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Circulating Tumor Cells in Breast Cancer: A Potential Liquid Biopsy

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Abstract

Circulating tumor cells (CTCs) have emerged as a new generation of liquid biomarker that allows for noninvasive longitudinal disease monitoring. CTCs represent a rare cell population in the blood, surrounded by billions of hematopoietic cells. Due to the rarity of CTCs in the blood, the isolation of pure CTCs' populations has proven to be challenging. However, a number of new technologies have emerged using CTCs cytometric/immunological and physical characteristics. Currently, patients with greater than 5 CTCs have a shorter progression-free survival, as compared with those with less than 5 CTCs per 7.5 ml of whole blood. Although the CTC count itself is an independent prognostic marker, the field is shifting toward understanding metastasis-relevant marker expression on CTCs for the improvement of the prognostic significance of CTCs. This chapter first introduces the principles of CTC isolation and detection methods, then the clinical utility of CTCs for prediction of prognosis and therapy response. Lastly, the heterogeneity of CTCs will be discussed.

Keywords: circulating tumor cell (CTC), breast cancer, liquid biomarker, metastasis

1. Introduction

Breast cancer is the most commonly diagnosed malignancy as well as one of the leading causes of cancer deaths. Treatment strategies in early-stage breast cancer are directed toward radical cure and prevention of recurrence or the development of metastatic diseases. However, once metastatic disease has been detected, alleviation of symptoms or palliative care becomes the focus, with the aim of extending overall and disease-free survival (DFS). Current methods of disease monitoring are limited to radio-imaging of detectable metastatic lesions and/or eleva-

tion of tumor markers in the serum. Due to a lack of sensitive and specificity, accurate disease monitoring remains a challenge. Recently, however, circulating tumor cells (CTCs) have received significant attention as a new class of "liquid biopsy" that would enable longitudinal and noninvasive disease monitoring in order to capture an overall snapshot of individual disease.

The presence of cancer cells in the circulation was recognized as early as the nineteenth century, when the Australian physician Thomas Ashworth detected the presence of cells in the blood that were similar to those from the primary tumor of a woman with metastatic breast cancer (MBC) [1]. The early 1900s yielded few descriptions of the isolation of tumor cells from the blood [2, 3]. In 1960, Alexander and Spriggs undertook cytopathologic analysis for the presence of CTCs in the blood of 140 cancer patients of various sites [4]. Although CTCs were detected in only seven of these cases, each of those patients had a markedly short survival of only several months, reinforcing the rarity as well as the potential clinical significance of CTCs. In addition to these early clinical investigations, parallel efforts using animal models to elucidate the process of cancer cell dissemination have further highlighted the clinical relevance of CTCs [5, 6]. Despite growing awareness throughout the twentieth century of CTCs' potential impact, their clinical implication was not robustly examined until the early 2000s. In 2002, Fehm et al. conducted cytogenetic analyses of cells obtained from the blood of cancer patients of several types, including breast, and compared chromosome profiles to those from their primary tumors, finding similar malignant features and chromosomal abnormalities between the two. The first large, multi-institutional clinical study evaluating the prognostic value of CTCs in patients with MBC was conducted in 2004 by Cristofanilli et al. Their study concluded that patients with 5 or more CTCs found in 7.5 ml of pretreatment blood have shorter progression-free survival (PFS) compared to those with less than 5 CTCs [7]. Since then, multiple studies have followed investigating the clinical validity of CTCs in the prediction of prognosis and therapy response.

These initial findings not only revealed that cancer cells in the circulation originate from the solid primary tumor but also provided new insight into the hematogenous metastasis pathway. Since the majority of breast cancer-related deaths are caused by distant organ metastasis rather than primary tumor burden, understanding this pathway is of great consequence to clinicians and researchers working to reduce breast cancer mortality. The completion of metastasis requires a sequential multistep process, the first step of which is local invasion. Increased motility facilitates the entry of cancer cells from the primary tumor into the blood stream. Vascular circulation is the interface between the primary tumor and the target organ for metastasis, making cancer cells disseminated in the blood a critical driver of metastasis. Although the drastic environmental change from a static solid tumor to dynamic blood flow eliminates many of the intravasated tumor cells, those that survive and adhere firmly to the vessel surface of a distant organ complete the next step in the metastasis pathway. The firm adhesion of CTCs to the endothelium under dynamic blood flow triggers permeabilization of endothelial tight junctions, subsequently allowing transendothelial migration of CTCs and eventual growth at distant organs [8]. Each of these steps is rate-limiting, and failure of even one inhibits metastasis. Thus, only a small fraction of the cancer cells disseminated from the primary tumor into circulation eventually give rise to overt organ metastasis.

