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Review article

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Circulating tumor cells in breast cancer: clinical validity and utility

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Circulating tumor cells (CTCs) have been extensively studied in breast cancer (BC), with large studies establishing CTCs as a robust prognostic biomarker in early and metastatic breast cancer (MBC). Several phase II and phase III trials have investigated the clinical utility of CTCs in BC. Here, we outline the current landscape for the use of CTCs in the clinic at different stages of BC, focusing first on early BC, then on MBC, with a particular focus on interventional clinical trials based on CTCs.

It has been 20 years since the seminal study by Cristofallini et al. on the prognostic value of CTCs[1], leading to the establishment of CTCs as a level of evidence I prognostic biomarker for MBC, reflected in current the AJCC Cancer Staging Manual[2] which identifies a M0 (i+) category for BC, defined as "no clinical or radiographic evidence of distant metastases, but deposits of detected tumor cells in the circulation fluids". Since then, the field of BC has seen considerable advances in therapeutics, with the advent of CDK4/6 inhibitors (CDK4/6i), PARP-inhibitors or antibody-drug conjugates (ADCs), and in treatment paradigms. In parallel, great progress has been made in single-cell technologies ranging from nucleic acid sequencing to multi-proteic and metabolic assays, with direct applications to CTCs[3–6]. The constant crosstalk between fundamental research in CTC biology and their applications in the clinic recently allowed both clinicians and researchers to provide evidence for CTCs as a clinically useful biomarker[7] in MBC. However, several obstacles hinder routine clinical use of CTCs. First, CTCs currently have no theragnostic value. For example, HER2 expression by CTCs cannot be used as a reliable surrogate for HER2 expression by the primary tumor or metastases[8]. Second, CTCs are rarely present in early breast cancer (EBC), limiting their use as a predictive or theragnostic biomarker or as a read-out of minimal residual disease in that population. Third, isolating CTCs entails additional costs, thus requiring an irrefutable demonstration of their clinical utility. Whilst this criterion has not been fully met yet, this review presents recent data supporting the clinical validity and utility of CTCs in BC.

Defining CTCs and their isolation techniques

One of the challenges in isolating CTCs is the ratio of white blood cells (WBC) and CTC concentration (1×10^7 WBC for a standard 7.5 mL blood tube, as opposed to sometimes one CTC per tube). Furthermore, some CTCs resemble monocytes in terms of size and morphology and can be concealed within aggregates of WBC. Thus, the study of CTCs is heavily dependent on the method used to isolate them from the bloodstream. CELLSEARCH®, the first FDA-cleared CTC isolation method, has demonstrated robust analytical (i.e., it is a sensitive and reproducible assay) and clinical validity in most large-scale studies on MBC[9]. For these reasons, most of the studies described below used CELLSEARCH®, with 7.5 mL CellSave® blood tubes specially designed for this method. Other CTC detection techniques, including antigen-agnostic methods, are reviewed extensively here[10].

Briefly, CTCs isolated with CELLSEARCH® are defined through immunochemical staining as entities with a nucleus (DAPI +), positive for markers of epithelial cells (cytokeratins 8-18-19 and EPCAM), negative for the CD45 marker of blood lineage[11]. Furthermore, CELLSEARCH® makes it possible to collect other features relevant to the metastatic process such as CTC-clusters, tumor-derived extracellular vesicles (tdEVs)[12], apoptotic bodies[13], and to label theragnostic markers such as HER2 or hormone receptors on CTCs, downstream of the pipeline. The advantages of this method include its reproducibility, since it now involves a semi-automated step for microscopy-based CTC enumeration (ACCEPT open-source software[14]); and its versatility, as the antibodies used to enrich CTCs can be replaced by antibodies targeting antigens of interest[15]. The technique can be adapted for use on other biologic liquids, like pleural effusions[16] or cerebrospinal fluid[17].

Yet, because the CELLSEARCH® system relies on an antigen-dependent method for the enrichment of CTCs with epithelial characteristics, it fails to isolate most CTCs that do not express epithelial markers, such as tumor cells with epithelial-to-mesenchymal transition (EMT) or basal-like phenotype[18]. Furthermore, cells processed with CELLSEARCH® have to be resuspended in a buffer containing a detergent to permeabilize them, before being stained with DAPI and antibodies targeting cytokeratins[19], limiting the range of analyses that can be performed on the collected CTCs. As a result, antigen-agnostic methods of CTC isolation have been developed[3,6]. For instance, the FDA-cleared PARSORTIX-system[20] is based on the microfluidic isolation of CTCs, using size and mechanical

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properties to isolate CTCs (such as their deformability). With this system, CTCs can be used for functional assays such as CTC-derived cell lines/organoids and patient-derived xenografts[20].

It has been debated whether CELLSEARCH® or antigen-agnostic methods can guarantee that the cells they retrieve are bona fide CTCs, since they are not analyzed beyond this step[21]. This is exemplified by the fact that both methods detect CTCs in patients without clinical evidence of BC[22,23]. However, the detection rate of CTCs in healthy subjects is very low: in the study by Allard et al.[22], only 5.5% of samples from healthy subjects had 1 CTC per 7.5 mL of blood, and 0% had ≥2 CTCs. Furthermore, it has been proposed that these cells could be genuine tumor cells, and that the lack of follow-up in those healthy subjects did not allow the detection of a recurring cancer.

Clinical validity of CTCs in EBC

The sensitivity of the CELLSEARCH® assay has enabled the isolation of CTCs in early BC[24] (EBC) for disease prognostication, in both neoadjuvant and adjuvant settings.

