

Chapter 2

CTCs in Solid Tumors. Clinical Applications of Circulating Tumor Cells in Breast Cancer



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2.1 Introduction

Breast cancer (BC) is one of the most studied types of cancer since the last century. For this reason, numerous studies have investigated the correlation between circulating tumor cells (CTCs) and BC [1].

When we consider using CTCs as a biomarker, it becomes necessary to differentiate early BC (eBC) from metastatic BC (mBC). About 70% of patients with mBC stage IV have >1CTC in 7.5 ml of blood, using CellSearch system to isolate and quantify CTCs. However, in eBC, using this system, we rarely detect CTCs, prompting doubts about its clinical use as a biomarker.

The objective of this chapter is to evaluate the validity and clinical applicability of CTCs in early and advanced BC [2].

2.2 Micrometastasis Biomarkers in BC

Before the use of CTC as a biomarker of micrometastasis in BC, various studies tried to use bone marrow tumor cells (BMTCs) as a viable biomarker.

In 4 of 8 studies analyzed by Bidard et al., in 2016 [1], there was a correlation between BMTCs and CTCs that reached up to 94%. This same study concluded that

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the dissemination of tumor cells in the patients' blood indicated an initial phase disease, while the detection of BMTCs indicated a more advanced disease [1].

In most mBC studies, the preferred method used to identify CTCs is the CellSearch system. This system relies on a semi-automated enrichment and immunostaining device that has been, to this day, the only validated method approved by the US Food and Drug Administration (FDA) to detect CTCs and for prognostication in metastatic colorectal, prostate, and breast carcinomas. This specificity was reliably documented in normal individuals and in patients with benign tumors [8]. CTCs were defined by the CellSearch system as those co-expressing EpCAM and CKs without expressing leukocyte common antigen CD45, and positive for 4',6-diamidino-2-phenylindole (DAPI) with a nucleus inside the cytoplasm and cell size $\leq 4 \mu\text{m}$. It is important to emphasize that CTC detection using the CellSearch system does not rely on any true morphological criteria, but rather on the magnitude of antibody fluorescent signal for CK, DAPI, and CD45. The CellSearch system is an epithelium-associated marker-dependent method; therefore, it faces technical problems similar to the PCR-based molecular method; its inability to identify epithelial–mesenchymal transition (EMT)-induced CTCs can give false-negative results [3–5].

Another well-cited method of detecting CTCs is the ISET (isolation by size of epithelial tumor cells) method. ISET methodology is a direct method for CTC and circulating tumor microemboli (CTM) identification, in which CTCs are isolated by filtration without use of tumor-associated markers, as a consequence of their large size relative to circulating blood leukocytes. This method is easy to perform, rapid, and inexpensive and makes it possible to directly isolate and count tumor cells in patients with different types of carcinomas, by cytopathological analysis [6].

A study commanded by Farace in 2011 [7] comparing CellSearch and ISET methods, using different metastatic carcinomas, demonstrated quite considerable discrepancies between the number of CTCs enumerated by the CellSearch and the ISET systems. In total, 30% of patients were negative according to CellSearch, while only 5% were negative using ISET. Interestingly, these discrepancies depended mostly on the patients' tumor type. Specifically, in patients with mBC, CTC counts were generally higher by CellSearch than by ISET. However, CTCs identified by CellSearch may not be true CTCs, because CTCs detected by CellSearch on the basis of the expression of an epithelial marker (EpCAM), which does not formally establish the malignant nature of circulating cells in the blood retained as CTC. Thus, the lower CTC counts obtained by ISET compared with CellSearch, most likely results from cell loss during the ISET procedure. It is important to state that this study did not compare the clinical relevance of both methods.

Although well-designed clinical trials are essential to further understand the clinical applications of ISET, this system could indeed represent a more accurate clinical tool for predicting patient's outcome in certain tumor types, and provide a significant advantage for performing molecular analyses in the era of personalized medicine.

A review conducted by Ma in 2013 [9], confirmed these results. They concluded that, overall, more CTCs were detected by ISET than by the CellSearch system, for two reasons: (1) the CellSearch system may not detect cells if they have undergone EMT (i.e., lack expression of CK and/or EpCAM), while ISET can be much more

efficient in isolating all rare cells of interest; (2) while ISET can isolate CTMs from metastatic cancer patients, the CellSearch cannot [10, 11]. Therefore, the detection of blood samples that only have CTMs will be underestimated by the CellSearch systems that use epithelial-marker-positive selection. However, the CellSearch system may overestimate CTCs in peripheral blood samples if they are contaminated with normal epidermal cells. In addition, the CTC detection efficiency varies in all relevant studies, whether by ISET or by CellSearch system. One of the main advantages of the CellSearch system is that it has the capacity to detect smaller CTCs than does ISET. On the other hand, the use of ISET for detection and identification of CTCs is more reliable than the CellSearch system and requires no expensive or special laboratory equipment. However, ISET is not sufficiently standardized in its current form to be routinely applicable in clinical practice (please see some pictures of CTCs isolated from metastatic breast cancer patients in Figs. 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 2.12, and 2.13).