In this chapter, we discuss the different platforms used to isolate CTCs from the blood as well as their clinical relevance in predicting prognosis and treatment response. The cytologi-

cal features and heterogeneity among CTCs will also be examined. Finally, throughout the chapter, we will explore new avenues of research on CTCs and their implications in establishing CTCs as the new "liquid biopsy."

2. Isolation and enumeration of CTCs

CTCs represent a rare cell population in the blood; therefore, they must be well distinguished from blood and other noncancerous cells (such as epithelial, fibroblast, and endothelial cells) present in the circulation [9–11]. Successful detection of CTCs is comprised of two consecutive steps: (1) enrichment, the separation of CTCs from blood cells and (2) confirmation, the identification of CTCs based on their unique biological characteristics. Currently, the main principles guiding CTC enrichment are based on the unique biological, morphological, and physiochemical characteristics that distinguish CTCs from other cells in circulation [12, 13]. However, each method faces its own difficulties. For example, CTC collection based on biological properties using surface markers will automatically eliminate CTCs without the marker expression. By contrast, enrichment by density, charge, or size collects any circulating cell with those prerequisite properties. A combinatorial approach may overcome current technology limitations. Additionally, a better understanding of the properties of CTCs may lead to the development of new enrichment criteria. Since strategies for CTC enrichment and identification vary in their respective strengths and weaknesses, the method of enrichment should be determined by the end point of individual studies. This section introduces commonly used CTC enumeration and identification methodologies and further discusses the advantages and potential pitfalls of each enrichment principle.

2.1. Isolation based on biological characteristics of CTCs

Breast cancer originates from epithelial cells in the mammary duct; thus, CTCs with positive expression of epithelial surface markers (cytokeratins and/or EpCAM) have traditionally been the focus of enrichment methodologies. CTCs are enriched by their affinity to bind antibodies against epithelial surface markers and excluded by the presence of the common leukocyte marker (CD45) as well as cytologic criteria [14]. This principle is the most widely adopted basis for enrichment techniques, and automated devices have been developed and commercialized for this application. While high reproducibility, specificity, and automation are major strengths, CTCs without epithelial marker expression likely escape inclusion by this method. Given the importance of epithelial mesenchymal transition (EMT) in invasion and metastasis, the potential exclusion of CTCs with weak or no epithelial marker expression should be considered [15, 16].

2.1.1. CellSearch® system (Veridex, LLC)

The CellSearch® system is a Food and Drug Administration (FDA)-approved CTC isolation device that is widely utilized for CTC enumeration in clinical studies. CellSearch's enrichment method relies on affinity binding of CTCs to magnetic ferrofluids attached to anti-EpCAM antibodies. EpCAM positive pools are then further used for enumeration by positive expression of cytokeratins $8^+/18^+$ and/or 19^+ that collocate with DAPI and the absence of CD45 [17]. Whole blood is processed using an automated blood cell diluting apparatus (CellPrep and Immunicon) and then

immunomagnetically labeled EpCAM$^+$ cells are concentrated using an external magnetic field. Finally, the immunomagnetically labeled cells are analyzed using either the CellSpotter Analyzer or the CellTracks Analyser II which examine cell morphology and staining patterns for CTC confirmation [17, 18]. The CTC criteria used in these methods are (1) an intact cell with a round to oval morphology and at least 4 μm in size; (2) positive for DAPI with a nucleus inside the cytoplasm (of at least >50%) and a nucleus area smaller than the cytoplasm, and (3) positive for cytokeratins and negative for CD45 [19]. One advantage of the CellSearch® System is that blood is collected in a CellSave tube which contains a mixed fixative. CTCs remain stable for 96 hours and can be transported at room temperature for later analysis. Since the CellSearch® system is semiautomated, the reproducibility of this study is high with minimal inter- or intrareader variability.

Using CellSearch®, Cristofanilli et al. were the first to report that circulating epithelial cells are rare in healthy women and those with benign tumors (0.1 ± 0.2 per 7.5 ml blood). Additionally, they found 5 CTCs per 7.5 ml blood to be a reliable cutoff for the prediction of patient survival among women with malignant breast tumors. A study of 517 breast cancer patients showed that patients at or above this cutoff had a shorter median PFS (2.7 months vs. 7.0 months; $p < 0.001$) and shorter overall survival (OS) (10.1 months vs. >18.0 months; $p < 0.001$) when compared to those with fewer than 5 CTCs. These data demonstrate that the number of CTCs prior to treatment is an independent predictor of PFS and OS in patients with MBC [7].

