

# Chapter 12

## Prognostic Implications of CTC in Breast Cancer

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**Abstract** Circulating tumor cells (CTCs) represent an important conceptual link between a primary tumor and the development of metastatic disease, and in the setting of metastatic disease CTC have the potential to reveal important insights into the biology and behavior of the cells undergoing the metastatic process and contributing to the resistance and progression of disease over time. In breast cancer the enumeration of CTC has been demonstrated to be a strong prognostic factor for both progression and survival. The finding of elevated CTC after one cycle of cytotoxic chemotherapy are associated with a particularly poor prognosis, suggesting the need for innovative drugs and treatment strategies. Although prognosis can be important in treatment planning, enumeration of CTC has not yet led to predictive models for the selection of specific drugs or for when to stop or switch the current therapy. CTC can be effective in defining when and how often to perform radiographic extent-of-disease scans. Given the relatively modest impact the CTC enumeration has had on clinical care, the focus has been the development of new platforms to increase sensitivity to allow their detection in a larger fraction of patients and to allow biologic interrogation of these cells such that CTC might allow marker-driven treatment choices.

**Keywords** Circulating tumor cell • CTC • Breast cancer • Prognosis • Prediction • Liquid biopsy • Tumor marker

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## 12.1 Detecting Circulating Tumor Cells

The hematogenous spread of a primary tumor to distant sites has long been at the core of oncologic dogma [1, 2]. Conceptually circulating tumor cells are the link between the primary tumor and metastasis. The first published report [3] was in 1869 when T.R. Ashworth, an Australian physician performing an autopsy on a patient who had died of metastatic cancer, noted cells seen by light microscopy that were morphologically identical to cells taken from the tumor. He commented, “that if they came from an existing cancer structure, they must have passed through the greater part of the circulatory system to have arrived at the internal saphena vein of the sound leg.” Clearly, to be clinically useful, these cells need to be detectable earlier in the course of the disease, and they must have at least prognostic significance and preferably a predictive role in the management of the treatment of the disease. Understanding the biology of CTCs should lead to a better understanding of the metastatic process, and in addition should lead to a better understanding of the behavior of metastatic disease after it has been established. Ultimately the goal is to improve clinical outcomes for patients with cancer.

The rarity of circulating tumor cells has been the central challenge in the development of CTC platforms. The technical limitations and the assumptions on how to define and quantify these cells affect the types of clinical questions that can be addressed and the outcomes the resulting clinical investigations. CTCs are estimated to occur at an average frequency of 1 in  $10^6$  nucleated blood cells. Early attempts at isolating tumor cells generally used physical properties of cells through either filtration or density gradient centrifugation to isolate CTCs, but these methods were labor intensive and had inadequate sensitivity and specificity. These earlier assays were associated with a significant loss in CTCs, with a recovery of only 10–65 % cultured tumor cells spiked into whole blood [4, 5]. There also remains a need to distinguish these cells from leukocytes. The majority of leukocytes are removed during the isolation process, but many still remain. The distinction is made even more challenging by the recognition that normal hematopoietic cells can transiently express epithelial markers such as EpCAM [5], cytokeratins [6], MUC-1 [7], and TAG-12 [8]. In the case of immunofluorescent cell identification this raises the importance of actively excluding cells by the use of leukocyte-specific markers such as CD45. For platforms that utilize rt-PCR to detect gene expression, the choice of target gene selection is important to reduce the chance of false positive results that could occur as a result of transient epithelial markers in the contaminating leukocytes.

The development of immuno-selection and automated image analysis were two major advances that allowed the field to move forward. Immunomagnetic techniques allow recovery rates of approximately 85 % [9, 10] from blood samples spiked with cultured epithelial tumor cells. Studies using patient samples have varied significantly in methodology, but in general 40–60 % of patients with metastatic breast cancer are defined by these assays as being positive for CTC (Table 12.1). Notably there are some patients who are CTC negative by immunomagnetic assays throughout the course of their disease, although the biologic significance of this observation is not well understood.

**Table 12.1** Selected CTC clinical studies in metastatic breast cancer

Reference	Technology	CTC threshold	Clinical scenario	N	Patients with elevated CTC (%)	mPFS (months)	mOS (months)
Cristofanilli [11]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	177	49	2.7 (≥5) 7.0 (<5)	10.1 (≥5) >18 (<5)
Budd [12]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	138	43	NR	8.5 (≥5) 22.6
Cristofanilli [13]	CellSearch	≥5 CTC	PS, first-line therapy.	83	52	4.9 (≥5)	14.2 (≥5)
Cristofanilli [14]	CellSearch	≥5 CTC	Any type of therapy.	151	44	9.5 (<5) NR	>18 (<5) 13.5 (≥5)
Bidard [15]	Density centrifugation, cytokeratin staining	≥1 CTC	RS, Any line or type of therapy.	37	41	NR	29.3 (<5) Approx. 15 (≥1)
Liu [16]	CellSearch	≥5 CTC	PS, Any type or line of therapy.	74	35	3.2 (≥5) 5.1 (<5)	22 (<1) NR
Pierga [17]	CellSearch	≥1 CTC	PS, first line, chemotherapy.	267	65	Approx 8 (≥5)	Approx 22.5 (≥5)
Muller [18]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	254	50	10 (1-4) 20 (0)	Not reached for 0 or 1-4 CTC 18 (≥5) 27 (<5)
	Adna Test (Immunomagnetic isolation, rt-PCR detection)	>0.15 ng/μL			40	8.8 (+) 10.7 (-)	19.3 (+) 23.8 (-)

(continued)