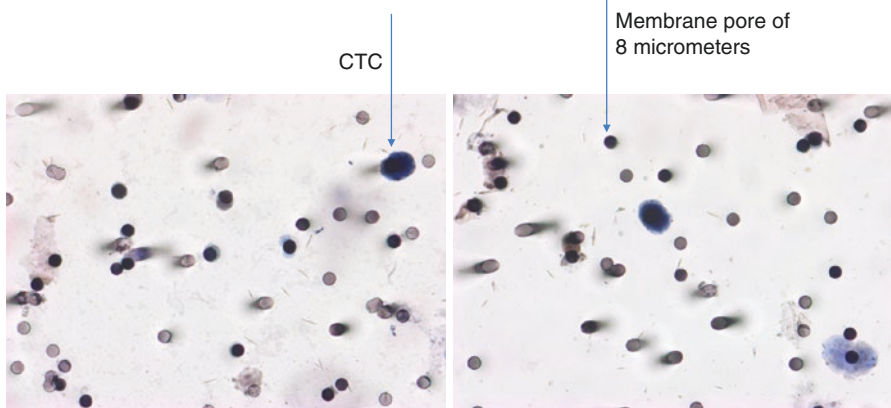


Fig. 2.1 CTCs from a woman, 53 years old, whose primary tumor was HER-2 positive. She had brain metastasis. The CTC count was 5 CTCs/mL. Her CTCs did not stain for HER-2

Fig. 2.2 CTCs from the same patient of Fig. 2.1. CTCs were collected around 4–5 weeks after radiotherapy for brain metastasis. CTC count: 3.0 CTCs/mL

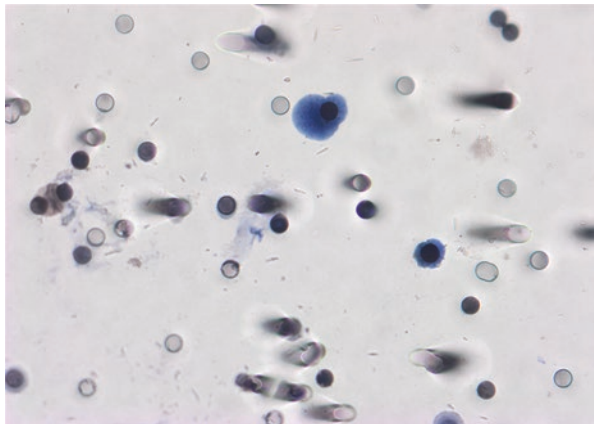


Fig. 2.3 Patient with 44 years old. CTCs were collected before the beginning of radiotherapy for brain metastasis. Her primary tumor was HER-2 positive. CTC count: 1.5 CTCs/mL, without HER-2 staining

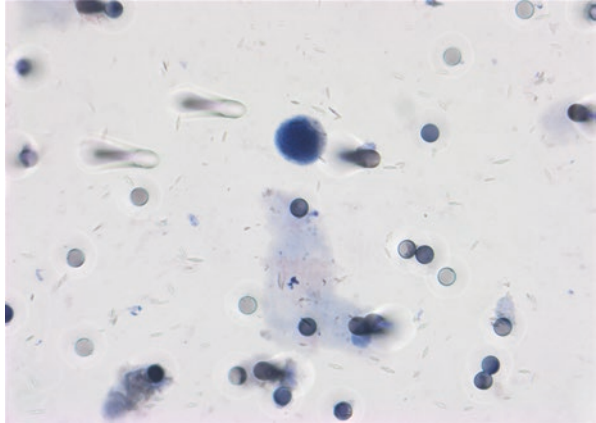


Fig. 2.4 CTCs from patient of Fig. 2.3. CTCs were collected around 4–5 weeks after radiotherapy for brain metastasis. CTC count: 1.5 CTCs/mL. We can observe the presence of a hyperchromatic nucleus, irregular, with irregular chromatin. Also note the abundant cytoplasm, not commonly seen in hematopoietic cells. In brown: positive staining with DAB for STGAL