2.1.2. Adnatest (AdnaGen AG)

Adnatest is an immunomolecular assay that combines immunomagnetic-based enrichment with multiplex reverse transcription polymerase chain reaction (RT-PCR). In the initial isolation step, EpCAM$^+$/MUC1$^+$ CTCs are enriched using magnetized antibodies and further identified by tumor-associated gene expression [20–22]. Since phenotypic changes occur in cancer cells throughout the disease course and in response to therapies, cancer cells in the primary tumor as well as in the circulation are diverse in their gene expression. Considering the heterogeneity of cancer cells, the most prominent advantage of Adnatest is that it allows use of a variety of antibody-based selection markers, thereby minimizing false negative and false positive results. Bredemeier et al. enriched CTCs in 62 MBC patients using immunomagnetic beads that target EpCAM, epithelial growth factor receptor (EGFR), and HER2. The enriched CTCs were then characterized by their expression of tumor-related genes using a multiplex qPCR assay (AdnaTest EMT-2/StemCellDetect™). Using this approach, authors of the study established a panel of nine genes able to identify differential expression of each phenotype— epithelial (*EpCAM*), EMT (*PIK3CA, AKT2*), stem cell (*ALDH1*), drug resistant (*ERCC1, AURKA*), receptor positive (*ERBB2, ERBB3,* and *EGFR*), and leukocyte control (*CD45*) [23]. Adnatest is capable of detecting as few as 2 CTCs in 5 ml of blood [20, 22] and in a comparative study showed greater sensitivity than the CellSearch® system in detecting CTCs (53 vs. 47% CTC positive, respectively, in a sample of 55 MBC patients) [24].

2.1.3. CTC-Chip

A surface coating of anti-EpCAM antibody enables the microfluidic CTC-Chip to capture EpCAM$^+$ cells in its channel while eliminating those that are negative under precisely controlled

laminar flow conditions. Etched in silicon and no larger than a standard microscope slide [25], the CTC-Chip contains an array of microposts functionalized with anti-EpCAM antibodies and a pneumatic pump to establish flow, all enclosed by a manifold. Once a blood sample has been pumped through, the microchip is gradually flushed with PBS to remove any nonspecifically bound cells. To identify CTCs, the microchip is then stained with DAPI, pan-cytokeratins (1, 4, 5, 6, 8, 10, 13, 18, and 19), and CD45 using immunocytochemistry. Cells meeting the morphological characteristics of malignant tumor cells (such as cell size, shape, and nuclear size) and positive for cytokeratins are considered CTCs. Assessment of cell membrane integrity following this method showed substantial viability (98.5 ± 2.3%). Additionally, the CTC-Chip captures cells with low EpCAM expression as efficiently as cells with high expression. The CTC-Chip successfully identified CTCs in the peripheral blood of patients with metastatic disease in 115 of 116 (99%) samples, with a range of 5–1281 CTCs per ml [26].

2.1.4. MagSweeper

The MagSweeper is another EpCAM-based immunomagnetic cell separator. It uses a round-bottom, magnetic rod covered with an ultrathin (25 μm) nonadherent plastic sheath. This assembly is robotically swept through a well containing a blood sample labeled with anti-EpCAM functionalized paramagnetic beads (CELLection Epithelial Enrich Dynabeads: Invitrogen). The EpCAM$^+$ cells captured on the covered magnetic rod (MagSweeper) are transferred to and washed in a well containing PBS, then released into another well of PBS by removing the magnetic rod from its sheath. Finally, EpCAM$^+$ cells are further confirmed by morphology and gene expression profiles [27, 28]. The gene expression profiles of MCF7 cells incubated with anti-EpCAM magnetic beads before and after MagSweeper isolation were analyzed using microarray analysis and compared with a similar number of MCF7 cells grown in culture media. Statistical analysis indicated that the MagSweeper isolation process does not induce any significant perturbation in the gene expression profile of cells. Additionally, the use of 4.5-μm magnetic beads permits isolation of target EpCAM$^+$ cells, even with single-bead attachment, making the procedure suitable for isolation of CTCs with moderate-to-low EpCAM expression. However, the attachment of large magnetic beads to the cell surface may interfere with certain applications. An additional drawback is that normal EpCAM$^+$ cells present in the circulation are also potentially selected and need to be distinguished from CTCs at a microscopic level. MagSweeper technology succeeded in isolating CTCs from all MBC patients ($n = 47$) at an average of 12 ± 23 CTCs per 9 ml of blood, while no CTCs were found in samples derived from healthy donors [28].