Neoadjuvant setting. In the neoadjuvant setting, several studies evaluated CTC count as a prognostic factor. In an ancillary study of the GeparQuattro neoadjuvant trial, Riethdorf and colleagues[25] assessed the CTC count before and after neoadjuvant chemotherapy. The detection of ≥1 CTC/7.5 mL or ≥2 CTCs/7.5 mL before neoadjuvant chemotherapy was associated with a lower disease-free survival (DFS) and overall survival (OS), while no significant association was found with the CTC count after neoadjuvant chemotherapy. A meta-analysis[26] then established CTCs as an independent, strong and quantitative prognostic factor, thus complementing previous models based on clinicopathological characteristics and response to treatment. This study showed the presence of CTCs in 25.2% of 1574 patients before neoadjuvant chemotherapy (NCT), with counts ranging from 0 to 559/7.5 ml of blood (median: 0 CTCs; third quartile: 1 CTC; 398 (25.2%), 199 (12.6%), and 93 (5.9%) patients had ≥1 CTC, ≥2 CTCs, and ≥ 5 CTCs detected, respectively).

Interestingly, the number of CTCs was associated with tumor size (particularly to cT4d inflammatory tumor), unrelated to pathologic complete response (pCR) and most importantly had a detrimental and decremental effect on distant DFS ($P < 0.001$), locoregional relapse-free interval ($P < 0.001$) and OS ($P < 0.001$). Indeed, the detection of CTCs before NCT had an independent prognostic value in multivariable analysis (notably independent of nodal status). CTC count as a continuous variable also had a prognostic value for all endpoints and was consequently a quantitative marker as each additional CTC detected correlates with a worse prognosis.

Hence, several CTC thresholds were used in this meta-analysis to best model patient prognostics. Interestingly, cut-off values of 1 (<1 CTC vs ≥ 1 CTC), 2 (<2 CTCs vs ≥ 2 CTCs) and 5 (<5 CTCs vs ≥ 5 CTCs) captured different survival trajectories with, in univariate analysis, a HR of death of 2.55, 95% confidence interval [CI] 1.91–3.39; 4.36, 95%CI 3.19–5.90; and 5.20, 95%CI 3.59–7.37; respectively. Given the paucity of CTCs in the localized setting, a historical threshold of 1 CTC/7.5 ml of blood has been used by most studies (whilst a threshold of ≥5 CTCs is used in metastatic disease).

The CTC count after NCT and before surgery was also prognostic: the HR of death was 2.01, 95%CI 1.40–2.84 (<1 CTC vs. ≥1); HR 2.20, 95%CI 1.28–3.57 (< 2 CTCs vs. ≥2) and HR 3.87, 95%CI 1.17–9.45 (<5 vs. ≥ 5). However, the evolution of CTC numbers after treatment did not significantly improve the model's prognostication. This is accounted for by Poisson's law[27,28], under which the low counts of CTCs fall in localized BC; this law states that, due to random fluctuations, a decrease from 1 CTC before therapy to 0 CTC after therapy is not considered a true decrease in CTC counts. This is in sharp contrast with MBC, where higher CTC counts make it possible to build models in which CTCs dynamics overlap closely with disease trajectories.

Upfront surgery/adjuvant settings. In patients eligible for upfront surgery, several studies have shown that CTCs have prognostic value. In a prospective single-center study of 403 patients with EBC[29], CTC counts were assessed before and at different timepoints after surgery. Almost all patients were treated by subsequent adjuvant radiotherapy ± chemotherapy (CT). With a cut-off of 1 CTC/30 mL of blood to separate patients of favorable (0 CTC) *vs*. unfavorable prognosis (≥1 CTC), relapse free survival (RFS) and OS were significantly different when CTCs were evaluated before surgery, after adjuvant therapy, one and two years after surgery, but not one week after surgery. This study thus indicates that the time point at which CTCs are collected impacts their prognostic value.

The SUCCESS A phase III trial[30,31] highlighted the prognostic relevance of CTCs before and after adjuvant chemotherapy (ACT): patients in whom CTCs were detected before ACT (≥1 CTC/30 mL of blood) had a reduced disease-free survival (DFS: HR 2.11, 95%CI 1.49–2.99; $P < 0.0001$) and OS (HR 2.18, 95%CI 1.32–3.59; P 0.002). The cut-off value of ≥5CTCs/30 mL of blood is of particularly poor prognostic value since it more than triples the risk of recurrence or death (DFS: HR 4.51, 95%CI 2.59–7.86; OS: HR 3.60, 95%CI 1.56–8.45). The authors also assessed the prognostic value of the CTC count two years after the initial chemotherapy[31], demonstrating that the persistence of CTCs at this time point was also associated with worse OS and DFS, in a multivariate analysis.

Further evidence comes from a pooled analysis focusing on the detection of CTCs at primary diagnosis in 3173 patients[32] with stage I-III BC and a median follow-up time of 62.8 months. Patients with ≥1CTC at primary diagnosis had a higher risk of death (HR 1.97, 95%CI 1.51–2.59) in a multivariate Cox regression model. In addition, this study confirmed the independent prognostic relevance of CTCs for BC-specific survival, DFS and distant DFS in EBC with a high level of evidence.

Likewise, a higher number of CTCs correlated with a worse prognosis in terms of OS. In subgroup analyses, CTC positivity was not a significant prognostic factor in low-risk patients defined by stage T1N0 primary tumors (HR 1.04, 95%CI 0.29–3.62), thus such patients may not require CTC-guided intervention. On the other hand, in high-risk patients, defined as those with primary tumors larger than 2 cm and lymph node involvement (HR, 2.46, 95%CI 1.78–3.39), CTC-guided intervention (treatment intensification or increased surveillance of relapse) could be relevant.

More specifically, a study published in 2018[33] based on the SUCCESS (1516 patients) population and on a National Cancer Database (1697 patients) cohort explored the predictive value of CTCs for patients with EBC who received radiotherapy. Importantly, in both cohorts, the study showed that radiotherapy, for patients who underwent breast-conserving surgery, correlates with longer OS in patients with detected CTCs (Time Ratio - describing the factor by which the time to event is associated between 2 groups—4.37, 95%CI 2.71–7.05) but not in patients without detected CTCs (Time Ratio 0.87, 95%CI 0.47–1.62). Moreover, radiotherapy did not statistically influence OS in patients after total mastectomy in CTC-positive or negative patients, prompting the conclusion that future trials should assess CTC-guidance for RT after breast-conserving surgery. To date, no subsequent study has complemented these initial promising results.

