuclear staining, 4,2-diamidino-2-phenylindole (DAPI) was applied. EpCAM and cytokeratin+, CD45− CTCs were further differentiated by nuclear and cell morphology: DAPI-positive intact nuclei were distinctive for morphologically iCTCs. DAPI-positive but disintegrated nuclei and spotted keratin staining were characteristic for aCTCs. In selected cases, this classification was proven by M30-staining (VLV bio, 1:100), tagging degraded keratin 18 typical for aCTCs. DAPI negativity distinguished acaryote fragments of CTCs also called enucleated CTCs.

CTCs were analyzed by two independent, trained observers, strictly following the manufacturer's instructions: CTC counts <5 or ≥ 5 CTCs per 7.5 ml blood were classified as negative or positive separately for each CTC subclass. In differing cases, a consensus was reached by reevaluation.

Course of CTC status: $Kinetics_{BL–PD}$

To further assess the prognostic effect of CTC_{PD} status, its development from study inclusion to further disease progression ($Kinetics_{BL–PD}$) was evaluated with i, a, or $fKinetics_{BL–PD}$, standing for the course of iCTCs, aCTCs, or fCTCs. An alternating $Kinetics_{BL–PD}$ was considered as a change in CTC status from baseline to PD: CTC_{BL}^{-} to CTC_{PD}^{+} ($Kinetics_{BL\text{-}PD}^{-/+}$) or CTC_{BL}^{+} to CTC_{PD}^{-} ($Kinetics_{BL\text{-}PD}^{+/-}$). Cases with CTC_{BL} and CTC_{PD} status being consistently both positive or negative were labeled stable $Kinetics_{BL–PD}$, double positive ($Kinetics_{BL\text{-}PD}^{+/+}$) or double negative ($Kinetics_{BL\text{-}PD}^{-/-}$).

Statistical analysis

Follow-up data of patients were gathered regularly, and the median follow-up time was calculated by the reverse Kaplan–Meier method immediately before data analysis in August 2015. This date also served as a cut-off for the follow-up completeness index [34, 35]. Demographic and clinicopathological characteristics were presented as the median (range) or number (percentage). For comparison of CTC_{PD}^{-} versus CTC_{PD}^{+} Wilcoxon and Fisher's exact test were used, as appropriate. Correlation of CTC count or status of different CTC subtypes was quantified by Spearman correlation coefficient (r_s) or φ-coefficient, respectively.

Since all patients had PD, OS was the primary endpoint of the analysis. OS was estimated using the Kaplan–Meier method, and groups were compared using the log-rank test. Patients without an event (death) were censored to the last registered date alive. OS and follow-up [median (95% confidence interval (CI)] were documented in months, starting from CTC_{PD} collection. Multivariate regression analysis with Cox proportional hazards models was performed to rate the prognostic impact of included factors following the methods suggested by Bidard et al. [22]. Concordance index, Bayesian information criterion (BIC), and the likelihood ratio test were used to compare different models.

Data analysis was conducted with R (The R Project for Statistical Computing, R version 3.3.2., 31.10.2016). The significance level for P values was set at <0.05, two-sided.

Results

Patients

From March 2010 to May 2015, 476 patients were eligible, 103 of whom exhibited a known $iCTC_{BL}$ and CTC_{PD} status (Fig. 1). Median follow-up was 29.9 [21.2, 40.0] months with a completeness index of 83.6%. Approximately two-thirds of the cohort had died by the end of follow-up. The majority (62%) was initially diagnosed with hormone receptor (HR)+ and human epidermal growth factor receptor 2 (HER2)− breast cancer, 13% with HER2+ breast cancer and 15% with triple-negative breast cancer (TNBC, Table 1). At baseline, 36% exhibited only nonosseous and 20% only osseous distant metastases; 44% exhibited both. Median ages at