2.1.5. Vita-Assay™ (Vitatex Inc.)

The Vita-Assay™ is a functional assay-based CTC enrichment method that takes advantage of invasive CTCs preferential adhesion to the cell adhesion matrix (CAM). Viable, invasive CTCs are captured on a plate coated with CAM-mimic, then further identified based on their proclivity to degrade and ingest the extracellular matrix. CTCs adhered to the Vita-Assay™ plate are released by the addition of an enzyme that dissolves the CAM coating, and then concentrated by centrifugation for cytologic analysis. This application's criteria for CTCs include positive CAM uptake (CAM$^+$) and negative hematopoietic lineage (HL) marker expression. Enumeration of CTCs by

flow cytometry may be further validated by microscopic comparison of CTC and immune cell morphology. The Vita-Assay™ CTC-enrichment platform is capable of enriching rare and invasive CTCs and is not biased by surface markers, morphology, or size. Rather, since it focuses on the adhesive properties of CTCs, it offers a potentially robust capture method for invasive CTCs. Additionally, Vita-Assay™ allows for sensitive multiplex flow cytometric and microscopic detection of CTCs. Vita-Assay™ successfully detected CTCs in all blood samples from MBC patients (n = 10) with a range of 18–256 CTCs per ml. Moreover, CTCs were detected in blood samples of 28 of 54 (52%) stage I–III breast cancer patients with a mean count of 61 CTCs per ml [29].

2.2. Isolation based on physicochemical properties of CTCs

Several isolation methods take advantage of differences between the physicochemical properties of CTCs and other circulating cells. Enrichment methods have been developed for properties including size, density, and surface charge [30]. For example, the well-documented fact that carcinoma cells have larger overall size and denser nuclei than normal epithelial and immune cells has been adopted for CTC isolation [31, 32]. Similarly, nuclear condensation of carcinoma cells has led to the development of density-based CTC enrichment [33]. Lastly, differential surface charges between carcinoma and normal epithelial or immune cells are also a strategy used for CTC enrichment [34]. Enrichment strategies based on physiochemical properties have emerged in order to minimize bias (i.e., exclusion of non-EpCAM+ cells); thus, the sensitivity of CTC isolation using these methods is high. However, their specificity is not always high due to the difficulty of completely eliminating potential leukocyte contamination during the enrichment step. Most of these methods rely on manual cytopathologic identification of CTCs, a highly laborious process with varied reproducibility depending on the pathologist. Despite this, the potential for versatile applications as well as live CTC collection remains major strengths of this method class.

2.2.1. Size and density

Since CTCs are larger in size than immune or red blood cells, two commercially available methods have used this principle to enrich CTCs. Using negative pressure or gravity, cells with diameter greater than the 6.5–8.0-µm pores are captured on porous membranes. This results in the acquisition of multiple types of cells, including CTCs, leukocytes, fibroblasts, normal epithelial, and endothelial cells. CTCs are then distinguished from immune cells by immunostaining and morphology. Although, different types of staining methods have been explored for reproducibility, no standard staining method for CTCs has been established thus far.

2.2.1.1. RareCells® system (Rarecells)

The RareCells® system allows performance of the Isolation by Size of Epithelial Tumor Cells test (ISET® test). The ISET® test enriches CTCs according to their size and subsequently identifies them based on their cytopathologic features. The RareCells® system is a negative depression-based filtration device. It consists of a 10-well filtration module that captures CTCs on a polycarbonate Track-Etch-type porous membrane [35–37]. Following red blood cell rupture and mild fixation, circulating cells smaller than 8 µm are filtered through the porous membrane, while those of a greater diameter are enriched on the membrane. The membranes may subsequently be stained for the detection of CTCs or stored for future analysis. The RareCells® system allows

for versatile applications, including both fixed and live CTC collection. Using ISET method, Hofman et al. conducted a blinded, multicenter study to assess the feasibility of CTC identification using cytopathologic criteria. CTCs were defined as circulating nonhematologic cells exhibiting at least 4 of the following criteria: irregular nuclei, anisonucleosis (ratio >0.5), high nuclear/cytoplasmic ratio, nuclei larger than 24 μm, or the presence of tridimensional sheets. Based on these criteria, CTCs were only detected in the blood of patients with malignant disease and were absent in healthy subjects [38]. The RareCells® system has shown successful detection of CTCs in breast cancer, melanoma, and nonsmall cell lung cancer cases [37, 39–41]. In a comparison between the RareCells® system and CellSearch®, the RareCells® system displayed greater sensitivity of detection (93% vs. 40%, respectively) and yielded higher median CTC counts [42].

2.2.1.2. OncoQuick® tube (Greiner Bio-One)

This separation device is composed of a centrifugation tube containing a liquid density separation medium and porous barrier membrane optimized for the enrichment of CTCs from blood. During the enrichment step, blood is layered on top of the gradient and then centrifuged. CTCs are enriched in the fluid above the porous barrier and collected in a tube by centrifugation. Following immunocytochemistry, CTCs are identified as cytokeratin-positive (7, 8, and 18) and CD45 negative with intact nuclei and an increased nuclear-cytoplasmic ratio [43, 44]. The purity and efficacy of CTC enrichment using OncoQuick® are higher than that with Ficoll, which traps up to 25 times more blood mononuclear cells [45]. However, detection sensitivity using OncoQuick® was found to be lower than CellSearch® (23% vs. 54%; $p < 0.001$) [43].