**Table 12.1** (continued)

Reference	Technology	CTC threshold	Clinical scenario	N	Patients with elevated CTC (%)	mPFS (months)	mOS (months)
Wallwiener [19]	CellSearch	≥5 CTC	PS, Any line or type of therapy.	486	42	4.8 (≥5) 7.6 (<5)	18.0 (≥5) Not reached
Bidard [20]	CellSearch	≥5 CTC	RS (pooled analysis of 20 studies), Any line or type of therapy.		47	Approx. 6.5 (≥5) 11 (<5)	Approx 16.3 (≥5) >34 (<5)
Smerage [21]	CellSearch	≥5 CTC on day 1 and day 22	PS (cooperative group), first line, chemotherapy.	595	54	4.9 (≥5, ≥5) 8.9 (≥5, <5) 11.1 (<5, <5)	13 (≥5, ≥5) 23 (≥5, <5) 35 (<5, <5)

Multiple studies of CTC have been performed in metastatic breast cancer, but they vary by technology used, CTC threshold used to define patients as positive, whether they were prospective studies (PS) or retrospective studies (RS), by allowed lines of therapy, and allowed types of therapy. Although most provided Kaplan–Meier curves, not all reported the actual mPFS or mOS. In cases without formal reporting the mPFS and mOS have been estimated visually and reported here as “approximate.” NR indicated data not reported

These techniques led to the development of CellSearch® (Janssen Diagnostics, LLC), which is currently the only FDA-cleared platform for the enumeration of CTCs in patient care. This system is highly analytically validated [10]. The blood sample is prepared by adding an anti-EpCAM antibody that has been labeled with a magnetically active ferric particle. The red blood cells are lysed and the tube is then placed into a magnetic field that pulls the EpCAM positive cells to the edges of the tube, allowing removal of the lysed red cells and most of the unlabeled leukocytes. The sample is then suspended in a small volume of media and placed into a cartridge for viewing. Fluorescent stains for DNA (DAPI), cytokeratin, and CD45 are added to the sample, and the sample is then visualized via automated fluorescent microscopy. The computer identifies “events” that are DAPI positive, cytokeratin positive, and CD45 negative, and then presents them as images for a technician to review. This review is important to make confirm that these images have the morphology of a cell, and to assure that they are not a contaminating leukocyte. As a result of the analytic validity and the commercial availability, it became the largest source of clinical data. The reality that after 10–20 years of research there is only one FDA cleared CTC device speaks to the difficulty in creating a platform with both analytic and clinical validity.

This assay was initially studied in patients with metastatic cancer, and it utilizes a threshold of 5 or more CTCs in 7.5 ml of whole blood to define a patient as being positive for CTCs. This threshold was selected because it best separated patient populations based upon median progression free survival (mPFS). It was at a threshold of 5 CTC that Cox proportional-hazards ratio between the populations with slow versus rapid progression reached a plateau [11]. The use of a 5 CTC threshold also minimized the false positive rate in a normal control population without cancer. In this original publication, single epithelial cells are found in only a small number of individuals with an average of 0.1 epithelial cells per 7.5 ml blood, and none of the normal controls had and two or more epithelial cells. Three or more epithelial cells are only rarely found in people without cancer. The selected threshold affects both the questions that can be asked and the clinical outcomes observed. For example, in patients with early stage breast cancer, the frequency of CTCs is much lower, and in using a threshold of 5 CTC in 7.5 ml blood, only 3 % would be considered positive [22]. To achieve adequate sensitivity in the early stage breast cancer most investigators began using larger volumes of blood and a lower threshold to define positivity.

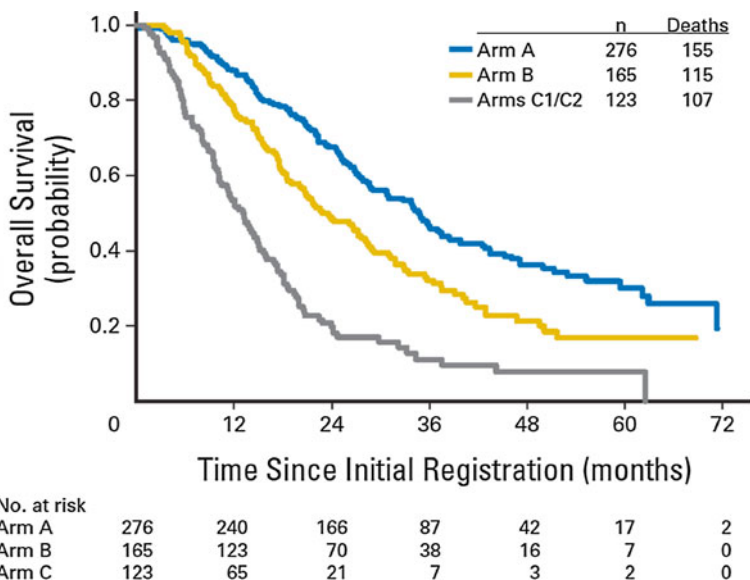
## 12.2 CTC and Prognosis in Metastatic Breast Cancer

Clinical decisions are a balance between risks and benefits, and in the care of patients with cancer prognosis is an important factor in this assessment. Cancer prognosis is important for many reasons. From the perspective of patients, prognosis is important so that one would know what to expect and so that appropriate planning can occur. From the perspective of clinical decision-making, it is important because prognosis is a significant component to the clinical assessment of risks and

benefits. In the adjuvant setting where the goal of therapy is cure, physicians and patients are more willing to accept increased short-term treatment toxicities if the disease risk is high. In the setting of incurable metastatic disease the goal of care is palliative, and if the prognosis is very poor with currently available drugs this might move physicians and patients toward either clinical trials of novel agents, or to less toxic therapy to maximize quality of life in the time that is remaining.