Table 1 Patient characteristics and CTC prevalence

Clinicopathological characteristics	Patients			
	Total $iCTC_{PD}$ ($n = 103$)	$iCTC_{PD}^{+}$ ($n = 38$)	$iCTC_{PD}^{-}$ ($n = 65$)	
	Median (range)			*P*
Age				
Age at first diagnosis (years)	50 (28–80)	48.5 (28–80)	51 (30–73)	0.732
Age at study entry (years)	58 (31–81)	57 (31–81)	60 (41–77)	0.424
iCTC count				
$iCTC_{BL}$ count	1 (0–650)	14 (0–250)	0 (0–650)	
$iCTC_{PD}$ count	2 (0–890)	27 (5–890)	0 (0–4)	
Clinicopathological characteristics	Patients			
	Total $iCTC_{PD}$ ($n = 103$)	$iCTC_{PD}^{+}$ ($n = 38$)	$iCTC_{PD}^{-}$ ($n = 65$)	
	No. (%)			*P*
Primary tumor characteristics				
ER status				0.343
ER+	72 (70)	24 (63)	48 (74)	
ER−	26 (25)	12 (32)	14 (22)	
Missing data	5 (5)	2 (5)	3 (5)	
PR status				0.186
PR+	63 (61)	20 (53)	43 (66)	
PR−	34 (33)	16 (42)	18 (28)	
Missing data	6 (6)	2 (5)	4 (6)	
HER2 status				0.357
HER2+	13 (13)	3 (8)	10 (15)	
HER2−	79 (77)	32 (84)	47 (72)	
Missing data	11 (11)	3 (8)	8 (12)	
Molecular subtypes				0.457
HR+HER2−	64 (62)	25 (66)	39 (60)	
HER2+	13 (13)	3 (8)	10 (15)	
TNBC	15 (15)	7 (18)	8 (12)	
Missing data	11 (11)	3 (8)	8 (12)	
Distant metastases at study entry				0.310
Nonosseous	37 (36)	10 (26)	27 (42)	
Osseous	21 (20)	9 (24)	12 (18)	
Nonosseous and osseous	45 (44)	19 (50)	26 (40)	
Therapy lines PD before study entry				0.964
1	43 (42)	15 (39)	28 (43)	
2	22 (21)	8 (21)	14 (22)	
≥ 3	38 (37)	15 (39)	23 (35)	

$iCTC_{PD}^{+}$ and $iCTC_{PD}^{-}$ patients were compared using Wilcoxon or Fisher's exact test

Therapy lines PD therapy lines due to progressive disease, *ER* estrogen receptor, *PR* progesterone receptor, *HER2* human epidermal growth factor receptor 2, *HR* hormone receptor, *TNBC* triple-negative breast cancer

diagnosis and at study inclusion were 50 (28–80) and 58 (31–81) years, respectively.

$iCTC_{PD}$ status and survival

Upon further progression of MBC, 37% ($n = 38$) of the participants were $iCTC_{PD}^{+}$ while 63% ($n = 65$) were $iCTC_{PD}^{-}$ (Supplementary Table 1). Thereby, CTC_{PD} status was