2.2.2. Electrical properties

CTCs have a unique surface charge that distinguishes them from other cells. Thus, a dielectrophoretic flow field can be used to fractionate CTCs from blood cells based on their differential electrical properties [34].

2.2.2.1. DEPArray™ technology (Silicon Biosystems)

Utilizing this principle, DEPArray™ is an automated instrument that can identify, quantify, and recover individual rare cells. It is used as a second purification step after initial EpCAM-based CTC enrichment methods. The individually isolated CTCs using DEPArray™ are then identified based on their morphological and immunocytochemical features. The system includes the DEPArray™ cartridge and DEPArray™ analysis platform. The single-use, microfluidic cartridge contains an array of individually controllable electrodes, each with embedded sensors. This circuitry enables the creation of dielectrophoretic cages around cells. After imaging, individual CTCs are gently moved into the holding chamber for isolation and recovery. The DEPArray™ analysis platform utilizes image-based selection to allow identification and isolation of CTCs on the DEPArray™ cartridge. The system uses a six-channel fluorescent microscope and a CCD camera to capture images and identify cells demonstrating the desired fluorescence labeling and morphological characteristics. The main advantage of DEPArray™ is its ability to eliminate mononuclear cell cross contamination from the preenriched CTC pool. Image-based selection enables the isolation of specific rare cells from other cell types. Moreover, the DEPArray™ system yields high-quality nucleic acids for molecular investigations, since the

Detection strategy		Detection method	Advantages	Disadvantages
Biological	EpCAM expression	CellSearch® System [17]	Semiautomated system	Only epithelial CTCs are captured and mesenchymal cells are discarded
			FDA approved	
			Quantification of CTCs	
		CTC-chip [25]	Captured cells are suitable for molecular analyses	
			High detection rate	
			Quantification of CTCs	
		IsoFlux	NSG applicable	
			Custom design for CTC isolation	
			Semiautomated system	
		Herringbone-Chip (HB-Chip) [98]	Enhanced platform for CTC isolation	
			Allows detection of microclusters of CTCs	
		MagSweeper [27, 28]	Automated immunomagnetic isolation	
			Allows for gene expression profiling analysis	
	EpCAM and MUC1 expression	AdnaTest [20–22]	High sensitivity	CTCs cannot be morphologically characterized
			Multiplex PCR assays	
	EpCAM expression and microfluidic	SIM-Chip [99]	Single-cell isolation High purity and recovery without cell damage	Possible cell damage
Physicochemical	Size	RareCells® system [35–37]	Single CTC morphological, immunocytological, and genetic analyses	Cross contamination with other rare blood cells such as megakaryocytes and large monocytes
			High sensitivity and specificity	
		ScreenCell® [100]	Isolation of live cells and allows for tissue culture experiments	
		Celsee PREP™ slide	Highly efficient CTC detection with high sensitivity and specificity	
			Immunohistochemistry, DNA, and mRNA analyses	
			Single cell analyses	
			Automatic imaging system	
	Surface charge	DEPArray™ [46]	Single cell isolation	
			High quality nucleic acids for molecular investigations (elimination of blood cells cross contamination)	

Detection strategy		Detection method	Advantages	Disadvantages
Functional	Secretion	EPISPOT [101]	Detects viable cells	Detects only Epithelial CTCs
	Matrix adhesion	Vita-Assay™ [29]	Enriches viable CTCs from blood up to one-million fold	Limited only to invasive CTCs which are able to ingest cell matrix

Table 1. Summary of approaches used for CTC isolation and their relevant devices.

cells receive minimal disturbance during capture or transport. Additionally, the DEPArray™ system allows for the isolation of single cells, making it a promising contributor to the understanding of CTC heterogeneity [46].

A summary for the CTC methods discussed in this chapter along with other methods is described in **Table 1**.

3. Clinical utility of CTCs as a biomarker

Liquid biopsy is a clinically amenable method to enable real-time and longitudinal disease monitoring. Currently, available serum markers lack the specificity and sensitivity needed for clinical management of breast tumors. Since the detection of CTCs became feasible, a number of clinical studies have undertaken exploration of whether CTCs could provide a new minimally invasive, longitudinal disease monitoring strategy in breast cancer [7, 47]. The ability of CTCs to predict disease progression in both early and MBC as well as in different tumor subtypes is currently under investigation. Moreover, several clinical trials have investigated the use of CTCs as an early therapy response marker. This section discusses the potential use of CTCs as a biomarker for prognosis and therapy response in breast cancer.