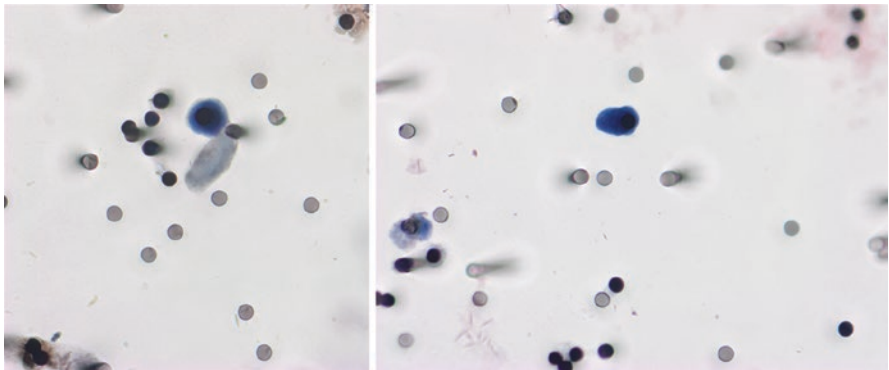
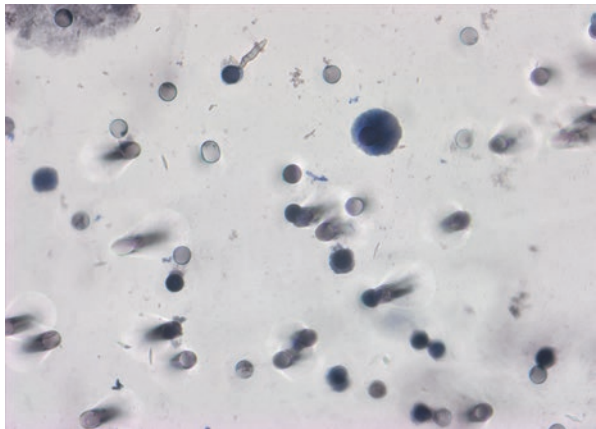


Fig. 2.5 Patient with 57 years old. CTCs were collected before the beginning of radiotherapy for brain metastasis. Her primary tumor was HER-2 positive. CTC count: 0.75 CTCs/mL. On the right, we can observe the presence of a hyperchromatic nucleus, irregular, with irregular chromatin. Also note the abundant cytoplasm, not commonly seen in hematopoietic cells. CTC stained with HER-2

Fig. 2.6 Patient with 56 years old. CTCs were collected around 4–5 weeks after radiotherapy for brain metastasis. Her primary tumor was HER-2 positive. CTC count: 2.0 CTCs/mL. CTCs did not stain for HER-2

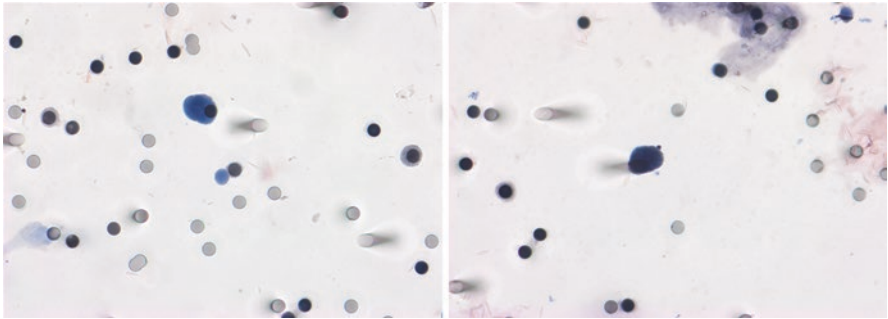
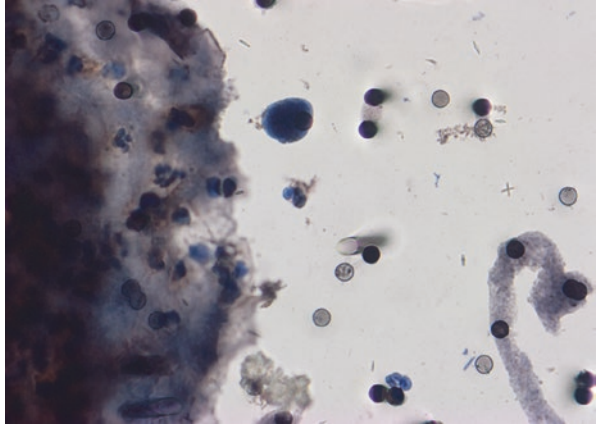


Fig. 2.7 Patient with 40 years old. CTCs were collected before the beginning of radiotherapy for brain metastasis. Her primary tumor was Luminal B. CTC count: 3.5 CTCs/mL (microscope: 20×)