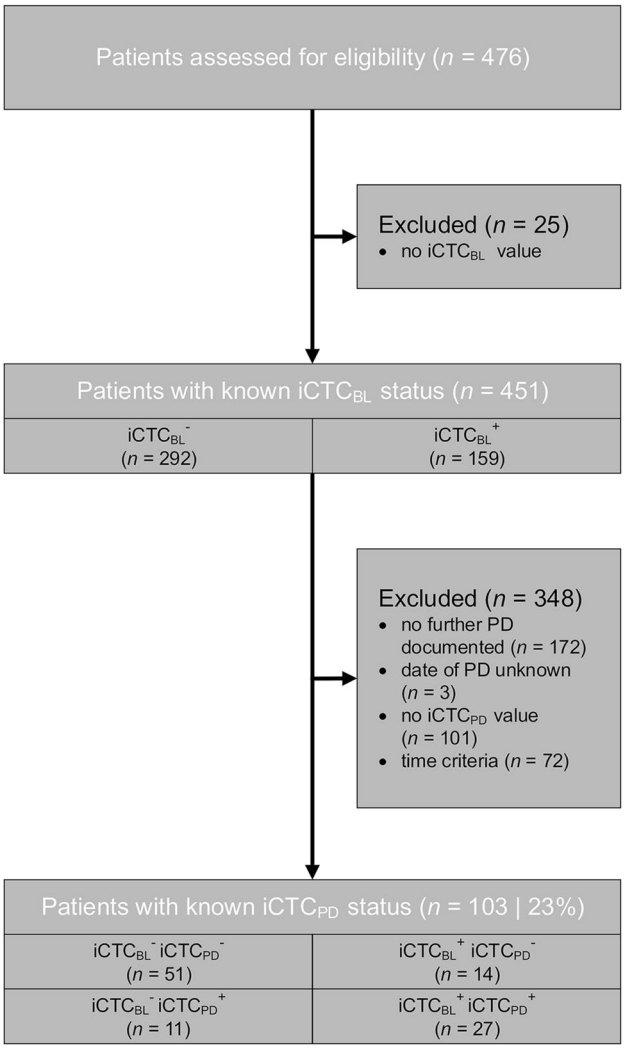

Fig. 1 Cohort derivation: $iCTC_{BL}$ and $iCTC_{PD}$ status were determined at study entry and within 4 weeks of MBC progression with a minimal interval of 6 weeks between the two blood collections (time criteria)

measured in 45% of $iCTC_{BL}^+$ and 33% of $iCTC_{BL}^-$ patients with PD. When comparing clinicopathological characteristics of $iCTC_{PD}^+$ versus $iCTC_{PD}^-$ patients, no significant differences were detected (Table 1). However, $iCTC_{PD}^+$ patients showed a significantly reduced OS in contrast to $iCTC_{PD}^-$ patients, with a median OS of 8.0 [5.1, 10.9] versus 22.6 [15.3, 39.8] months; $P < 0.001$ (Fig. 2a). The difference in median OS between those two subgroups was 14.6 months. Moreover, $iCTC_{PD}$ positivity was associated with a notably higher percentage of events (87%, $n = 33$) than $iCTC_{PD}$ negativity (49%, $n = 32$).

$iKinetics_{BL–PD}$ and survival

Evaluating the development of iCTC status from study inclusion to further tumor progression, the log-rank test revealed a significant difference in OS, depending on $iKinetics_{BL–PD}$ ($P < 0.001$; Fig. 2d). Of the total cohort, 76% ($n = 78$) showed a stable $Kinetics_{BL–PD}$ of iCTCs: the OS of $Kinetics_{BL\text{-}PD}^{+/+}$ or $Kinetics_{BL\text{-}PD}^{-/-}$ was almost identical to that of $iCTC_{PD}^+$ or $iCTC_{PD}^-$ with a median OS of 7.6 [4.4, 11.5] ($iKinetics_{BL\text{-}PD}^{+/+}$) versus 8.0 [5.1, 10.9] months among $iCTC_{PD}^+$ and 22.6 [18.9, not available (NA)] ($iKinetics_{BL\text{-}PD}^{-/-}$) versus 22.6 [15.3, 39.8] months among $iCTC_{PD}^-$ The same applied to the event rate, accounting for 93% (double positive, $n = 25$) and 47% (double negative, $n = 24$) (Supplementary Table 1).

One out of four patients ($n = 25$) displayed an alternating $iKinetics_{BL–PD}$, meaning that the iCTC status changed between the two timepoints. A shift from $iCTC_{BL}^-$ to $iCTC_{PD}^+$ ($iKinetics_{BL–PD}^{-/+}$: 11%, $n = 11$) was associated with a median OS of 8.4 [4.0, NA] months, almost identical to that of $iCTC_{PD}^+$ and $iKinetics_{BL\text{-}PD}^{+/+}$. In contrast, patients with $iKinetics_{BL\text{-}PD}^{+/-}$ (14%, $n = 14$) represented an intermediate group with a median OS of 11.4 [9.7, NA] months.