3.1. Prediction of prognosis of metastatic breast cancer by CTC

In a landmark study, Cristofanilli et al. conducted the first large, multi-institutional clinical study to evaluate the utility of CTCs as a predictive biomarker for disease progression in patients with MBC. Using the CellSearch® system, CTCs were enumerated from 177 patients with measurable MBC from 20 clinical centers across the United States. Of these, 49% had ≥5 CTCs per 7.5 ml of blood at baseline prior to treatment. When compared to patients with fewer than 5 CTCs at baseline, these patients had shorter median PFS (2.7 months vs. 7.0 months; p < 0.001) and OS (10.1 months vs. >18 months; p < 0.001). After 4 months, 10 of the 177 patients had died, each showing an average of 3000 CTCs per 7.5 ml of blood at baseline [7]. This data clearly indicated that the presence of CTCs is strongly associated with poor outcomes in MBC patients. When another study, conducted by Nakamura et al., examined the correlation between CTC count and OS, increased risk was found for patients with higher pretreatment

counts Hazard Ratio (HR): 2.4 for patients with 5–10 CTCs; (95% CI, 0.72–8.24, p = 0.1481); HR: 13.95 for patients with 21–100 CTCs; (95% CI, 4.57–42.55, p < 0.0001). Furthermore, when the OS of patients with ≥5 CTCs was compared to those with <5 CTCs, the HR was 3.1 times higher (95% CI, 1.49–6.29, p = 0.002) [47]. This relationship has been further confirmed by both meta- and pooled analyses [48–50]. Comprehensive meta-analysis of 49 studies, including a total of 6825 patients (Early stage [M0, n = 2993], metastatic [M1, n = 3069], and pooled patients with I–IV stages [n = 763]) found that CTC count was significantly associated with shorter PFS and OS in both early stage and MBC patients (HR: 1.78; 95% CI, 1.52–2.09, p < 0.001 for PFS; HR: 2.33; 95% CI, 2.09–2.60, p < 0.001 for OS in MBC patients) [48]. Additionally, pooled analysis of 20 studies from 17 European breast cancer centers found similar trends. In this cohort of 1944 MBC patients, 46.9% had a CTC count of ≥5 CTCs per 7.5 ml at baseline, which was associated with both decreased PFS (HR: 1.92; 95% CI, 1.73–2.14, p < 0.0001) and OS (HR: 2.78; 95%CI, 2.42–3.19, p < 0.0001) compared to those with <5 CTCs [49]. In another recent meta-analysis of 24 studies including 3701 MBC patients, in 1 and 2 years PFS and OS rates (respectively), higher CTC counts correlated with shorter PFS [50].

Since the studies included in these analyses varied in CTC detection method and time point of blood draw, Zhang et al. evaluated whether these differences affect the prognostic value of CTC counts. Using a subgroup analysis stratified by detection method and time point of blood sampling, their meta-analysis found that the prognostic value of CTCs in PFS was significant in studies using RT-PCR (HR: 2.58; 95% CI, 1.99–3.35) or CellSearch® methodologies (HR: 1.85; 95% CI, 1.53–2.25). This demonstrated that CTCs are a reliable prognostic marker from pre-dose/preoperative blood in patients with MBC, regardless of other differences in study design [51]. Similarly, CTC counts have also been shown to be a prognostic marker in the postdose blood of MBC patients receiving their first cycle of first-line treatment [7, 52].

Together, these studies lay the foundation for CTCs as a valid and reliable prognostic indicator both before and after breast cancer treatment. Given their promising clinical application, the question of whether CTCs are superior to other prognostic factors has also been addressed. A multivariate, Cox proportional hazards regression analysis showed that CTC count at baseline was the most significant predictor of both PFS and OS, regardless of histology grade, recurrence, de novo stage IV breast cancer, or hormone and HER2 receptor status. Moreover, in a retrospective analysis of MBC patients, comparison of the prognostic significance of CTCs with tumor burden, therapy type, and receptor subtype showed that CTCs were an independent predictor of prognosis [7, 53].

3.2. Prediction of prognosis of nonmetastatic breast cancer by CTCs

As mediators of metastasis, CTCs' presence and role in advanced breast cancer cases have received much attention. However, their implications in nonmetastatic cases have been a focus of investigation as well. Using mRNA expression of cytokeratin 19 (CK19) to identify CTCs, Stathopoulou et al. detected CTCs in the blood of 148 patients with operable breast cancer prior to initiation of adjuvant therapy. For stage I and II breast cancer, the presence of CK19+, CTCs was an independent prognostic factor associated with early relapse (p < 0.001) and disease-related death (p = 0.01) during a median follow-up of 28 months [54]. Using the same approach, Xenedis et al. analyzed 167 node-negative breast cancer patients before the

initiation of therapy and found 21.6% of the patients had CK19$^+$ CTCs, which were associated with early relapse ($p < 0.001$) and disease-related death ($p < 0.008$). Multivariate analysis further confirmed the detection of CK19$^+$ CTCs in the blood of node-negative patients is an independent prognostic factor [55].