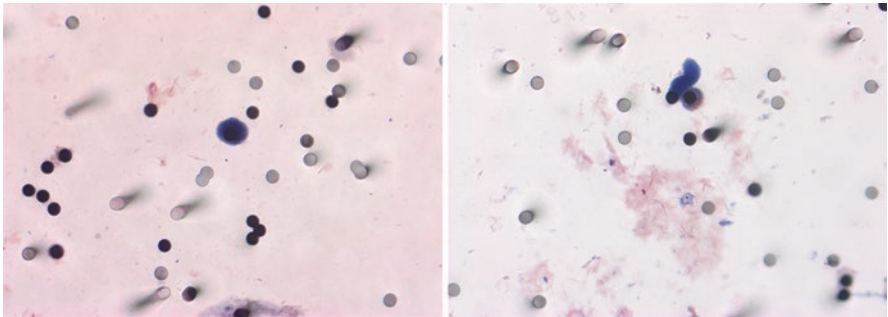


Fig. 2.8 Patient with 46 years old. CTCs were collected around 4–5 weeks after radiotherapy for brain metastasis. Her primary tumor was Luminal B. CTC count: 3.5 CTCs/mL (microscope: 20×)

Fig. 2.9 Photo from same patient Fig. 2.8 showing a cohesive group of neoplastic cells, with planetary aggregation, forming neoplastic impaction. Individually, isolated neoplastic cells are noted with alteration of the nuclear/cytoplasmic ratio and irregularity of chromatin (microscope 40x)

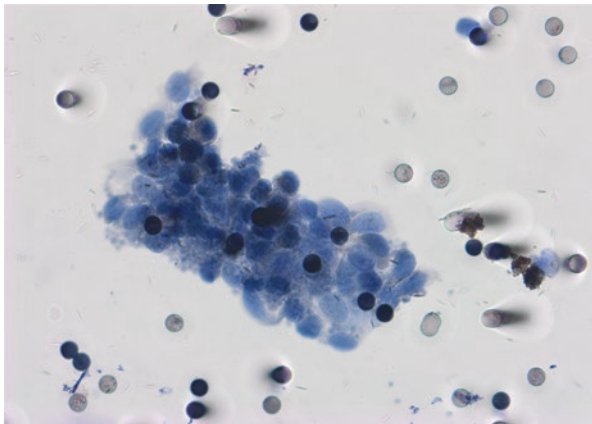


Fig. 2.10 CTM from a patient with 42 years old. CTCs were collected before the beginning of radiotherapy for brain metastasis. Her primary tumor was Luminal B. CTC count: 1.75 CTCs/mL

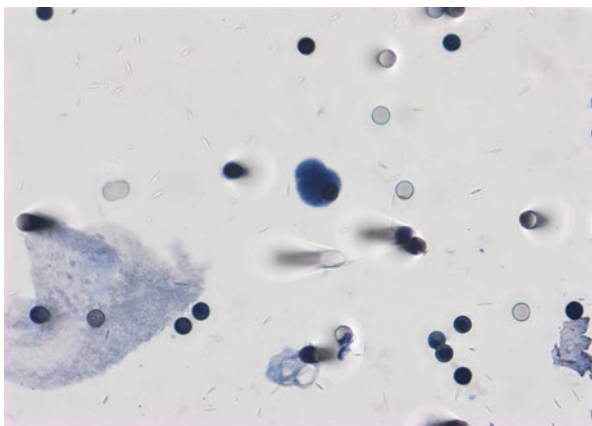
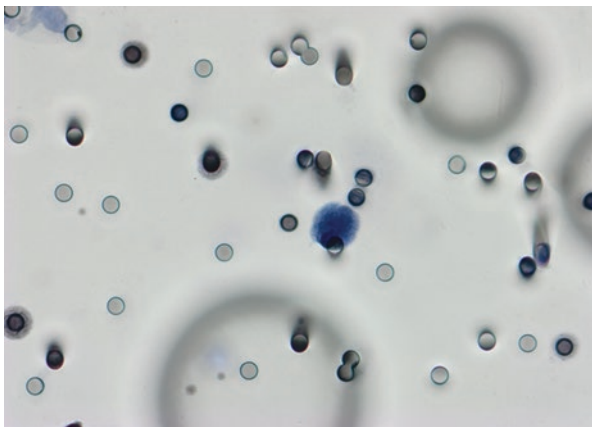


Fig. 2.11 Patient with 61 years old. CTCs were collected around 4–5 weeks after radiotherapy for brain metastasis. Her primary tumor was Luminal B. CTC count: 8.75 CTCs/mL