Clinical utility of CTCs in EBC

To date, no phase III clinical study has demonstrated the clinical utility of CTCs in EBC. Several obstacles currently hinder CTCs from being a clinically useful biomarker in this setting. First, at this early stage, most patients have undetectable levels of CTCs and for those for whom CTCs can be detected, the low numbers of CTCs render molecular testing particularly challenging. Yet, the presence of CTCs at baseline in EBC remains a strong pejorative prognostic factor and suggests treatment escalation in high-risk groups. More stringent surveillance of relapse could also be offered for CTC-positive patients at baseline or after treatment, since CTC detection does not necessarily imply relapse (not all CTCs have metastatic potential and even those that do may fail to survive their journey to distant organs)[30].

Second, the small number of patients with >1 CTC in EBC limits the evaluation of CTC dynamics (before *vs*. after NCT/ACT/adjuvant

radiotherapy/surgery): 25.2% of patients eligible for NCT had ≥1 CTC, but only 12.6% had ≥ 2 CTCs in the 2018 meta-analysis[34]. Furthermore, the prognostic value of the CTC count has clearly been demonstrated but its utility to predict the benefit of CT is unclear.

However, the cT4d subgroup, in which CTCs are more likely to be detected, might be a niche for further investigation and demonstration of clinical utility in EBC. In the adjuvant setting, patients for whom treatments fail to eradicate CTCs might benefit from enrolment in clinical trials assessing new molecules, treatment intensification regimens, or from a more stringent follow-up[35,36].

Clinical validity of CTCs in MBC

The study of CTCs has been most extensive in MBC and demonstrates their clinical validity in this setting. In 2004, Cristofanilli et al. showed in 177 patients with a metastatic BC that the baseline CTC count (≥5/7.5 mL or < 5/7.5 m) was an independent predictor of OS and progression-free survival (PFS)[1]. Other studies evaluating CTCs in MBC have confirmed that, independently of other variables, higher numbers of CTCs correlate with poorer prognosis, and that the measurement of CTC dynamics across time is more informative than at a single time point[9]. The OS prognostic value of the CTC count at different time-points, as determined by the main meta-analyses in both EBC and MBC, is presented in Fig. 1.

A threshold for the baseline CTC count (i.e., before the first line of chemotherapy or other systemic treatment) was established by an international consensus[37] with a pooled analysis of individual data of 2436 MBC patients from 18 cohorts; it separates MBC patients in two groups: (1) a "stage-IV indolent" group (roughly 50% of MBC patients) defined by <5 CTCs/7.5 ml with a favorable prognosis (median OS: 36.3 months) and (2) a "stage-IV aggressive" group defined by ≥5 CTCs/7.5 ml with a poor prognosis (median OS: 16.0 months). The strong and independent prognostic value of this CTC threshold could be used at baseline to better homogenize patient cohorts in prospective clinical trials.

Thus, CTCs were among the first biomarkers to be validated as a robust and objective independent prognostic factor in MBC. Further, within subgroups of MBC previously thought to be of more favorable prognosis (bone only metastatic disease), CTC enumeration identifies approximately 50% of patients with poor prognosis (for instance, 198 patients in a cohort of 399 MBC patients with bone-only metastases[38]).

Several studies have demonstrated that CTC dynamics in MBC after a line of treatment improves prognostication[9]. In fact, patients with high baseline CTC counts, but with rapidly decreasing CTCs during treatment have a favorable prognosis. A study[39] showed no statistically significant difference in terms of OS and PFS between 1) patients having <5 CTCs both at baseline and after starting therapy and 2) patients with ≥ 5 CTCs at baseline but with a decrease in CTCs (<5 CTCs) after therapy. Conversely, OS/PFS in patients with ≥5 CTCs, both at baseline and during therapy were not significantly different from OS/PFS of patients with < 5 CTCs at baseline and ≥ 5 CTCs after starting therapy.

In another study, the authors sought to enhance the criteria for the identification of patients with a poor prognosis. The CTC count of 56 MBC patients was determined before the start of a third line of chemotherapy, and before the second cycle of chemotherapy for patients who had at least one CTC detected at baseline. A composite criterion consisting of (1) <5 CTC/7.5 ml or (2) relative decrease ≥−70% of the baseline CTC count, showed better prognostication for PFS[40]. Similarly, a study that included 469 MBC patients undergoing a first line of CT identified four distinct prognostic groups, defined by at least three iterative CTC assessments, including baseline[41]. Patients within the most pejorative prognosis could benefit from clinical trials assessing the efficacy of new treatments.

Interestingly, CTCs and ctDNA have non-overlapping detection profiles and complementary prognostic values in patients with MBC, as shown in two comparative studies which are summarized in Table 1[42,43].

The ability of CTCs to identify patients with a poor prognosis and to predict disease trajectories based on CTC enumeration in first or later lines of treatment prompted the design of trials attempting to demonstrate the clinical utility of CTCs, which has only recently been confirmed.

Clinical utility of CTCs in MBC

Currently, MBC is the main setting in which the clinical utility of CTCs has been explored in several phase II/III randomized trials.