Role of apoptotic and enucleated CTCs

Figure 2b, c illustrates the Kaplan–Meier curves according to $aCTC_{PD}$ and $fCTC_{PD}$ status. Group size, number of events, and survival of $aCTC_{PD}^+$ or $fCTC_{PD}^+$ and $aCTC_{PD}^-$ or $fCTC_{PD}^-$ were almost identical to those of $iCTC_{PD}^+$ and $iCTC_{PD}^-$ only the percentage of $fCTC_{PD}^+$ (45%) was slightly higher (Supplementary Table 1). This is plausible since multiple fragments could emerge from one iCTC. The difference in median OS between positive and negative CTC $_{PD}$ status in all three subtypes accounted for over 1 year of survival (14.2–15.1 months). Beyond that, patients positive or negative for both $iCTC_{PD}$ and $aCTC_{PD}$ or $fCTC_{PD}$ showed an OS comparable to that in $iCTC_{PD}^+$ or $iCTC_{PD}^-$ patients. Whereas the limited number of patients and events precluded conclusions concerning a differing $iCTC_{PD}$ and $aCTC_{PD}$ or $fCTC_{PD}$ status (Supplementary Table 1; Supplementary Fig. 1).

In line with these results, $aKinetics_{BL–PD}$ and $fKinetics_{BL–PD}$ showed an OS equivalent to that of $iKinetics_{BL–PD}$ (Fig. 2e, f).

The strong correlation of CTC status and absolute count of all three subtypes at study inclusion and upon progression (Table 2) are reasons for the almost congruent survival curves and for the lack of an additional prognostic impact of aCTCs or enucleated fCTCs, which was irrespective of whether the threshold for CTC positivity was doubled.