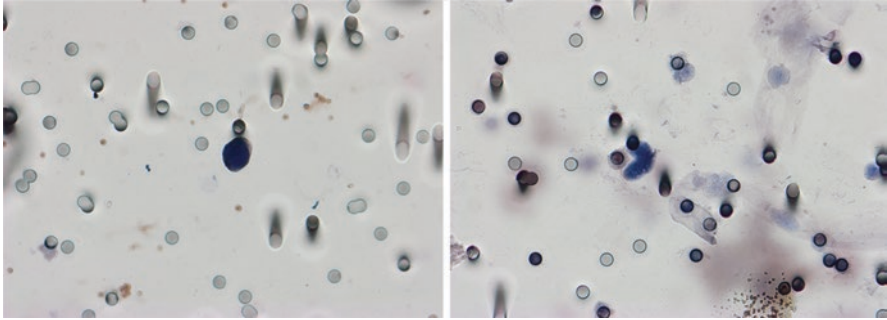


Fig. 2.12 Same patient of Fig. 2.11 in brown : immunocytochemistry with anti-Notch antibody visualized with DAB. Here, we can see a CTC without any staining

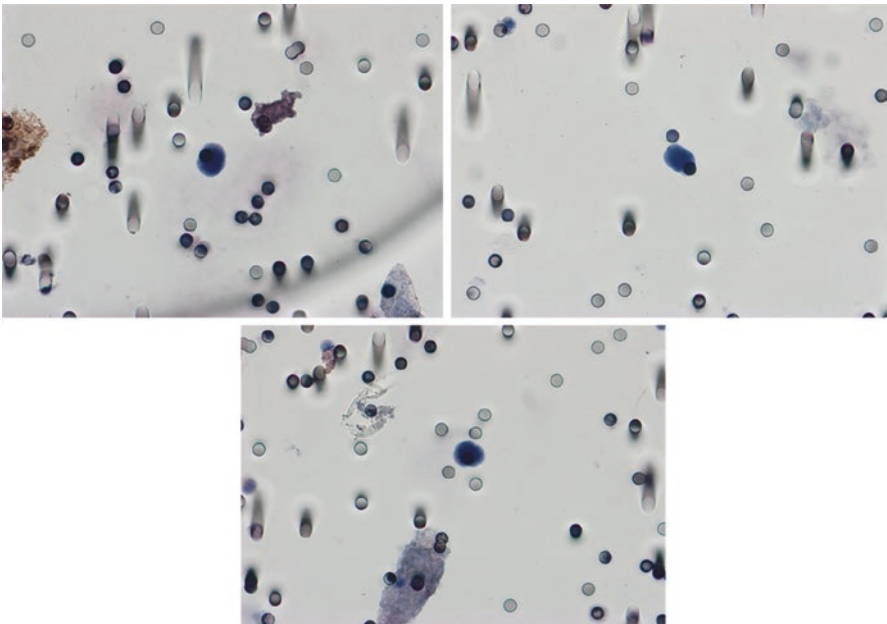


Fig. 2.13 Same patient of Fig. 2.11

2.3 Metastatic BC

2.3.1 Clinical Validity of CTCs in mBC

In contrast to that observed in eBC, there is enough evidence to utilize CTCs as a biomarker in mBC.

A study conducted by Cristofanilli in 2004 [12], utilizing the CellSearch® system to detect CTCs, analyzed the number of CTCs in patients with mBC. Before

initiating a new treatment, patients underwent an evaluation of metastatic sites by means of standard imaging studies and the collection of a blood sample to be used for the enumeration of circulating tumor cells. A different blood sample was collected at the first follow-up visit, approximately 3 to 4 weeks after the initiation of the new therapy. Disease status follow-ups were made every 9 to 12 weeks, utilizing the same techniques used at baseline. This disease status was assessed without knowledge of the levels of CTCs. An alternate control group made up of 72 premenopausal healthy women and 73 postmenopausal healthy women without known illnesses and no oncologic history, 99 women with benign breast diseases, and 101 women with other nonmalignant diseases. The respective testing laboratories were aware that the samples were from a control group, but were unaware to the difference between no known illness and benign conditions.

A worse prognostic relation was established in patients with a high number of CTCs in both instances, when compared to those with a low number of CTCs pre-CT and after one cycle. Interestingly, patients with a high CTC count pre-CT, but with a low count after one cycle, had a similar prognostic value to those with a low pre-CT count. These results were corroborated by Hayes in 2006 [13].

Finally, an analysis of 1944 individuals indisputably established the superiority of using CTC count in comparison to traditional tumor markers, such as CEA and CA15, as a treatment response biomarker in patients with mBC [14].