Trials based on CTC baseline enumeration. The first phase III randomized trial based on CTC-driven management of MBC to reach its primary endpoint is the STIC-CTC study[44]. Using a non-inferiority design in a population of HER2-negative, HR + MBC patients, it compared a clinician-based choice of first-line therapy: CT or single-agent endocrine therapy (ET), to a CTC-based choice (CT for patients with a

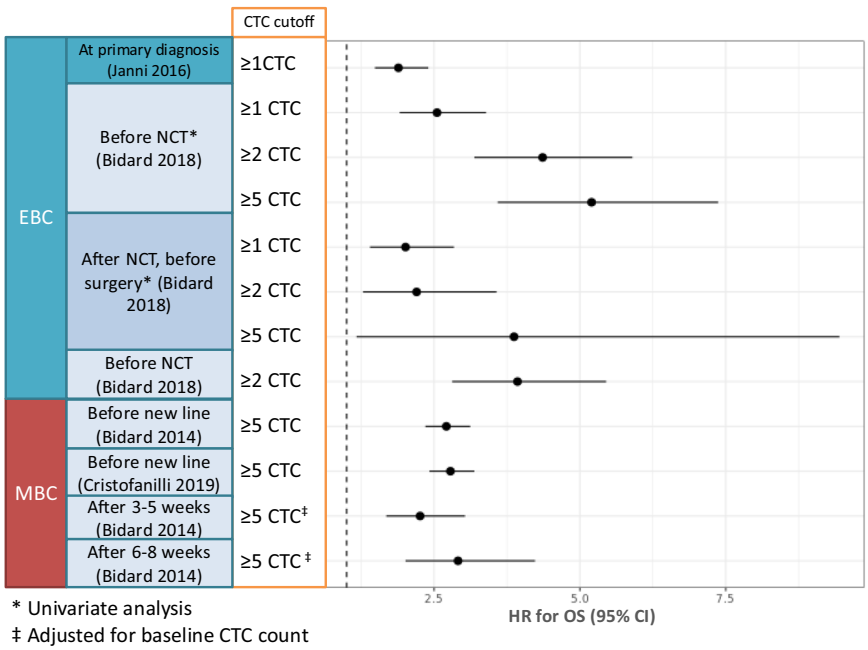

Fig. 1 | Hazard ratios (HR) for overall survival (OS) with their 95% confidence interval (CI 95%) in main meta-analyses assessing the clinical validity of circulating tumor cells (CTCs), according to the CTC count cut-off; in both early breast cancer (EBC) and metastatic breast cancer (MBC). Analyses are multivariate unless specified otherwise. NCT: neoadjuvant chemotherapy.

* Univariate analysis
‡ Adjusted for baseline CTC count

Table 1 | Summary of two large-scale studies comparing CTC detection (with CELLSEARCH®) and ctDNA

Original publication	Bortolini Silveira et al.[42]	Gerratana et al.[43]
Design and number of patients analyzed	In a prospective cohort (n = 198) of HER2-negative MBC patients, matched blood samples were collected at baseline (BL) and before the 2nd cycle of a first line CT with paclitaxel + bevacizumab. ctDNA detection by capture-based targeted next-generation sequencing (NGS).	Retrospective cohort (n = 107), any line of treatment for MBC, matched blood samples collected at BL, first evaluation (EV1) and at progression. ctDNA detection by targeted NGS. Prospective cohort (n = 48, HR + HER2-, first line for MBC), matched blood samples collected at BL and EV1. ctDNA detection though the detection of short fragments of the *ACTB* gene (associated with ctDNA).
CTC-related results	Prognostic value of the CTC count confirmed. As a continuous variable, BL CTC count has a log-linear impact on PFS and OS.	The CTC count increased between EV1 and progression. No CTC count decrease was observed between BL and EV1.
ctDNA-related results	Higher ctDNA levels, assessed by variant allele frequency (VAF) are associated with a worse prognosis. As a continuous variable, BL VAF has a linear impact on PFS and OS. The detection of variants in *TP53* is associated with a worse prognosis.	Retrospective cohort: VAF decreased between BL and EV1. VAF increased between BL/EV1 and progression. Prospective cohort: patients with a high proportion of short/all size fragments of ACTB at baseline had a shorter PFS. An increase of $ACTB_{short}$ between BL and EV1 was also associated with a decreased PFS.
Prognostic value of the multimodal liquid biopsy	CTC counts and ctDNA levels were moderately correlated. Both improved a prognostic model based on clinicopathological characteristics. Overall, both decreased between BL and week 4, but an opposite trajectory was observed in 15% of patients. The best prognostic model for PFS included both BL VAF and CTC count at 4 weeks.	Not formally investigated, although the different trajectories of CTC count and VAF between BL and EV1 suggest a complementary role.

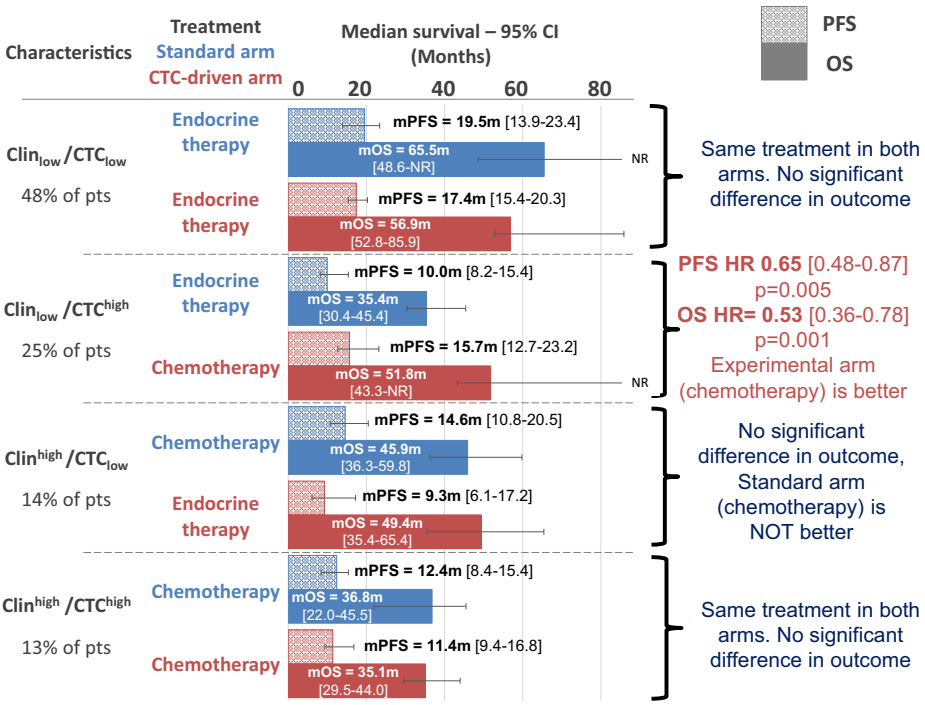

Fig. 2 | Median progression-free survival (mPFS) and median overall survival (mOS) results with their 95% confidence interval (CI 95%) for the STIC CTC trial, in the four subgroups defined by the clinician's choice of therapy ($Clin^{high}$ or $Clin_{low}$) and the CTC count (CTC^{high} or CTC_{low}). HR: hazard ratio.