The largest study on CTCs conducted in an adjuvant setting was the SUCCESS study, which included 2026 patients (median follow-up of 35 months) and collected CTCs after surgery and chemotherapy. The presence of even one CTC before adjuvant systemic treatment was associated with poor disease-free survival and OS. Additionally, node-positive patients were found to have CTCs more frequently than node-negative patients. In a subgroup analysis, the presence of CTCs was not significantly associated with DFS in node-negative patients; however, in node-positive patients, CTC was proportionally associated with number of node involvement and poor prognosis (positive node number of 1–3; $p = 0.008$), 4–9; $p < 0.001$; ≥10; $p = 0.001$). Patients who had CTCs present both before and after systemic chemotherapy had the worst 3 years DFS of any group in this study. Multivariate analysis further confirmed that detection of CTCs prior to chemotherapy was an independent prognostic factor for DFS (HR: 2.11; 95% CI,1.49–2.99, $p < 0.0001$) and OS (HR: 2.18; 95% CI,1.32–3.59, $p = 0.002$) in early breast cancer patients [56]. Although the study provided valuable prognostic data, its clinical significance remains uncertain since it only provided a short follow-up time. Two separate studies have found the presence of one or more CTCs to be of prognostic significance in nonmetastatic, chemotherapy-naïve (HR: 4.62 for PFS; 95% CI, 1.79–11.9, p = 0.005; HR: 4.04 for OS; 95% CI, 1.28–12.8, $p = 0.011$; [57]) and surgery-naïve patients (HR: 2.72 for relapse-free survival (RFS); 95% CI,1.57–4.72, $p < 0.001$; HR: 2.29 for OS; 95% CI, 1.12–4.67, p = 0.02;[58]). Moreover, a meta-analysis of 49 studies ($n = 6825$ of both early breast cancer and MBC patients) showed that in early breast cancer patients, CTC count correlated with both shorter DFS (HR: 2.86; 95% CI, 2.19–3.75, $p < 0.001$) and OS (HR: 2.78; 95% CI, 2.22–3.48, $p < 0.001$) [51]. Similar to MBC patients, posttherapy CTC count was shown to be an independent prognostic factor in non-MBC patients. In a study of stage I–III node-negative breast cancer patients ($n = 175$) who had completed adjuvant chemotherapy, Ignatiadis et al. detected CTCs using a panel of three biomarkers (CK19, mammaglobin (MGB1), and HER2) using RT-PCR. The detection of all three markers was associated with shorter DFS (for CK19$^+$; HR: 2.967; 95% CI, 1.64–5.34, $p < 0.001$, for MGB1+; HR: 3.275; 95% CI, 1.58–6.76, $p = 0.001$, and for HER2+; HR: 2.869; 95% CI,1.63–5.02, $p < 0.001$) in univariate analysis [59]. While these studies suggest the independent prognostic significance of CTCs in non-MBC patients both before and after adjuvant chemotherapy, the data remains somewhat controversial.

Kuniyoshi et al. reported no correlation between PFS and the presence of CK19 or HER2 CTCs in non-MBC patients ($n = 167$) at baseline or the first two subsequent follow-ups during chemotherapy [60]. This data was further supported by the recently published results from the SUCCESS-A trial, a randomized, multicenter trial (EudraCT2005000490-21) that evaluated the prognostic value of CTCs in 1221 early-stage (94% of patients were stages I and II) breast cancer patients prior to adjuvant chemotherapy. Using a density gradient followed by labeling with the anticytokeratin antibody, the SUCCESS-A trial detected CTCs in only 20.6% of all patients, and univariate analyses demonstrated that the presence of one or more CTCs had no significant impact on DFS or OS over a median follow-up of 64 months [61]. The inconsistent results regarding the prognostic utility of CTC counts in non-MBC patients may be partially attributable to the use of different CTC detection methods. While studies using the

CellSearch® system have consistently reported CTCs as a significant independent prognostic marker in non-MBC patients, those using other methods (such as mRNA or cytokeratin-protein expression) yielded data to the contrary. These conflicting reports underscore the need for standardization of CTC-detection methodologies.