2.4 Clinical Applicability of CTC in mBC

In a retrospective study conducted by Cristofanilli in 2018 [15], 2436 patients with mBC from 18 cohort studies were analyzed. These patients were arranged in accordance to their tumor's biomolecular type, location, and previous treatments. A cut-off point of 5 CTCs per 7.5 ml of blood was established. Thus, a $> 5\text{CTC}/7.5\text{ mL}$ count was determined as IV aggressive (IVa) and $<5\text{CTC}/7.5\text{ mL}$ count as IV indolent (IVi).

Patients IVi had a higher median overall survival, when compared to those stage IVa (36.3 months vs. 16.0 months, $p < 0.0001$). Furthermore, patients IVi had a higher overall survival in all tumor subtypes when compared to IVa: positive hormone receptor (44 months vs. 17.3 months, $P < 0.0001$), HER2-positive (36.7 months vs. 20.4 months, $P < 0.0001$), and triple-negative (23.8 months vs. 9.0 months, $P < 0.0001$). Similar results were obtained independent of previous treatment or tumor location [15].

2.5 Early BC

2.5.1 *CTCs as a Micrometastasis Marker in Patients with eBC Treated with Neoadjuvant Therapy*

Measuring CTCs in patients, submitted to neoadjuvant chemotherapy (CT), intents on evaluating if the micrometastasis process has started and possibly evaluating its response to QT.

The IMENEO meta-analysis observed a significant association between T staging and CTCs ($P < .001$), using CellSearch system. Excluding tumors T4d from analysis, they observed that a positive CTC result was detached from clinical or pathological characteristics of the initial tumor. The positivity was 21.4% and 24.2% in patients with negative and positive lymph nodes, respectively. This study also showed that there was a statistically significant drop of CTC count at the end of neoadjuvant QT ($p < 0.001$). Furthermore, the CTC count pre-QT presented itself as a strong independent indicator of distant metastasis (hazard ratio [HR]: 3.73, 95% confidence interval [CI] = 2.82–4.90), overall survival (HR: 3.93, 95% CI = 2.81–5.45) and local relapse (HR: 3.02, 95% CI = 1.88–4.75) [16]. Curiously, the survival impact was directly related to the number of CTCs detected, suggesting the use of CTCs as a quantitative biomarker in BC (see some examples in Table 2.1).

2.5.2 *CTC as a Micrometastasis Marker in Patients with eBC Treated with Adjuvant Therapy*

In the context of adjuvant therapy in eBC, a multicentric randomized German study, SUCCESS-A, which tested CTCs in patients eligible to receive adjuvant CT, correlated the positivity of CTC to the lymph node status. This study confirmed that CTCs are an independent factor for disease-free survival (HR: 2.11, 95%CI = 1.49–2.99) and overall survival (HR:2.18, 95%CI = 1.32–3.59). Finally, a high CTC count was associated with worse prognosis, validating the use of CTCs as a quantitative biomarker [17]. The recently published 2-year follow-up of this study showed that those patients that had a positive CTC count after 2 years of treatment had a risk 3.9 times higher of death and 2.3 times higher of relapse in the multivariate models, when compared to those that had a negative result; all these results were true in those patients with HER2-negative BC [18].

In 2018, Sparano et al. [19] conducted a study that analyzed the recurrence of CTC detection after 4.5–7.5 years of follow-up in patients with HER2-negative BC

Table 2.1 Studies with CTCs in breast cancer

Tumor	Stage	No. Patients	Method	Markers	Authors	Year	Main results	Abbreviations
Breast	IV	177	CellSearch™ method (Menarini Silicon Biosystems, LLC)	CD45 and expressing cytokeratin	Cristofanilli et al.	2004	CTCs \geq 5 per 7.5 ml of whole blood, compared with the group with $<$ 5 circulating tumor cells per 7.5 ml, had a shorter median progression-free survival ($P < 0.001$) and shorter overall survival ($P < 0.001$). At the first FU visit after the initiation of therapy, this difference between the groups persisted ($P < 0.001$). The levels of CTCs at baseline and at the first FU visit were the most significant predictors of progression-free and overall survival	CTC: circulating tumor cells
Breast	IV	177	CellSearch™ method (Menarini Silicon Biosystems, LLC)	CD45 and expressing cytokeratin	Hayes et al.	2006	Median PFS times for patients with $<$ 5 CTC were 7.0, 6.1, 5.6, 7.0, and 6.0 months, respectively. For patients with \geq 5 CTC, median PFS was significantly shorter: 2.7, 1.3, 1.4, 3.0, and 3.6 months, respectively. Median OS for patients with $<$ 5 CTC was all $>$ 18.5 months. For patients with \geq 5 CTC, median OS was significantly shorter: 10.9, 6.3, 6.3, 6.6, and 6.7 months, respectively. Median PFS and OS times at baseline and up to 9 to 14 weeks after the initiation of therapy were statistically significantly different	FU: follow-up
Breast	IV	1944	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HER2 and HR	Bidard et al.	2014	46-9% patients had a CTC count of 5 per 7.5 mL or higher at baseline, which was associated with decreased progression-free survival ($p < 0.0001$) and overall survival ($p < 0.0001$) compared with patients with a CTC count of less than 5 per 7.5 mL at baseline. Increased CTC counts after start of treatment, adjusted for CTC count at baseline, was associated with shortened progression-free survival ($p < 0.0001$) and overall survival (HR 2.26)	PFS: progression-free survival