baseline CTC count of ≥5 CTCs/7.5 mL, ET for patients with a baseline CTC count <5). Designed before the CDK4/6i era, it aimed to show that the CTC-based choice of first-line treatment, more reproducible and objective than the clinician's choice, was non-inferior in terms of PFS, its primary endpoint. It reached its primary objective, with a median PFS of 15.5 months (95%CI 12.7–17.3) in the CTC arm vs.13.9 months (95%CI 12.2–16.3) in the standard arm (HR 0.94; 90%CI 0.81–1.09). Recently, results for OS (and updated PFS) were published[44] with a median follow-up of 4.7 years: a median OS of 51.3 months (95%CI 46.8–55.1 months) was reached in the CTC arm vs. 45.5 months in the standard arm (95%CI 40.9–51.1 months), with a HR for death of 0.85 (95%CI 0.69–1.03, p = 0.11).

Importantly, in the planned subgroup analysis for ClinLow/CTCHigh group (i.e. patients with ≥5 CTCs/7.5 mL but an ET recommended by the clinician); patients randomized in the CTC arm (who had their treatment escalated from ET to CT), had a significantly higher PFS and OS: median PFS 15.7 months, 95%CI 12.7–23.2 months, HR 0.64, 95%CI 0.47–0.87, median OS 51.8 months, 95%CI 43.3–not reached, HR 0.53, 95%CI 0.36–0.77; and for patients in the standard-arm: median PFS 10.0 95%CI 8.2–15.2 months and median OS 35.4 months 95%CI 30.2–45.4 months (Fig. 2). Thus, STIC-CTC sheds light on ClinLow/CTCHigh patients (25% of patients, n = 189) for whom a CTC-driven therapeutic strategy is relevant. Planned subgroup analyses according to baseline characteristics also identified a PFS benefit in >60-year-old patients.

This initial demonstration of the clinical utility of CTCs in MBC did not translate into a change in clinical practice because of the now preferred combination of a CDK4/6i with ET as the first-line treatment for HR + HER2-negative MBC patients. However, the dilemma of the choice between

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Table 2 | Past clinical trials based on CTC-driven treatment allocation in MBC

Trial, recruitment period, trial design and description	Number patients screened + analyzed	Primary endpoint	Results	Clinical relevance
CTC enumeration				
STIC-CTC[44] –Recruitment period: 2012-2016 –Randomized phase III trial, non-inferiority of CTC-driven intervention tested, open-label –Goal: in HER2-negative, HR + MBC patients, to compare a clinician-based choice of first-line therapy (CT or single-agent ET) to a CTC-based choice (CT for patients with a baseline CTC count of ≥5 CTCs/7.5 mL, ET for patients with a baseline CTC count < 5)	797 pts screened 778 pts analyzed	PFS	–First trial to reach its primary endpoint, demonstrating the non-inferiority of a CTC-driven intervention vs. a clinical-driven intervention –Higher rate of upfront chemotherapy in the CTC-driven arm	–CTC-based choice is more reproducible than the clinician's choice –In the "CTC count ≥5/low clinical risk" subgroup, benefit in PFS and OS –However, results not applicable to current practice because of current guidelines for HR + MBC management: combination of CDK4/6 inhibitors + ET
Treat-CTC[36] –Recruitment period: 2013-2016 –Randomized phase II trial –Goal: in subjects with HER2-negative BC with≥1/15 mL CTC after surgery, to evaluate an adjuvant treatment by 6 cycles of trastuzumab, or observation	1317 pts screened 58 pts analyzed	% of patients with CTC count ≥ 1 per 15 mL at week 18	–Stopped for futility –5 patients had ≥1 CTC at week 18 in the trastuzumab arm *vs*.4 patients in the standard arm	–No benefit of treating CTC-positive patients with trastuzumab without molecular characterization of CTCs –Most patients (76%) had HER2-negative CTCs
CTC level variation				
SWOG S0500[57] –Recruitment period: 2006-2012 –Randomized phase III trial, superiority of CTC-driven intervention tested, open-label –Goal: 22-days after start of 1st line of CT, to assess the benefit of a CTC-driven early change to a 2nd line of CT in metastatic BC patients with ≥5CTCs/7.5 ml	595 pts screened 123 pts analyzed	OS	–Failed to demonstrate benefit in OS:median OS in the CTC-driven arm: 12.5 months; in the standard arm: 10.7 months, $p = 0.98$ –Difference in median PFS not statistically significant: 4.6 months in CTC-driven arm *vs*. 3.5 months in standard arm	Despite the lack of OS benefit in the CTC arm, confirmed high prognostic value of CTCs. Limits: Selection of patients with a very poor prognosis –Short lead time for treatment switch in experimental arm
CirCe01[40] –Recruitment period: 2010-2015 –Randomized phase III trial, superiority of CTC-driven intervention tested, open-label –Goal: in patients with MBC after 2 lines of treatment and with ≥5CTCs/7.5 ml, randomization between: 1) CTC driven arm: if persistence of ≥5CTCs or <70% of decrease in initial CTCs, switch to subsequent line of CT; 2) standard arm: treatment according to CT-scan evaluation	207 pts screened 101 pts analyzed	OS	Did not demonstrate benefit in median OS and PFS	Despite negative result: –Refined criterion for CTC based intervention with composite criterion including a 70% of decrease in initial CTCs –In a limited post-hoc analysis, benefit for the patients who effectively switched CT upon lack of CTC decrease
Molecular characterization				
LAP10559[47] –Recruitment period 2008 - 2010 –Randomized phase II trial –Goal: to assess the efficacy of lapatinib in patients with metastatic HER2-negative BC but HER2-positive CTCs (≥ 2 CTCs/7.5 m, with a HER2 status evaluated by immunofluorescence and FISH)	139 pts screened 96 pts analyzed 7 pts treated with lapatinib	ORR	–Seven of the 96 patients (7%) had HER2-positive CTCs and were treated with lapatinib –No objective tumor responses were observed in this population	–Low rate of HER2-positive CTCs –No signal for lapatinib efficacy
CirCe T-DM1[45] –Recruitment period: 2013-2016 –Randomized phase II trial, superiority of CTC-driven intervention tested, open-label –Goal: In patients with metastatic HER2-negative BC but HER2-amplified CTCs (at least one HER2 amplified CTC/7.5 ml, as determined by CELLSEARCH® and FISH), who had received at least 2 lines of CT, to assess the tumor response rate to trastuzumab-emtansine	155pts screened 11 pts analyzed	ORR	–Did not demonstrate a benefit in ORR and secondary endpoints –≈10% of MBC patients with HER2-negative tumor have HER2amp CTCs: subsequent DETECT III trial found similar proportions	HER2-amplified CTCs are rare in metastatic BC with HER2-negative primary tumor: the trial did not reach its required accrual (among the 155 screened patients, only 11 received T-DM-1, 1 patient had a partial response)