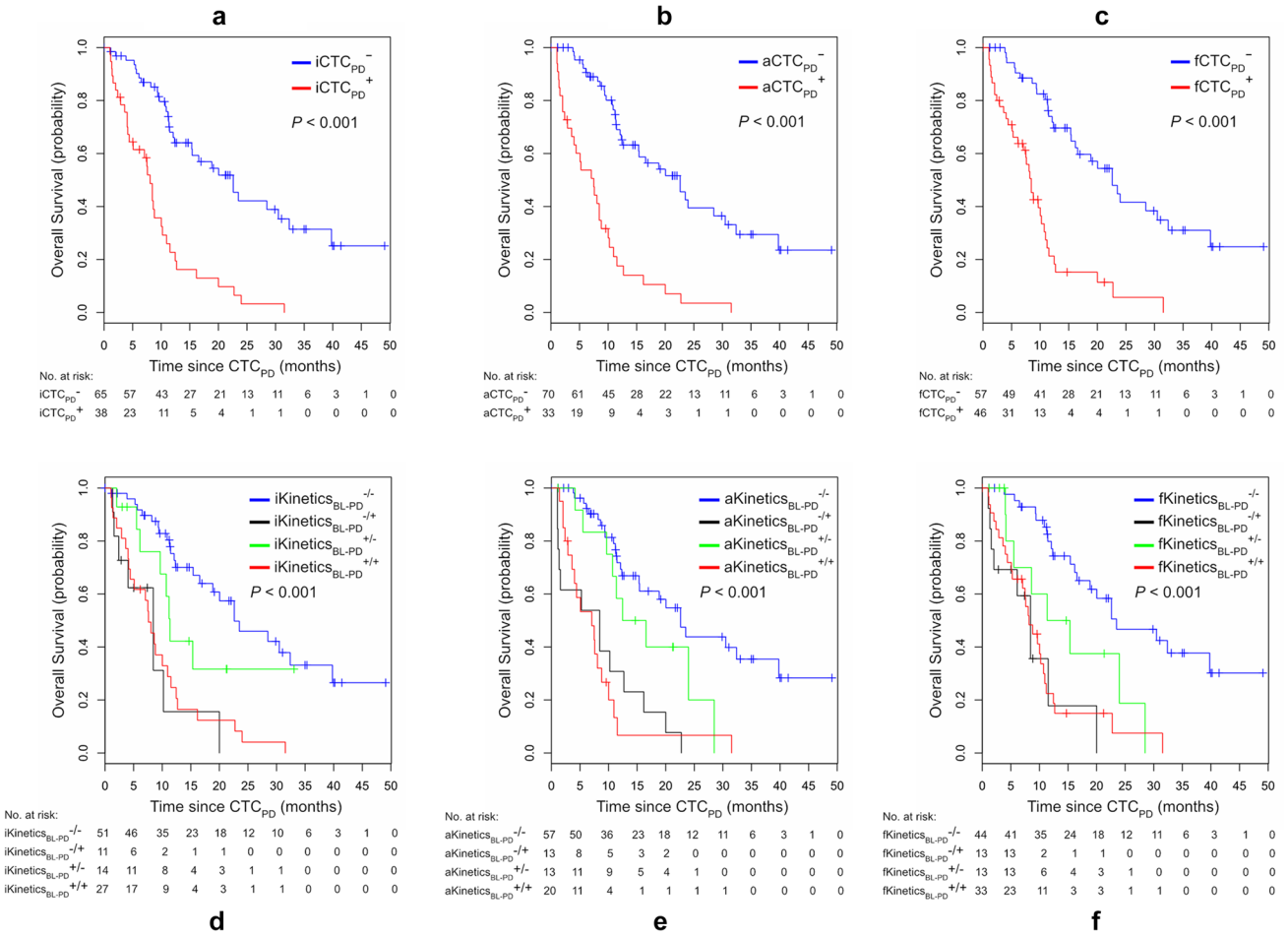

Fig. 2 Kaplan–Meier plots of overall survival in patients with progressive MBC based on the status of **a** intact, **b** apoptotic, **c** enucleated CTCs and on the kinetics from BL to PD of **d** intact, **e** apoptotic, and **f** enucleated CTCs (*P*, log-rank *P* value)

Table 2 Correlation indices of CTC subtypes at PD and BL

Categories	CTC count	CTC status	
	Spearman r_s	φ-coefficient	
		Cut-off 5	Cut-off 10
PD			
$iCTC_{PD}$ and $aCTC_{PD}$	0.76	0.73	0.71
$iCTC_{PD}$ and $fCTC_{PD}$	0.77	0.61	0.54
$aCTC_{PD}$ and $fCTC_{PD}$	0.80	0.72	0.66
BL			
$iCTC_{BL}$ and $aCTC_{BL}$	0.71	0.67	0.68
$iCTC_{BL}$ and $fCTC_{BL}$	0.74	0.65	0.59
$aCTC_{BL}$ and $fCTC_{BL}$	0.75	0.60	0.60

CTC count (absolute CTC values) versus CTC status (< 5 or ≥ 5 CTCs per 7.5 ml blood as negative or positive)

Serial enumeration of CTCs

OS of patients with alternating $iKinetics_{BL–PD}$ implied an alteration in prognosis depending on the $iCTC_{PD}$ status. Hence, $iCTC_{BL}^{+}$ and $iCTC_{BL}^{-}$ patients were analyzed separately according to their $iCTC_{PD}$ status. Figure 3a, b demonstrates the significant change in OS prediction of $iCTC_{BL}^{+}$ ($iCTC_{BL}^{+}iCTC_{BL}^{-}$ vs. $iCTC_{BL}^{+}iCTC_{PD}^{+}$: 11.4 [9.7, NA] vs. 7.6 [4.4, 11.5] months; $P = 0.044$) and $iCTC_{BL}^{-}$ patients ($iCTC_{BL}^{-}iCTC_{PD}^{+}$ vs. $iCTC_{BL}^{-}iCTC_{PD}^{-}$: 8.4 [4.0, NA] vs. 22.6 [18.9, NA] months; $P < 0.001$) due to an iCTC status shift.