Breast	Early	2026	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HR	Rack et al.	2014	<p>Before chemotherapy, CTCs were detected in 21.5% of patients, with 19.6% of node-negative and 22.4% of node-positive patients showing CTCs ($P < .001$). No association was found with tumor size, grading, or hormone receptor status. After chemotherapy, 22.1% of patients were CTC positive. The presence of CTCs was associated with poor disease-free survival (DFS; $P < .0001$), distant DFS ($P < .001$), breast cancer-specific survival ($P = .008$), and overall survival (OS; $P = .0002$). CTCs were confirmed as independent prognostic markers in multivariable analysis for DFS ($P < .0001$) and OS ($P = .002$). The prognosis was worst in patients with at least five CTCs per 30 mL blood (DFS: HR = 4.51, OS: HR = 3.60)</p>	OS: overall survival
Breast	NI or High-risk node-negative HER2-negative	4994	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HR	Sparano et al.	2018	<p>A positive CTC assay result was associated with a 13.1-fold higher risk of recurrence (hazard ratio, 13.1). 30.4% patients with recurrence had a positive CTC assay result at a median of 2.8 years before clinical recurrence. The CTC assay result was also positive for 4.1% patients with hormone receptor-negative disease, although only 1 patient experienced disease recurrence (this patient was CTC negative).</p>	
Breast	IV (indolent and aggressive)	2436	CellSearch™ method (Menarini Silicon Biosystems, LLC)	Estrogen receptor, progesterone receptor, and HER2	Cristofanilli et al.	2018	<p>Stage IVi patients had longer median OS than those with Stage IVa ($P < 0.0001$) and similarly for de novo MBC patients ($p < 0.0001$). Moreover, patients with Stage IVi disease had significantly longer OS across all disease subtypes compared to the aggressive cohort: hormone receptor-positive ($P < 0.0001$), HER2-positive ($P < 0.0001$), and triple negative ($P < 0.0001$)</p>	IVi = indolent // IVa = aggressive

(continued)

Table 2.1 (continued)

Tumor	Stage	No. Patients	Method	Markers	Authors	Year	Main results	Abbreviations
Breast	I, II, III	2774	CellSearch™ method (Menarini Silicon Biosystems, LLC)	Hormone receptor +/- [HER2-], HER2+, triple-negative	Bidard et al.	2018	CTC detection revealed one or more CTCs in 25.2% of patients before NCT; this was associated with tumor size ($P < .001$). The number of CTCs detected had a detrimental and decremental impact on OS ($P < .001$), distant disease-free survival ($P < .001$), and locoregional relapse-free interval ($P < .001$), but not on pathological complete response. Patients with one, two, three to four, and five or more CTCs before NCT displayed hazard ratios of death of 1.09 (95% confidence interval [CI] = 0.65 to 1.69), 2.63 (95% CI = 1.42 to 4.54), 3.83 (95% CI = 2.08 to 6.66), and 6.25 (95% CI = 4.34 to 9.09), respectively. In 861 patients with full data available, adding CTC detection before NCT increased the prognostic ability of multivariable prognostic models for OS ($P < .001$), distant disease-free survival ($P < .001$), and locoregional relapse-free interval ($P = .008$)	Neoadjuvant chemotherapy (NCT)
Breast	Early stage, high risk	1087	CellSearch™ method (Menarini Silicon Biosystems, LLC)		Trapp et al.	2019	Two years after chemotherapy, 198 (18.2%) patients were CTC-positive. The median follow-up after this timepoint was 37 months. Cox regressions that included CTC status at baseline revealed that CTC status 2 years after chemotherapy had statistically significant and independent prognostic relevance for OS (hazard ratio [HR] = 3.91, 95% confidence interval [CI] = 2.04 to 7.52, $P < .001$) and DFS (HR = 2.31, 95% CI = 1.50 to 3.55, $P < .001$)	HR: hormone receptor