Table 2 (continued) | Past clinical trials based on CTC-driven treatment allocation in MBC

Trial, recruitment period, trial design and description	Number patients screened + analyzed	Primary endpoint	Results	Clinical relevance
DETECT III[48] –Recruitment period: 2012-2019 –Randomized phase III trial, superiority of CTC-driven intervention tested, open-label –Goal: in patients with HER2-negative BC but HER2-positive CTCs, randomization between: 1) Standard therapy (eribulin) and 2) lapatinib + eribulin. Assess efficacy of CTC-driven (HER2-positive CTCs) lapatinib administration	2090 pts screened 105 pts analyzed	PFS, CTC clearance rate	Did not demonstrate a benefit in terms of: –Revised primary endpoint (PFS) –CTC clearance rate (initial primary endpoint) –However, showed a significant benefit in OS	–Confirmed rarity of HER2-positive CTCs in HER2-negative MBC –Argues in favor of targeting HER2-positive CTCs with lapatinib in HER2-negative MBC; yet caution required because: 1) Lack of statistical power 2) Biases between 2 arms of trial 3) Results inconsistent with the previous data on lapatinib

CTCs were detected by the CELLSEARCH® technology

ET and CT has moved to the second line, a setting in which a CTC-based choice may be relevant.

The ECLECTIC (NCT06195709) phase III trial, currently conducted in patients with ER + HER2-negative MBC progressing on first-line treatment with aromatase inhibitor + CDK4/6i, is aimed at evaluating the combined predictive value of a 16α-[18 F]-fluoro-17β-estradiol Positron Emission Tomography-Computed Tomography ([18 F]FES PET/CT) complemented by the prognostic value of CTCs to guide the second line treatment decision between an investigator's choice of second line ET ± targeted therapy, and CT.

Besides, the clinical utility of CTCs in the context of CDK4/6i+ET vs. 1st line CT is one of the endpoints of the ongoing AMBRE trial (NCT04158362). This trial will, as a secondary endpoint, evaluate the clinical value of CTCs in HR + HER2-negative MBC patients (with visceral metastases and high tumor burden) receiving either first-line CT or the combination of the CDK4/6i abemaciclib with ET.

Table 2 lists all published clinical trials based on CTC-driven treatment allocation in MBC. Ongoing clinical trials based on CTC-driven interventions in MBC are presented in Table 3.

Trials based on CTC molecular assessment. Several trials based a therapeutic intervention on CTC count and HER2 phenotype[45–48] in patients with HER2-negative MBC but with HER2-positive status on CTCs, as determined by immunohistochemistry and fluorescent in-situ hybridization (FISH).

Historically, several studies have demonstrated that HER2-positive CTCs were detected in HER2-negative MBC[49,50]. Pestrin and colleagues analyzed samples from 40 patients with advanced BC and detectable CTCs and found that 29% of patients with an HER2-negative BC had HER2-positive CTCs, and that 42% of patients with an HER2-positive BC had HER2-negative CTCs. It has been suggested that targeting these HER2-positive CTCs may improve patient outcomes. The rationale for using HER2 also came from previous results of the DETECT study group[51], showing that the isolation of ≥1 CTC with strong HER2 staining in MBC patients with HER2-negative primary tumors, was associated with shorter OS. This hinted towards a role for HER2 expression on CTCs and supported the use of anti-HER2 agents for these patients.

The LAP105594 trial[47] was a phase II trial using lapatinib (an anti-HER2 TKI) in patients with HER2-negative MBC and HER2-positive CTCs. HER2-positive status in CTCs was defined 1) by immunofluorescence: as expression of HER2 in at least 50% of CTCs when HER2 was not amplified or non-evaluable in FISH or 2) by FISH, if less than 50% of CTCs expressed HER2 by immunofluorescence but had amplified HER2 status. The primary endpoint was objective tumor response. Of the 96 patients with detectable CTCs (≥2 CTCs/7.5 mL) only 7 (≈7%) had HER2-positive CTCs. Of these 7 patients treated with lapatinib, only one presented durable stable disease and the trial was stopped for futility.

CirCe T-DM1[52], another phase II trial, assessed the efficacy of T-DM1 (trastuzumab-emtansine, an antibody-drug conjugate targeting HER2) in HER2-negative MBC patients treated with ≥2 prior lines of CT and with ≥1 HER2amp CTC. The primary endpoint was the overall response rate. Of the 154 screened patients, 14 (9.1% of patients with ≥ 1 CTC/7.5 ml) had ≥1 HER2amp CTC. Of the 11 patients treated with T-DM1, one achieved a partial response. Trastuzumab-Deruxtecan[53] instead of TDM-1 in the same patient population could possibly yield more favorable results.