Multiple studies have demonstrated and confirmed the prognostic implications of CTC in patients with metastatic breast cancer. Patients with elevated CTC have a worse prognosis as measured by PFS and by overall survival (OS) (Table 12.1). Comparing the outcomes between different studies is difficult due to the use of different populations, different thresholds to define positivity, and difference in reporting of clinical outcome. The first published clinical study using CellSearch [11] evaluated 177 patients representing a wide cross-section of metastatic breast cancer, including patients starting hormonal therapy or chemotherapy and patients receiving first-line therapy or later-line therapy. Median PFS was significantly worse for patients with elevated CTC prior to initiation of a new therapy. Patients with elevated CTC at baseline had a mOS of 2.7 months compared to 7.0 months ( $p < 0.001$ ) for patients with low CTC at baseline. Similarly, survival was significantly worse with a mOS of 10.1 months vs. >18 months ( $p < 0.001$ ) for patients with elevated versus low CTC respectively. When looking at the subset of patients starting a new line of chemotherapy, the mPFS was 2.3 months vs. 6.8 months and mOS was 8.3 months vs. >18 months for patients with elevated versus low CTC. Original interpretation of the non-chemotherapy group was not as clear because it was a much smaller subset ( $n = 54$ ) and because the reported population received hormonal therapy, “immunotherapy,” or both. The largest group represented by “immunotherapy” was trastuzumab for HER2 positive cancer. The difference in PFS and OS in this heterogeneous small subgroup was not as great and was not statistically significant ( $p = 0.44$ ). However in a subsequent analysis [13] it was found that patients starting first-line endocrine therapy ( $n = 23$ ) had a notable difference in mPFS of 11.3 months vs. >18 months, although due to the small sample size it remained nonsignificant ( $p = 0.15$ ). It was this data that led to the FDA clearance of CellSearch. Subsequently, similar CTC data have been published in colon cancer [23] and prostate cancer [24] has led to the expansion of the FDA clearance to those cancers.

The prognostic significance of CTC is also true for patients initiating first-line chemotherapy. A retrospective subset analysis of the original study [13] evaluated patients starting first-line cytotoxic chemotherapy and revealed a mPFS of 2.7 months vs. 7.0 months ( $p < 0.001$ ) and mOS of 10.1 months vs. >18 months ( $p < 0.001$ ) for patients with elevated versus low CTC respectively. A separate study by Pierga and colleagues [17] evaluated 267 patients initiating first-line chemotherapy and showed qualitatively similar results. In this analysis patients were grouped by CTC levels into cohorts of patients with  $\geq 5$ , 1–4, and 0 CTC per 7.5 ml of whole blood. In this analysis the mPFS were 8 months, 10 months, 20 months respectively. Median OS had not been reached for the 0 and 1–4 CTC populations so comparison of mOS was not possible. These differences in prognosis for patients initiating first-line chemotherapy were confirmed by the prospective SWOG S0500



**Fig. 12.1** SWOG S0500 Overall survival by CTC group—Patients had CTC levels drawn at baseline. Patients with low CTC (<5 CTC per 7.5 ml whole blood) at baseline were observed on Arm A. Patients with elevated CTC at baseline had CTC level repeated on day 22. Those that converted to low after one cycle of first-line chemotherapy were observed on Arm B. Patients with elevated CTC at both baseline and after one cycle of therapy were randomized to different treatment strategies on Arms C1 and C2. There were no differences in outcome between C1 and C2 (see Fig. 12.3), and the overall survival presented here is the combined C1 and C2 population. Adapted from Smerage, J. et al., JCO (2014) 32: 3483–3489 [21] with permission. Copyright® 2014 Journal of Clinical Oncology. All rights reserved

study. In this study of 595 patients, 123 had elevated cells at both baseline and after one cycle of chemotherapy, 165 had elevated cells ( $\geq 5$  CTC/7.5 ml) at baseline but converted to low after the first cycle of chemotherapy, and 276 had low CTC at baseline. The mPFS were significantly different at 3 months, 9 months, and 11 months ( $p < 0.001$ ) and mOS were significantly different at 13 months, 23 months, and 35 months ( $p < 0.001$ ) respectively for the three groups (Fig. 12.1). Similar prognostic differences were seen in subgroups with ER positive, HER2 positive, or triple negative disease.

CTC may also be a better predictor of OS than radiographic staging studies. In a study 138 patients [25] with metastatic breast cancer, CTCs drawn about 4 weeks after starting a new therapy were compared to imaging done after a median of 10 weeks after the initiation of therapy. Inter-reader variability was greater for the radiologic evaluation compared to CTCs. For radiology there was a 15.2 % disagreement between interpreting radiologists when assessing radiographic status between indeterminate, stable disease or partial response, or progressive disease. There was only a 0.7 % disagreement in the assessment of CTC being <5 versus being  $\geq 5$ . Patients with non-progression (stable disease or response) on scans but

low CTC had a better mOS of 26.9 months compared with the patients who had non-progression on scans but elevated CTC with a mOS 15.3 months. Notably patients with progressive disease on scans but low CTC also had longer mOS of 19.9 months when compared to patients with both radiographic progression and elevated CTC with a mOS of 6.4 months. As with the results from the SWOG S0500 study, this suggests that elevated CTC after initiating therapy is a clinically important finding that reflects a particularly unfavorable tumor biology and general resistance to current therapeutic options.