Breast	IV	254	AdnaTest BreastCancer and CellSearch	HER2	Fehm et al. DETECT III	2010	Using the CellSearch assay, 50% of patients had ≥ 5 CTCs, and HER2-positive CTCs were observed in 41% of these patients. 39% patients were CTC positive using AdnaTest BreastCancer, and HER2 positivity rate was 47%. The rate of breast cancer patients with HER2-negative primary tumors but HER2-positive CTCs was 32% and 49% using the CellSearch assay and AdnaTest BreastCancer, respectively. Concordant results regarding HER2 positivity were obtained in 50% of the patients (31/62) ($P = 0.96$, $\kappa = -0.006$)	RFS: recurrence-free survival
Breast	High risk early breast cancer	206	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HR	Janni et al. SUCCESS A	2018	The CTC status was positive in (7.8%) patients. Overall, 13 late recurrences were observed; 11 in 153 hormone receptor-positive patients and 2 in 53 hormone receptor-negative patients. In hormone receptor-positive patients, CTC status was a significant prognostic factor for RFS in univariable (hazard ratio [HR] 5.14, 95% confidence interval [CI] 1.47–18.03, $p = 0.011$) and in multivariable cox regressions adjusted for age, tumor stage, nodal stage, grade, histological type, and HER2 status (HR 5.95, 95%CI 1.14–31.16, $p = 0.035$)	NT: neoadjuvant treatment

(continued)

Table 2.1 (continued)

Tumor	Stage	No. Patients	Method	Markers	Authors	Year	Main results	Abbreviations
Breast	III	287	CellSearch™ method (Menarini Silicon Biosystems, LLC)	HER2	Riehdorf et al. GearQuattro Trial	2010	We detected ≥ 1 CTC/7.5 mL in 21.6% of patients before NT and in 10.6% after NT ($P = 0.002$). 15.0% initially CTC-positive cases were CTC-negative after NT, whereas 8.3% cases were CTC-positive after NT. CTC detection did not correlate with primary tumor characteristics. Furthermore, there was no association between tumor response to NT and CTC detection. HER2-overexpressing CTC were observed in 24.1% CTC-positive patients, including 8 patients with HER2-negative primary tumors and 3 patients after trastuzumab treatment. CTC scored HER2-negative or weakly HER2-positive before or after NT were present in 11 of 21 patients with HER2-positive primary tumors. HER2 overexpression on CTC was restricted to ductal carcinomas and associated with high tumor stage ($P = 0.002$).	
Breast, Prostate and Lung Carcinomas	IV	60	CellSearch™ method (Menarini Silicon Biosystems, LLC) and ISET (Rarecells, France)		Farace et al.	2011	Concordant results between CellSearch and ISET were obtained in 55% (11 out of 20) of the patients with breast cancer, in 60% (12 out of 20) of the patients with prostate cancer and in only 20% (4 out of 20) of lung cancer patients	

that received primary surgical treatment, followed by adjuvant CT. In the multivariate models, a positive CTC was associated with a risk 13.1 times higher of recurrence in patients with positive hormone receptors (HR: 13.1, 95% CI = 4.7–36.3). No patients with negative hormone receptors and positive assay had a recurrence of CTC (0%, 95% CI = 0% to 37%).

The TREAT-CTC trial was the first attempt to try to demonstrate the clinical applicability of CTCs in patients with eBC. This study also tried to evaluate if the addition of a new adjuvant therapy (Trastuzumab) would help to elongate the relapse-free interval in patients with a positive CTC count. This study, therefore, concluded the following: (1) CTC-based screening is feasible in the adjuvant setting of early breast cancer. (2) CTC-positive patients do have a higher risk of relapse. (3) Trastuzumab has no effect on CTCs in HER2-negative BC [20–23].

Therefore, the use of CTCs as an evaluating tool of metastatic risk in eBC still needs further scientific comprobaton. However, it is highly probable that the number of CTCs will have a significant impact as a prognostic and metastatic biomarker in eBC [1].

2.6 Conclusion

The use of CTCs as a prognostic factor in early and mBC has been shown to be quite significant. Despite the detection of CTCs in eBC being a rare event, its clinical validity as a prognosis marker has reached the highest level of scientific evidence. However, its clinical applicability is still a subject to be studied.

Focusing on adjuvant treatments such as radiotherapy, QT, and hormonal therapy, and associating these with new detecting techniques and with new biomarkers such as circulation tumor DNA, will possibly reveal new treatments and early micrometastasis diagnosis [24, 25].

And finally, when we are talking about patients with mBC, the quantitative and qualitative CTC analysis must be considered an important tool with prognostic and therapeutic implications.

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