In multivariate regression analysis of $iCTC_{PD}$ status and clinicopathological characteristics, $iCTC_{PD}$ positivity was identified as the only significant predictor of OS independent of the addition or exclusion of $iCTC_{BL}$ status among all qualities tested: hazard ratio, HR 3.18 [1.53, 6.59]; $P = 0.002$ or HR 3.67 [2.02, 6.67]; $P < 0.001$ (Table 3).

The comparison of Cox proportional hazards models showed that the addition of $iCTC_{PD}$ status to clinicopathological characteristics and $iCTC_{BL}$ status significantly improved OS prediction ($P = 0.002$). This was supported by a concordance index increase of 0.052 (from 0.642 to 0.694)

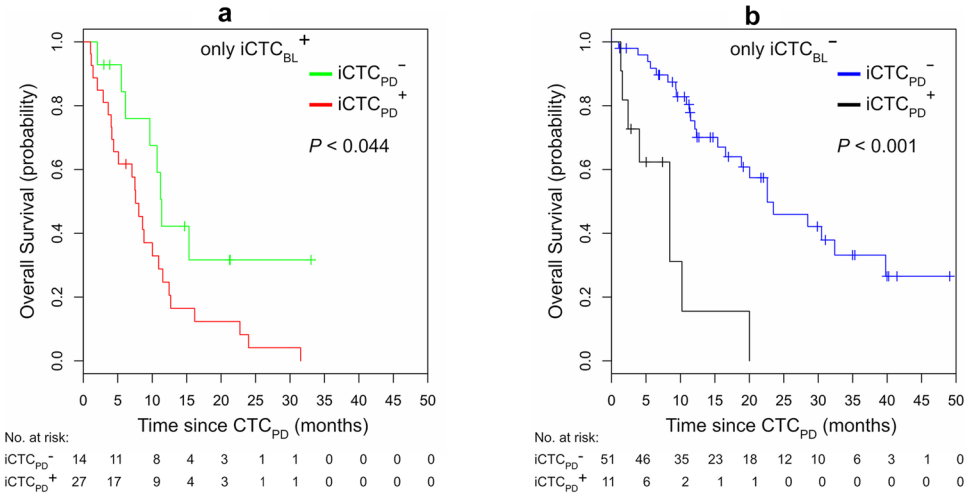

Fig. 3 Overall survival in progressive MBC separately analyzed for patients with **a** positive or **b** negative CTC status at study entry. Kaplan–Meier curves within **a** and **b** based on the status of intact CTCs upon further progression (*P*, log-rank *P* value). For median OS also refer to $iKinetics_{BL–PD}$ (Supplementary Table 1)

Table 3 Multivariate Cox regression analysis for prediction of OS—comparison of different models

Variables	Model 1: $iCTC_{BL}$ status		Model 2: $iCTC_{PD}$ and $iCTC_{BL}$ status		Model 3: $iCTC_{PD}$ status	
	HR [95% CI]	*P* (log-rank)	HR [95% CI]	*P* (log-rank)	HR [95% CI]	*P* (log-rank)
iCTC status						
$iCTC_{BL}^-$	1.00	1.00	–	–		
$iCTC_{BL}^+$	2.55 [1.37, 4.74]	0.003	1.28 [0.62, 2.66]	0.507	–	–
$iCTC_{PD}^-$	–	–	1.00	1.00		
$iCTC_{PD}^+$	–	–	3.18 [1.53, 6.59]	0.002	3.67 [2.02, 6.67]	< 0.001
Age at study entry	0.99 [0.96, 1.01]	0.321	0.99 [0.97, 1.02]	0.480	0.99 [0.97, 1.02]	0.491
Molecular subtypes: primary tumor						
HR+HER2−	1.00	1.00	1.00			
HER2+	1.04 [0.48, 2.28]	0.916	1.27 [0.57, 2.83]	0.556	1.29 [0.58, 2.87]	0.534
TNBC	1.35 [0.61, 2.98]	0.458	1.22 [0.55, 2.71]	0.625	1.25 [0.57, 2.76]	0.572
Distant metastases at study entry						
Nonosseous	1.00	1.00	1.00			
Osseous	0.54 [0.22, 1.32]	0.177	0.56 [0.22, 1.39]	0.210	0.59 [0.24, 1.45]	0.254
Nonosseous and osseous	1.11 [0.51, 2.44]	0.795	1.08 [0.50, 2.33]	0.842	1.16 [0.56, 2.43]	0.691
Therapy lines PD before study entry						
1	1.00	1.00	1.00			
2	1.13 [0.51, 2.50]	0.766	1.57 [0.69, 3.56]	0.281	1.67 [0.75, 3.70]	0.209
≥ 3	1.32 [0.65, 2.65]	0.444	1.28 [0.64, 2.59]	0.486	1.27 [0.63, 2.54]	0.500
Concordance index	0.642		0.694		0.683	
BIC	447.9		442.2		438.6	
Likelihood ratio test model 2/3			$P = 0.504$			
Likelihood ratio test model 1/2	$P = 0.002$					