### **12.3 CTC in the Monitoring of Metastatic Disease for Progression**

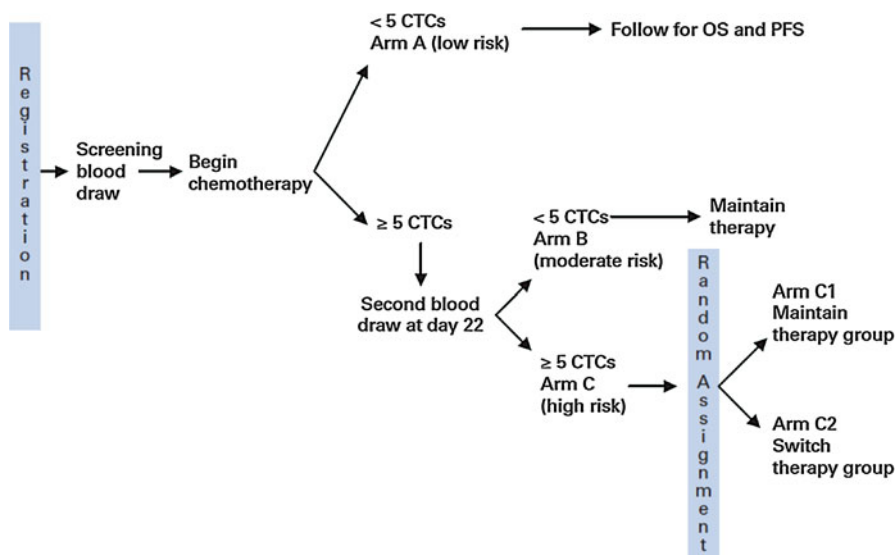
Several analyses have shown the monitoring of CTCs can predict patients whose cancers are beginning to progress or will progress in the near future. In a prospective study of 68 patients [16], CTC were collected at monthly intervals for 6 months and then every 3 months afterwards. Radiographic staging was performed every 3 months. Looking back from each staging scan, the investigators looked to see if the preceding CTC predicted the results of the imaging. Patients with elevated CTC 7–9 weeks prior to performing scans were 70 % likely to have progression on those scans. When performed 3–5 weeks prior to planned scans, CTC predicted a 60 % chance of progression on the scans. From the clinical perspective it is important to note that this means that 30–40 % did not have progression. Thus an elevated CTC value raises the concern for progression now or in the near future, but it does not define progression. A second similar study [26] followed 177 patients starting a new therapy for metastatic breast cancer and obtained CTC with each clinic visit (every 3–5 weeks). Of patients with elevated CTC at 3–5 weeks or 6–8 weeks after the first dose of therapy, 68 % and 53 % experienced progression within 3 months respectively. This population included both patients starting hormonal therapy and patients starting chemotherapy. It is possible that these two populations have different temporal patterns in their CTC levels related to the initiation of a new therapy and in their time to progression. Again, it is noted that a significant portion of these patients did not have progression on their subsequent scans. So the elevated CTC raised the possibility of progression but did not define progression. For patients with low CTC the risk of progression is much lower. If a patient continues to have low CTC, it might be reasonable to delay future scans. One clinical strategy would be to use CTC to determine how often to perform staging scans. Patients without significant symptoms or laboratory abnormalities might be able to delay scans as long as their CTC remain low, but as soon as the CTC rise to 5 or greater this would be an indication for further radiographic evaluation, even in the absence of symptoms. Such a strategy has the potential to reduce side effects from contrast agents, to reduce radiation exposure, to reduce patient inconvenience, and to save costs associated with radiographic scans.



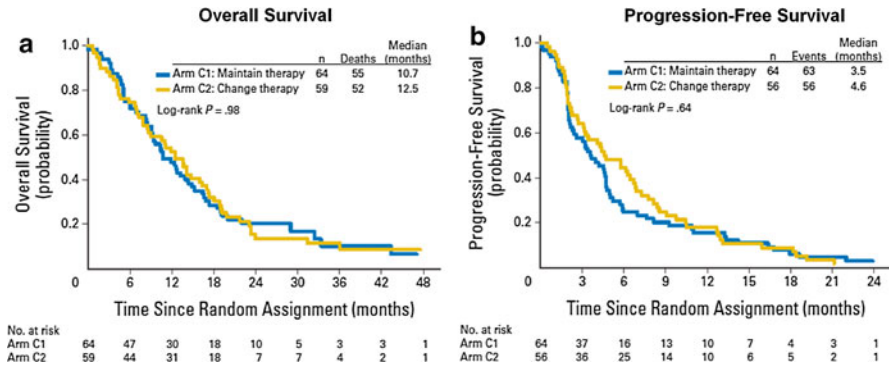
## 12.4 CTC in Randomized Interventional Studies

The SWOG S0500 clinical trial, referred to above in the discussion about CTC and prognosis, was a randomized phase III study testing the hypothesis that patients who have elevated CTC after starting a new therapy are likely on ineffective therapy and that they would experience improved outcomes by switching to an alternative treatment. The basis for this hypothesis came from the observation from the original IMMC-01 trial [13] that patients who have elevated CTC after one cycle of first-line chemotherapy had a very short mPFS of 2.0 months and mOS of 9.2 months. It appeared that these CTC were identifying patients who had cancers that were very likely resistant to that first therapy. Thus it was hypothesized that patients would have improved outcomes if they switched immediately to an alternate chemotherapy in a different drug class. By switching after one cycle of therapy, they would potentially avoid cumulative toxicities from the initial (and presumed ineffective) chemotherapy, and would have a greater chance of having a response by switching to a drug with a different mechanism of action.

SWOG S0500 enrolled a total of 595 patients who were about to initiate first-line chemotherapy for metastatic breast cancer. The choice of therapy was determined by the treating physician. The schema is shown in Fig. 12.2. Patients had CTCs measured prior to starting chemotherapy using CellSearch. Patients who had low cells were believed to have lower risk disease and were observed for outcome without intervention. Patients who had elevated CTC at baseline ( $\geq 5$  cells/7.5 ml



**Fig. 12.2** SWOG S0500 Clinical trial schema—Adapted from Smerage, J. et al., JCO (2014) 32: 3483–3489 [21] with permission. Copyright® 2014 Journal of Clinical Oncology. All rights reserved