These two trials show that a small proportion of patients with an HER2-negative MBC display HER2-amplified or even HER2-positive CTCs. Furthermore, both of these trials were designed before a seminal paper suggested that HER2 expression on CTCs was not a valid surrogate for therapeutically actionable HER2 addiction in these CTCs when cultured in vitro[54]. In this study, beyond the fact that cultured HER2-positive/HER2-negative CTCs spontaneously interconverted phenotypes, lapatinib was not able to refrain proliferation in either of these CTC populations.

Retrospectively, failure of these proof-of-concept studies to demonstrate results of potential clinical benefit might be attributed to the lack of

Table 3 | Ongoing clinical trials based on CTC-driven interventions in MBC

Trial, recruitment period, trial design, status and description	Number patients planned	Primary endpoint	Clinical relevance
ECLECTIC, NCT06195709 –Recruitment period: started in 2024 –Randomized, multicentric, open-label, phase III trial –Goal: In patients with ER + HER2-negative metastatic breast cancer progressing under a first line with a CDK4/6 inhibitor and an aromatase inhibitor, to evaluate the combined value of 18F-FES PET/CT and circulating biomarkers to guide second line treatment: • In case of low 18F-FES PET/CT uptake, or high uptake but CTC ≥ 5/7.5 ml, patients will be randomized between ET or CT • In case of high 18F-FES PET/CT uptake + low CTC count <5: ET	300 pts	PFS	Will, in the context of ER + HER2-negative metastatic BC, evaluate the benefits of a combined use of: 18F-FES PET/CT imaging and circulating biomarkers (CTCs) to guide second-line treatment decisions
HER2Cell, NCT04993014 –Recruitment period: 2021-2024 (2028 for study completion) –Randomized phase II unicentric trial, superiority of CTC-driven intervention tested, open-label –Goal: Patients with early HER2-positive BC eligible to neoadjuvant therapy with trastuzumab + pertuzumab will have CTCs collected at baseline. Only patients with a complete pathological response will then be randomized in 2 cohorts: • HER2 positive CTCs at baseline and • HER2 negative/absent CTCs at baseline In each cohort, patients will be randomized in 1:1 ratio for adjuvant trastuzumab *vs*. adjuvant trastuzumab + pertuzumab	80 pts	DFS	Will further document the prognostic/predictive value of HER2-positive CTCs

statistical power, and possibly to the absence of HER2 addiction in HER2-positive CTCs.

However, DETECT III, a phase III randomized trial[48], also exploited discrepancies between HER2 expression on CTCs and the primary tumor (approximately the same patient population as in LAP105594 and CirCe T-DM1).

This is the first study to suggest that treatment decisions leading to improved patient outcomes can be based on the CTC phenotype. DETECT III randomized patients with HER2-negative MBC (HER2 staining on the primary tumor) and HER2-positive CTCs in one group receiving standard therapy (eribulin) and in another treated with lapatinib + eribulin. Of 2090 patients with a valid CTC detection test, 154 patients (14%) had HER2-positive CTCs, and the intention-to-treat population comprised 105 patients. Consequently, the authors suggested caution in the interpretation of the results because of this limited patient accrual. Although PFS (primary endpoint of the trial) was not statistically superior in the lapatinib + eribulin group (HR 0.69, 95%CI 0.42-1.14, $p = 0.14$), OS was increased in this arm with a median of 20.5 months *versus* 9.1 months in the standard arm (HR 0.54, 95%CI 0.34–0.86; $p = 0.008$). Possible biases include imbalances between the two arms of the trial in terms of disease subtype, bone-only metastases, previous therapies at inclusion and subsequent lines of treatment. Furthermore, a discordance of HER2 status between primary tumor and metastases might be considered (many patients did not undergo re-evaluation/confirmation of HER2 status on metastases). This could reflect the targeting of a particularly aggressive clone of cancer cells by lapatinib, the fact that PFS is an imperfect surrogate for OS[55] or simply be due to biases in the evaluation of PFS (as suggested by the authors). Results obtained regarding the relation between CTC dynamics and OS are discordant with several previous studies (OS and decrease of CTCs are uncorrelated in both arms). Hence, results from DETECT III could serve as ground for future randomized trials with OS as a primary endpoint and outline the challenge of HER2 targeting in MBC patients with HER2 negative primary tumors.

Future studies could also take advantage of the molecular analyses that can be performed on CTCs isolated with antigen-agnostic methods. For instance, RNA sequencing can be performed on CTC-enriched samples obtained with the Parsortix system[56], at different time points, making it possible to monitor the level of expression of clinically actionable biomarkers or of gene signatures.

Trials based on CTC level variation

Following the discovery of the strong prognostic potential of CTCs, the first randomized phase III trial[57] (SWOG S0500) was aimed at demonstrating the clinical utility of CTCs. It assessed, in patients with persistent increase in CTCs despite one cycle of first-line CT, whether switching to a second line of CT would prolong OS. This strategy did not improve OS or PFS (median OS for CTC-driven arm: 12.5 months *vs*. standard arm: 10.7 months $p = 0.98$). Yet careful analysis of this study provides precious insights for efficient use of CTCs as a predictive biomarker.

Several reasons for the negative results of the trial were raised: 1) the CTC-based switch to a second line of CT preceded the clinically-driven switch at best by 2 months, which was unlikely to impact OS for the 60 patients in the CTC arm; 2) a selection of patients with a very poor prognosis, highlighted by the low OS observed in this subgroup (median OS of 13 months) for a first line of chemotherapy; 3) the limited statistical power of the trial 4) the threshold of 5 CTCs used in the trial, although relevant for clinical validity, could be inappropriate for clinical utility and 5) at the time of this study (late 2000s), the strong prognostic relevance of CTCs was unlikely to result in clinical benefit due to the limited therapeutic options after resistance to first-line CT.