Number of patients (events) included in models 1–3: 92 (59). $iCTC_{PD}$ status in addition to clinicopathological markers and $iCTC_{BL}$ status significantly improved OS prediction (model 1 vs. 2; $P = 0.002$), whereas the exclusion of $iCTC_{BL}$ status was not associated with a significant difference in survival prediction (model 2 vs. 3; $P = 0.504$). Further, the BIC of model 3 was slightly lower than the BIC of model 2. However, the concordance index suggested that the addition of iCTC status at both BL and PD (model 2) was superior to $iCTC_{PD}$ status alone (model 3). The effect of iCTC history could statistically be underestimated due to the limited sample size of patients with favorable, alternating $Kinetics_{BL–PD}$

HR hormone receptor, *HER2* human epidermal growth factor receptor 2, *TNBC* triple-negative breast cancer, *Therapy lines PD* therapy lines due to progressive disease, *BIC* Bayesian information criterion

and a BIC decrease of 5.7 (from 447.9 to 442.2) including $iCTC_{PD}$ status.

Discussion

This analysis demonstrates that iCTC status upon progression of MBC is prognostic for OS, significantly enhancing established prognostic models. Advancement of these models is of high importance considering the prognostic and therapeutic limitations in MBC.

Multiple studies have outlined the prognostic impact of CTCs and their potential role in tailoring therapy efficiency [12, 22–24, 36–39]. A European multicenter study of 17 centers and 1944 MBC patients [22] determined that serial cyclic CTC enumerations every few weeks significantly improved survival prediction. Furthermore, iCTC status after one cycle of a new systemic therapy line and the related transition from baseline was shown to be prognostic of clinical outcome in MBC [12]. Liu et al. [36] presented a strong correlation between CTC results and radiographic disease progression in stage IV breast cancer. CTCs seem to hold great potential in tumor prevention and response prediction by indicating breast cancer or minimal residual disease [18]. Budd et al. [39] suggested that CTCs are an earlier, more robust and reproducible prognostic indicator that better reflects disease status than conventional imaging results. To our knowledge, this was also the only study so far that separately analyzed CTC status around disease progression of MBC, with a significantly longer OS of <5 versus ≥ 5 CTCs per 7.5 ml of blood (19.9 [12.5, 23.6] vs. 6.4 [3.5, 12.9] months; $P=0.0039$). Nevertheless, prediction was restricted by the small sample size ($n=42$), and the gap between blood collection and detection of progression.

Deutsch et al. [26] reported an unfavorable OS dependent on the $aCTC_{BL}$ status of $iCTC_{BL}{}^{+}$ patients (median OS 10.3 vs. 16.4 months; $P=0.012$). This effect was not resilient to increasing cut-off values and did not apply to this study upon progression of MBC (Supplementary Table 1; Supplementary Fig. 1). Likewise, the assessment of fCTCs provided no additional prognostic evidence compared to iCTCs.