**Fig. 12.3** SWOG S0500 Clinical outcomes for the randomized population—Patients with elevated CTCs ( $\geq 5$  CTC per 7.5 ml whole blood) at baseline and after one cycle of first line chemotherapy were randomized to either continue current therapy (Arm C1) or switch immediately to a new class of chemotherapy drug (Arm C2). Kaplan–Meier curves are presented for (a) overall survival and (b) progression-free survival. Adapted from Smerage, J. et al., *JCO* (2014) 32: 3483–3489 [21] with permission. Copyright® 2014 Journal of Clinical Oncology. All rights reserved

whole blood) had a repeat CTC evaluation 3 weeks after the first dose of chemotherapy. Patients who had converted to low CTC (4 or less) were observed for outcome without intervention. Patients who continued to have elevated CTC were randomized to continue their current chemotherapy (standard of care) or to switch immediately to a new class of cytotoxic chemotherapy. Both arms were then followed until progression. Disappointingly there was no difference in outcome for PFS ( $HR=0.92, p=0.64$ ) or OS ( $HR=1.0, p=0.98$ ) (Fig. 12.3). Given the differences in prognosis based upon CTC, the investigators concluded that patients who have elevated CTC after the first cycle of chemotherapy are highly likely to have cancers that are generally resistant to cytotoxic mechanisms. These patients need better treatment options and might derive more benefit from early consideration of clinical trials of novel agents rather than simply following standard sequential lines of cytotoxic therapies.

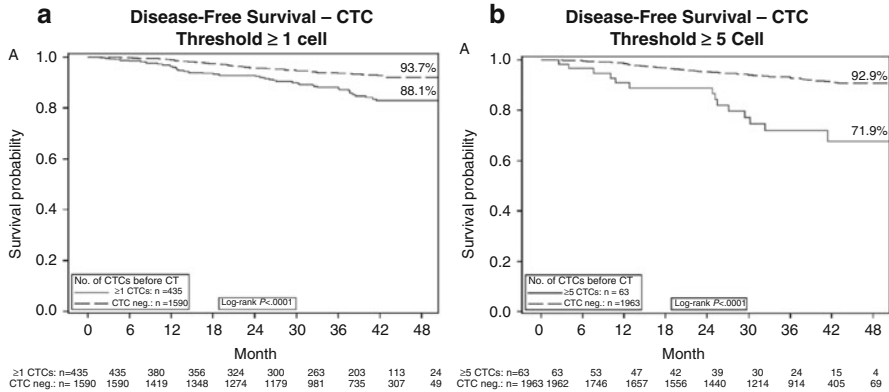
There are additional randomized clinical trials for which results have not yet been reported [27]. The *STIC CTC METABREAST Study* is using CTC to assess prognosis in patients with newly diagnosed metastatic ER positive, HER2 negative breast cancer who have not received prior treatment for metastatic disease. Enrolled patients are randomized between standard therapies versus CTC-directed therapy. For the standard therapy arm, the choice of initial treatment is made by the treating physician based upon clinical judgement. For patients on the CTC-directed treatment arm, patients with low CTC are considered at lower risk and proceed to endocrine therapy, and the patients with elevated CTC are considered high risk and are started on cytotoxic chemotherapy. The *CirCe01 Study* also randomized patients between a standard therapy arm and a CTC-directed therapy arm. For the standard arm, patients receive standard clinical and radiographic evaluations during the course of their therapy, and changes in therapy are based upon clinical definitions of

progression. For patients on the CTC-directed therapy arm, CTC are obtained after cycle 1 of any new therapy. If CTC are elevated after one cycle they would switch immediately to an alternate therapy without waiting for clinical signs of progression. This same assessment would occur after starting each new line of therapy. Thus unlike SWOG S0500, which only used CTC to direct therapy for the first line of therapy, CirCe01 uses CTC to evaluate all lines of therapy. The *DETECT III Study* is evaluating patients with metastatic HER2 negative breast cancer starting first through third lines of therapy, and CTC are being tested for HER2 expression. Patients whose CTC are found to overexpress HER2 are randomized to receive or not receive the oral anti-HER2 tyrosine kinase inhibitor lapatinib. The goal is to determine whether HER2 on CTC predicts response to anti-HER2 therapy. The *Treat CTC Study* is evaluating patients with HER2 negative early stage (non-metastatic) breast cancer who have completed neoadjuvant chemotherapy and breast surgery. Patients with detectable CTC are randomized to receive 18 weeks of adjuvant trastuzumab versus observation. The CTC will be tested for HER2 expression and correlated with clinical outcomes. The goal is to determine whether HER2 over-expression on CTC is predictive for benefit from anti-HER2 therapy. Randomized studies will be key to knowing whether directing therapy based upon CTC numbers or based upon CTC marker expression provides clinical benefit. All four of these studies utilize CellSearch for CTC evaluation. The results of these studies are anxiously awaited.

## 12.5 Predicting Recurrence in Early Breast Cancer

The use of CTC in early breast cancer (stages 1–3) has been more limited due to the lower number of patients with detectable cells and due to the overall lower concentration of cells in those patients that do have detectable CTC. The CellSearch platform typically requires either a lower threshold to define positivity or increased sample volumes of 30 ml when used in the early breast cancer setting. Other technologies such as rt-PCR may be more sensitive and may play a role in the adjuvant setting [28].

The SUCCESS study evaluated CTC using CellSearch in 2026 women starting adjuvant chemotherapy for stage I–III breast cancer. All women had CTC samples drawn prior to starting therapy, and 1492 had samples drawn at the completion of therapy. To achieve adequate sensitivity, this study used 30 ml of whole blood. Prior to starting chemotherapy 21.5 % had  $\geq 1$  CTC and 3.1 % had  $\geq 5$  CTC. Across the population the number of CTC ranged from 0 to 827 per 30 ml blood. After completing chemotherapy, 22.1 % of patients had one or more CTC, but only 1.9 % had 5 or more cells. With a median follow-up of 35 months, the presence of CTC prior to chemotherapy identified a population of women at significantly higher risk of recurrence. In the primary analysis using 1 CTC as the threshold of positivity, the 3-year recurrence-free survival was significantly worse for those with  $\geq 1$  CTC at 88 % versus those with no cells at 94 %,  $p = < 0.0001$  (Fig. 12.4, panel a). In an exploratory analysis, they looked at the threshold of  $\geq 5$  CTC and found a 3-year recurrence-free