The subsequent Circe-01 prospective randomized trial[52] was conducted in patients with ≥5 CTC/7.5 mL starting a third (or subsequent) line of chemotherapy in MBC: in the CTC-driven arm, a switch of chemotherapy was indicated if the decrease in CTC count was deemed insufficient during the first cycle of each new line of treatment (≥5 CTC/7.5 ml after one cycle or a <70% decrease of the baseline CTC count). In the interventional step, 207 patients were included, and 101 patients with CTC ≥ 5 were randomized in the CTC-driven arm ($n = 51$) and in the standard arm ($n = 50$). The trial was negative, with no difference between the CTC-driven arm or standard of care arm (PFS HR of 0.9 (95%CI 0.6–1.3, $p = 0.6$) and OS 0.95 (95%CI 0.6–1.4, $p = 0.8$)), but in the CTC arm, only 18 of the 29 patients who had an insufficient CTC response complied with the early treatment switch. The data supporting the clinical validity and utility of CTCs at different stages of breast cancer is summarized in Fig. 3.

Future applications of CTCs in breast cancer

First, multi-modal single-cell assays could enable a holistic approach of CTC biology[58]. Such methods can determine the genome, epigenetic landscape,

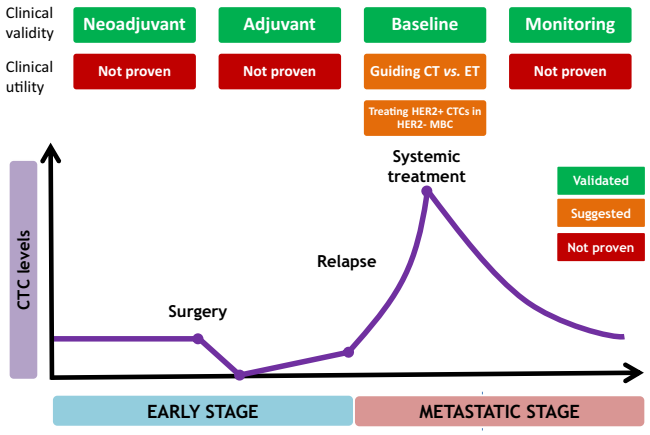

Fig. 3 | Summary of the data supporting the clinical validity and utility of circulating tumor cells (CTCs) at different stages of breast cancer. CT: chemotherapy, ET: endocrine therapy, MBC: metastatic breast cancer.

transcriptomic and proteomic profiles of CTCs simultaneously, thus capturing a "fuller" picture of CTCs. Second, the future of CTCs in the clinic probably resides in multiparametric approaches exploiting other aspects of liquid biopsies such as ctDNA[43], tdEVs and biomarkers such as CA15-3, LDH and inflammatory cytokines. Interestingly, genomic studies of CTCs have shown non overlapping mutational profiles with ctDNA[59]. Indeed, robust evidence now shows that CTCs and ctDNA contribute differentially to the understanding of BC biology as they are generated by distinct cellular events[42,43]. The very short life of CTCs in the bloodstream (the estimated maximum being of a few hours[60]) suggests constant seeding into the circulation, while ctDNA mostly reflects tumoral death and passive shedding of cellular debris into the bloodstream. Further, the detection of actionable genetic events is more sensitive with ctDNA than with CTCs[43].

Therefore, ctDNA is more suited to identify minimal residual disease than CTCs. Importantly, multiparametric approaches are most likely better suited to early stages of BC where tdEVs and ctDNA are more abundant than CTCs and sometimes better reflect characteristics of the primary tumor (HER2 status of the primary tumor better correlates with that of tdEVs than CTCs[8]). Importantly, seminal studies establishing the prognostic value of CTCs did not consider qualitative aspects of CTC biology such as CTC clusters (homotypic or heterotypic with WBC) and apoptotic status, which could hold relevant information.

Third, with the advent of new therapeutics, CTCs could provide useful information on the detection of treatment efficacy and/or resistance. Importantly, there are currently no predictive biomarkers available for treatments such as CDK4/6i. Consequently, clinical trials are now investigating the use of CTCs to detect early resistance to CDK4/6i (NCT04158362). In the context of patients harboring *BRCA/PALB2* germline mutations, CTCs could also inform clinicians on the efficacy of PARP inhibitors. For instance, scores based on the immunostaining of RAD51 foci[61] (thus reflecting restoration of the ability to detect DNA damage) have the potential to inform on the BRCA-ness phenotype (conferring eligibility to platinum-based chemotherapy and PARP-inhibitors) in a dynamic manner and in patients afflicted by genetic alterations other than *BRCA1/2* mutations. The advent of ADCs broadens considerably the portfolio of potential biomarkers[62]. Unlike other targeted therapies, ADCs do not require the expression of the target antigen on all cancer cells. It is thus conceivable that the expression of an antigen targeted by ADCs on only a fraction of CTCs may suffice as a read-out for its potential efficacy. With the emergence of new therapies and the need for personalized treatment, the detection of CTC levels to enhance patient prognostication could be utilized in the future. For example, some patients with a poor prognosis may require more aggressive treatment, as assessed in the ongoing ECLECTIC (NCT06195709) trial in the post-CDK4/6 setting, where the choice between further endocrine therapy and chemotherapy can be challenging in clinical practice. The negativity of treatment switch trials based on CTC dynamics should also be reevaluated with the availability of these new effective drugs, as the primary reason for the negativity of these trials was likely the lack of effective drugs in a resistant setting.

Conclusion

CTCs are a highly validated prognostic biomarker in BC, at baseline or during treatment. In addition to its prognostic value, CTC detection could be used in the future, especially in multiparametric liquid biopsy approaches, for analyses that are difficult with ctDNA, such as large molecular characterization (whole genome sequencing, RNA, epigenome, proteome, metabolome), or for the establishment of CTC-derived ex/in vivo models.

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Author contributions

T.T.B., J.Y.P., F.C.B., L.C., and N.K. wrote the manuscript. T.T.B., L.C., and N.K. designed the figures, which were approved by all authors. All authors have read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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