Noting the inclusion criteria, the overall sample size of the present study was adequate. However, considering the subgroup analysis of alternating $Kinetics_{BL–PD}$, the quantity and proportion of events were limited, which should be considered when interpreting the results. Concurrent inclusion of correlated variables such as CTC_{BL} and CTC_{PD} status implicates the challenge of multicollinearity addressed by Cox model comparison following the methods proposed by Bidard et al. [22]. Moreover, only patients experiencing further progression of MBC during the observed period were accessible for blood collection. This could entail an over- and underestimation of OS for both $iCTC_{PD}{}^{+}$ and $iCTC_{PD}{}^{-}$.

CellSearch is an extensively utilized, well-established, FDA-approved method to quantify CTCs, a heterogeneous cell population. However, by enriching only for EpCAM+ CTCs, the CellSearch system excluded, for example, CTCs undergoing epithelial–mesenchymal transition, which might be associated with disease progression, although this remains controversial in breast cancer [40, 41].

Even if CTCs were ineffective as a surrogate for treatment conversion, as suggested by the controversially discussed SWOG0500 trial [42], they still might be an effective marker for therapeutic management, sparing patients from the toxicity of futile therapies, diagnostics, and the associated side effects and therefore improve quality of life [24, 36]. Furthermore, OS prediction alone could be highly important for patients in a palliative situation. Our data clearly demonstrate the prognostic impact of iCTC status upon further progression of MBC, with CTC_{PD} positivity being a statistically significant negative prognostic factor. A transition of iCTC status under progression compared to baseline significantly altered survival prediction. Thus, the current iCTC status and thereby the serial enumeration of these cells seem to be crucial. Nevertheless, iCTC history might still be important when applied to the small subgroup of patients with a favorable, alternating $iKinetics_{BL\text{-}PD}{}^{+/-}$. Indeed, shifting from $iCTC_{BL}{}^{+}$ to $iCTC_{PD}{}^{-}$ was associated with an improved overall outcome with regard to $iCTC_{BL}$ status. At the same time, however, median OS was almost 1 year shorter than $iCTC_{PD}$ negativity alone would have predicted. Multivariate Cox regression analysis and the contrast of different models supported the additional prognostic impact of $iCTC_{PD}$ status and the relevance of repeating CTC counting over time.

These results together with other studies [12, 22–24] imply that the additional prognostic impact of serial longitudinal CTC measurements is independent of the point in time of blood collection. Instead, contemporary iCTC counts reflecting the actual disease state seem to be decisive. Of course, these hypotheses must be verified in large, prospective, multicenter trials.

CTCs are of high biological and clinical significance, especially in the absence of superior prognostic markers. As the interface between the primary tumor and different metastases, CTCs are an essential target in understanding the mechanisms of tumor progression and metastasis. Along with other markers, such as circulating tumor DNA (ctDNA) or exosomes, CTC characteristics could greatly contribute to a more personalized medicine, depicting both inter- and intratumor heterogeneity via simple liquid biopsy [43].

In conclusion, our study demonstrated the prognostic impact of iCTC status in progressive MBC. It supports the significance of CTCs as an easily accessible, prognostic biomarker and emphasizes the importance of serial CTC

quantifications. Future intensive investigations are needed to explore the subsequent clinical implications of these cells and their potential contribution to precision medicine.

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Compliance with ethical standards

Conflict of interest FS has served as a Consultant/Advisor for Roche and Novartis and has received Honoraria from Roche, Novartis, and Amgen. FM has served as a Consultant/Advisor for Roche, Pfizer, Novartis, AstraZeneca, and Tesara and has received travel, accommodations, and expenses from Roche, Novartis, Pfizer, AstraZeneca, and Honoraria from Roche, Pfizer, Novartis, Amgen, AstraZeneca, PharmaMar, Genomic Health, and Tesara. MW has provided Expert Testimony for Novartis, has received Honoraria from Novartis and Celgene and travel, accommodations, and expenses from Novartis. AS has received Honoraria from Roche, Celgene, AstraZeneca, Novartis, and Pfizer; research funding from Celgene; and travel, accommodations, and expenses from Roche and Celgene. All remaining authors have declared no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the Ethical Standards of the Institutional and/or National Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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