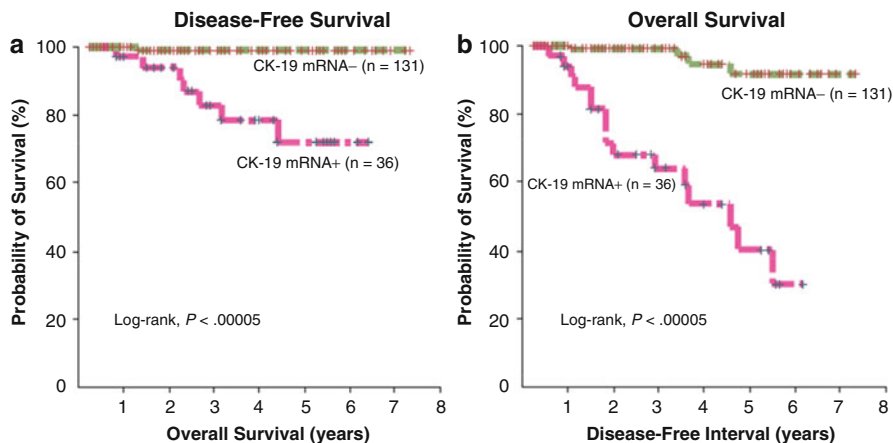


**Fig. 12.4** SUCCESS study—Prognostic value of CTC in early stage breast cancer—Patients with stage I–III breast cancer had CTC samples drawn prior to starting adjuvant chemotherapy. Sample volume of 30 ml is larger than volumes used in metastatic setting. Presented are Kaplan–Meier curves based on different thresholds of CTC positivity. (a) When CTC threshold of  $\geq 1$  CTC per 7.5 ml whole blood is used 24 % of population is positive. (b) When CTC threshold of  $\geq 5$  CTC per 7.5 ml whole blood is used 3 % of population is positive. Adapted from Rack, B. et al., JNCI (2014) 106: dju066 [22] with permission. Copyright® 2014 Journal of the National Cancer Institute. All rights reserved

survival of 72 % versus 93 %,  $p < 0.0001$  (Fig. 12.4, panel b). Notably 14 % of the patients with  $\geq 5$  CTC had died within 3 years, which is a very high 3-year mortality for early stage breast cancer. The choice of CTC threshold also had a significant affect on the number of patient considered high risk. In this study population, 435 patients (21 % of total population) had  $\geq 1$  CTC, whereas only 63 patients (3 % of total population) had  $\geq 5$  CTC.

Another study reported outcomes for 302 patients treated at MD Anderson for stage I–III breast cancer. All patients had a biopsy-proven diagnosis of invasive breast cancer but had not yet undergone definitive surgery. None received neoadjuvant chemotherapy. CTCs were drawn prior to surgery. Analysis was based upon a 7.5 ml volume of blood using the CellSearch platform. Twenty four percent of this patient population had  $\geq 1$  CTC. Patients with increasing numbers of CTC had higher risks of recurrence. When using a cut-off of  $\geq 1$  CTC the hazard ratio for recurrence was 4.0 ( $p = 0.02$ ), for  $\geq 2$  CTC the hazard ratio for recurrence was 8.2 ( $p < 0.0001$ ), and for  $\geq 3$  CTC the hazard ratio for recurrence was 11.5 ( $p < 0.0001$ ). The Kaplan–Meier recurrence-free survival estimates also demonstrated significant separation. This study also showed significant decrease in the number of patients deemed to have elevated cells based on the threshold used. Patients with  $\geq 1$  CTC represented 24 % of the population, but patients with  $\geq 2$  or  $\geq 5$  CTC only represented 10 % and 5 % of the population respectively.

Other technologies may be more sensitive in early stage breast cancer, and one study using rt-PCR to detect cytokeratin-19 (CK-19) transcripts was able to separate patients into low and high risk of recurrence. A population of 167 patients with node negative breast cancer were evaluated for CK-19 mRNA with samples collected prior to initiation of adjuvant systemic therapy [29]. The population was mixed in



**Fig. 12.5** Prognostic value of CTC detected by rt-PCR in patients with early stage breast cancer— (a) Progression-free survival and (b) Overall survival for 167 patients with lymph node negative early stage breast cancer based on the presence or absence of cytokeratins-19 messenger RNA (CK-19 mRNA) by quantitative RT-PCR (qRT-PCR) (Adapted from Xenidis, N. et al. JCO (2006) 24: 3756–3762 with permission. Copyright® 2006 American Society of Clinical Oncology. All rights reserved [29])

that some received chemotherapy, some endocrine therapy, and some both based upon tumor characteristics and standard of care risk assessment. Clinical outcomes were dramatically different depending upon the presence or absence of detectable CK-19 mRNA (Fig. 12.5). With a median follow-up of 32 months, 44 % of patients with detectable CK-19 mRNA experienced relapse compared to 3 % for those patient that were CK-19 mRNA negative. In addition, seven of the eight patients that died during follow-up were in the group with detectable CK-19 mRNA.

The threshold for defining positive CTCs is potentially more critical in this setting of early breast cancer because “circulating epithelial cells” can be identified in “normal” women and in women with benign breast diagnoses. Note that for the purpose of this discussion, these will be called epithelial cells rather than CTC because they are being isolated from women not known to have cancer. This finding complicates the interpretation of CTC in the early breast cancer setting because it introduces the risk of false positives when a low threshold is used to define patients as being positive for CTC. The numbers are generally small in patients without cancer, commonly being reported as an average of 0.1 CTC per 7.5 ml whole blood [10, 11, 30]. In one population of 145 “healthy” women CellSearch revealed that 5.5 % had one epithelial cells and none had two or more cells, and of 199 woman with benign breast diagnoses 7.5 % had one cell, 1 % had two cells, and 1 % had three cells [10]. In a second population of 84 individuals without cancer CellSearch revealed that 4.9 % had detectable cells, one with one cell, two with two cells, and one with three cells [22]. In a third population of 89 “healthy” women rt-PCR for CK19 revealed 2.2 % to be positive for the circulating epithelial marker [31]. There is no consensus on what level of CTC should define positivity in the early breast cancer, but caution should be used in using the low threshold of 1 CTC.

While CTC can identify early stage breast cancer patients at higher risk of recurrence, there are no studies demonstrating benefit from changing therapy. Notably, in both the SUCCESS study and the MD Anderson study all patients received adjuvant chemotherapy, and if appropriate, adjuvant endocrine therapy. Thus there are either few or no additional treatments within standard of care that could be added to augment the therapy that these patients received. The studies also differ in the timing of the CTC evaluation. The MD Anderson study evaluated the patients prior to surgery, and the SUCCESS study evaluated the patients after surgery. It is not known which time point is more clinically relevant. Thus this is a group of patients for which CTC are prognostic but do not yet drive therapy decisions. This is clearly a group that would benefit from new treatment options. Given their relatively high risk of recurrence, they would be good candidates for clinical trials of novel therapeutic approaches.

Despite the increased risk of recurrence for patients with detectable CTC, it is important to note that not all patients with detectable CTCs will have recurrence of disease. This is clear from the low recurrence rates seen in the SUCCESS and MD Anderson studies. This is an area of CTC biology that is poorly understood. One study by Meng and colleagues [32] suggests that CTC can persist for long periods of time in the circulation without progression to clinically evident disease. They evaluated CTC in a group of long-term survivors of early breast cancer who were all without evidence of recurrent disease. Included were 36 women who had been treated with mastectomy for early stage breast cancer 7–22 years prior to enrollment in this study. None had clinical or radiographic evidence of recurrence. Of 36 patients, 13 (33 %) had 1–2 Circulating Epithelial Cells in 10–25 ml of blood. Fluorescence in situ hybridization (FISH) demonstrated abnormalities in these CTC including aneusomy in Chromosomes 1, 3, 8, 11, and/or 17. These abnormalities suggest that these are cancer cells. A control population without a history of cancer was also investigated, and only 1 of 26 control individuals had a single detectable epithelial cell, and that cell did not have any detected aneusomy. All of the breast cancer patients in this study were far enough out from their original diagnosis that it is reasonable to conclude that most of them were cured of their breast cancer. However, it is unknown if any of these patients might experience a recurrence in the future. Breast cancer is a disease known to have some very late recurrences [33]. These persistent CTC might be an explanation for these late recurrences. If these are tumor cells, they may have acquired the needed mutations for uncontrolled growth and migration, but they need a “second hit” to be able to form actual tumor masses at distant sites.

## 12.6 New Technology Platforms

While determining prognosis is clearly important in cancer care, the true vision for CTC is to use them to predict sensitivity to therapeutic agents and to help determine the best next therapies. Many investigators would describe this as using CTC as a

minimally invasive biopsy or as a liquid biopsy. CTC represent the potential of a biopsy that can be repeated over time, such that changes in tumor biology can be monitored as the cancer mutates [34, 35]. In that context CTC research is currently at a crossroads between the enumeration of CTC versus the biologic characterization of CTC.

The majority of current clinical outcomes data is based on platforms and techniques that primarily had the capability of quantifying cells. These platforms have the limitation that only about half of the patients have detectable cells. This is an advantage if the goal is to count cells because it separates patients into two populations that can be compared based upon the number of cells. However, if the goal is to understand the biology of the patient's cancer, then at sensitivity of only 50 % is a disadvantage. The ideal assay for phenotype determination would be able to isolate cells from all patients.

The majority of assays developed over the past 1–2 decades relied on epithelial cell surface markers such as EpCAM and cytokeratins to identify CTC. The rationale was that most tumors are of epithelial origin and that hematopoietic cells should not express epithelial markers. While this is generally true, there is growing evidence that some of the most important cancer cells may not express these markers, including tumor initiating “stem cells” and cells that have undergone epithelial–mesenchymal transition (EMT). Given the loss of epithelial markers, these cells would not be identified by assays that rely on epithelial markers for either isolation or detection/visualization [36, 37]. There is also data to suggest that CTC that are captured in clusters may represent these more stem-like cells and may be associated with a worse prognosis [38].

To circumvent this limitation there are several new strategies in development that aim to increase the sensitivity of CTC isolation such that a higher proportion of patients have detectable cells and to collect CTC in ways that are not reliant on epithelial marker alone for the isolation. Most new platforms utilize microfluidics or microfiltration to increase yield. Early generation microfluidic devices continued to use anti-EpCAM antibodies for capture, but had significantly higher capture rates. For one device using microposts [39], CTC were recovered from 115 of 116 patients tested, across an array of cancer types including breast, prostate, lung, colon, and pancreatic cancer. CTC counts ranged from 5 to 1281 cells/ml of blood, and these cells were at 50 % purity, which represents a significant improvement in purity over the immunomagnetic platforms. The average volume of blood used per patient was 2.7 ml. None of 20 healthy volunteers had detectable cells by this assay. A second device using graphene oxide nanosheets [40], detected CTC in all patients of a 20-patient cohort with metastatic breast cancer, early stage lung cancer, and metastatic pancreatic cancer. All patients had  $\geq 2$  CTC per ml, and the average was 5 CTC per ml. None of six healthy controls had cells by this assay. In a third platform using a herringbone surface pattern to create microvortexes [41], 14 of 15 patients with metastatic prostate cancer or lung cancer had isolated cells with a median of 63 CTC/ml and a mean of 386 CTCs/ml. This platform was also described as having rare “false positive” findings in healthy volunteers. They reported a median of 1 cell/ml and a mean of 3 cells/ml in these normal volunteers. As a result of these

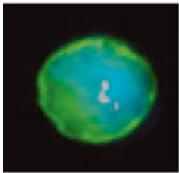
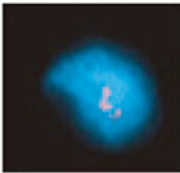
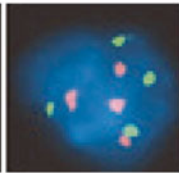
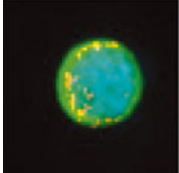
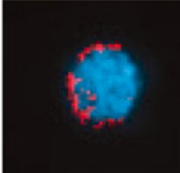
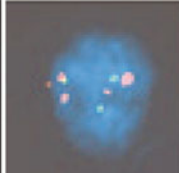
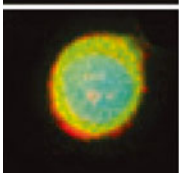
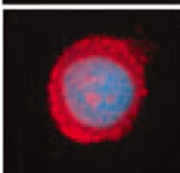
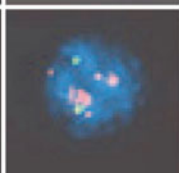
findings, the investigators set an initial threshold for positivity at 10 cells/ml of whole blood. These false positives also raise the concern for false positive marker analysis if noncancer cells are captured and evaluated for RNA or protein expression. Other microfluidic platforms are using novel mechanisms to isolate CTC without the use of epithelial markers. This includes techniques such as deterministic lateral displacement, inertial focusing and magnetophoresis, immunomagnetic depletion of leukocytes, dielectrophoresis, as well as a large number of novel materials and nanostructures [42–45]. In addition, microfiltration devices are also in development allowing sized-based isolation of CTC without reliance on epithelial markers for the isolation process. One such device is was able to identify CTC in 51 out of 57 patients testing, compared to 26 of 57 matched samples evaluated by CellSearch [46]. Cells isolated with this platform are also highly viable and easily cultured after isolation from spiked whole blood samples [47]. All of these platforms are still very early in the analysis of their analytic and clinical validity, but if successful these technological advances have the potential to transform the CTC from a prognostic marker to a predictive marker that allows individualized selection of therapy based upon the biology of an individual patient’s tumor.

## 12.7 Assessing Markers on CTC

Many biological markers have been detected on CTC. These include ER [48, 49], HER2 [32, 50–53], EGFR [54], MAGE [55], phosphorylated FAK [55], PI3K [55], androgen receptor [56], insulin-like growth factor [57], BCL2 and M30 [58, 59], and others. The comparisons of CTC immunohistochemistry and FISH analysis are quite remarkable. Meng and colleagues demonstrated the ability to perform both assays in breast cancer CTC (Fig. 12.6). With advances in technology even complex evaluations such as whole-genome sequencing and expression profiling are now becoming possible on single cells [61, 62], raising the possibility very sophisticated analyses of CTC, even when few in number.

The biologic interrogation of CTC raises the possibility of using CTC as a predictor of response to targeted therapies. As noted above, the *DETECT III Trial* and *Treat CTC Trial* are attempting to do this with HER2-targeted therapies. Another group is using this approach to develop a CTC-based assay to predict response to endocrine therapy in breast cancer [49]. In an ongoing prospective clinical trial, they are evaluating women initiating a new line of endocrine therapy for ER positive metastatic breast cancer. CTC are being collected and the cells stained for proteins associated with estrogen signaling, including ER, BCL-2, HER2, and Ki67. These markers are being combined to create an “endocrine therapy index.” Expression of these proteins will be correlated with time to progression and OS. The clinical goal is to develop a test that will help clinicians know whether to continue with sequential lines of endocrine therapy because the index predicts that the tumor cells remain sensitive, or whether to switch to chemotherapy because the index predicts resistance to endocrine therapy. This is another example of an innovative clinical trial that is attempting to move beyond just enumeration of CTC.



HER2 Ab and Cytokeratin Ab	HER2 Ab Alone	HER2 and CEP17 by FISH	IFI	FISH ratio
			1+	1.0
			2+	1.7
			3+	3.5

**Fig. 12.6** Evaluation of protein expression and chromosome aneusomy using CTC. Displayed are three different CTC, each in a horizontal row. Cells were evaluated for HER2 protein expression by fluorescent immunohistochemistry, cytokeratin protein expression by fluorescent immunohistochemistry, and HER2 and Chromosome 17 centromere (CEP17) by FISH. All cells are counterstained with the nuclear stain DAPI. Adapted from Meng, S. et al., PNAS (2004) 101: 9393–9398 [60] with permission. Copyright® 2004 Proceeding of the National Academy of Sciences. All rights reserved

## 12.8 Summary

The technology in the detection of CTC has evolved quickly over the past decade. The majority of current clinical data is from platforms primarily designed to count cells based upon expression of epithelial markers, and it has been clearly demonstrated that elevated CTC are associated with a worse prognosis for women with breast cancer. This includes both early stage cancer and advanced cancer. While understanding prognosis is an important aspect of clinical decision-making, the counting of CTC has not been able to provide insight into what drugs to use and when to change therapy. Based upon the results of the SWOG S0500 study, it is clear that women starting first-line chemotherapy who have elevated cells after one cycle of chemotherapy have a very poor prognosis with currently available treatment options. This suggests that this population should be considered for clinical trials of novel agents early in their course of treatment. Ongoing platform development now focuses on the biologic characterization of CTC with the hope that such characterization will allow rational selection of targeted agents. The newer

platforms allow characterization to be done more easily, and they have a much higher sensitivity, allowing detection and characterization of cells in almost all patients with metastatic breast cancer. The true clinical value of these innovations awaits further analytic and clinical validation.

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