3.3. Subtype-dependent prognostic significance of CTCs

Breast cancer is a heterogeneous disease, and large-scale gene expression analysis of primary tumors has made it possible to stratify clinical cases into four intrinsic subtypes based on receptor expression—Luminal A, Luminal B, HER2+, and triple negative (TN). These subtypes are significantly associated with differences in clinical outcomes and define a patient's course of therapy. In a large retrospective study using CellSearch®, Giordano et al. addressed subtype-specific differences among CTCs in 517 MBC patients prior to first course of therapy. CTC counts were predictive of prognosis in Luminal and TN breast cancer subtypes but were less so in the HER2-positive subtype. In Luminal A patients, the median OS and PFS of those with ≥5 CTCs (n = 292) were significantly shorter than those with <5 CTCs (OS, 18.8 vs. 48.7 months; p < 0.001; and PFS, 5.9 vs. 7.1; p = 0.004). Within the TN subtype, patients with >5 CTCs (n = 124) had a median OS significantly shorter than patients with <5 CTCs (10.4 vs. 17.8 months respectively; p = 0.001); however, there was little difference in median PFS for these patients (PFS, 5.1 vs. 4.8, respectively; p = 0.274). By contrast, among HER2+ patients, there was no significant association between CTC count and OS or PFS (median OS, 27.2 vs. 21.4 months; p = 0.991; median PFS 7.6 vs. 8.6; p = 0.458) [62]. Likewise, another retrospective study using CellSearch® found similar trends in the relationship between CTCs and subtype among MBC patients. Patients were stratified into groups based on their CTC count at baseline (0, 1–4, or ≥ 5 CTCs) and subtype. Similar to Giordano's findings, CTCs were predominately found in patients with Luminal-A/Luminal-B/HER2-negative subtypes. Moreover, patients of all subtypes, except HER2+, with no CTCs detected in the blood had a better prognosis compared with those with 1–4 or >5 CTCs [63]. However, a large, multicenter study conducted in Germany found that CTC count at baseline was positively associated with shorter OS in all tumor subtypes, including HER2+ patients [64]. Additionally, two recent reports have investigated the prognostic role of CTCs, specifically in TN subtype. A meta-analyses including 10 studies with a total of 642 metastatic and nonmetastatic TN breast cancer patients found the presence of CTCs, predicted aggressive disease progression (HR: 2.18; 95% CI, 1.59–2.99, p = 0.010) and reduced OS (HR: 2.02; 95% CI, 1.59–2.57, p = 0.169) [65]. Additionally, Karhade et al. evaluated CTCs at baseline in 113 stage I–III nonmetastatic TN patients and found that presence of ≥2 CTCs predicted shorter PFS (HR: 8.30; 95% CI, 2.61–26.37, p < 0.001) and OS (HR: 7.19; 95% CI, 1.98–26.06, p < 0.0004) [66].

In summary, the prevalence and clinical relevance of CTCs vary by breast cancer subtype. Currently, data indicates that the prevalence of CTCs is high among metastatic Luminal A as well as both metastatic and nonmetastatic TN breast cancers. However, data regarding the prognostic significance in HER2+ tumors remains inconclusive.

3.4. Prediction of therapy response by CTCs

Neoadjuvant therapy is increasingly popular among patients (e.g., TN, HER2+, or large tumor burden, etc) who would qualify for adjuvant chemotherapy. Several studies have explored

the clinical validity of CTCs in this setting, specifically the REMAGUS02, GeparQuattro, and BEVERLY-2 trials. Each of these studies enumerated CTCs before and after neoadjuvant therapy, yet produced contradictory findings. The REMAGUS02 trial found no correlation between the presence of CTCs and pathological complete response, tumor size, grade, or lymph node status. However, multivariate analysis revealed that patients without CTCs before and after neoadjuvant therapy had better distant metastasis-free survival (DMFS) (before; Relative Risk (RR): 2.4; 95% CI,0.9–6, $p = 0.06$), although no difference was noted in DMFS or OS after a median follow-up of 70 months [67]. On the other hand, the GeparQuattro trial failed to show any correlation between the presence of CTCs and, worse, DFS or OS [68]. The BEVERLY-2 study, however, showed that the presence of CTCs at baseline was an independent prognostic factor for poor DFS (HR: 4.75; 95% CI,1.56–14.50, $p = 0.006$) at 3 years of follow-up [69]. Another study compared CTC enumeration with CT scan results in MBC patients following therapy. CTCs were measured at baseline and 4 weeks following therapy, and CT scans were obtained at 9–12-week intervals to assess response to therapy using RECIST criteria. CTC counts were reviewed by a local and central laboratory, while two central radiologists reviewed the CT scans. Superior interreader agreement for CTCs was observed at 0.7% variability, and radiological responses showed 15.2% variability. Patients with <5 CTCs following 4 weeks of therapy who had stable or partial response on the CT scans demonstrated the best median OS of 26.9 months. After these results, however, it is still unclear whether the change in therapy course can be based on CTC detection following chemotherapy in MBC patients [70]. In 2014, the first interventional study based on postchemotherapy CTC detection was launched by the SWOG trial. The main goal of the S0500 SWOG study was to demonstrate an OS benefit in CTC-positive patients who were nonresponsive to therapy by switching them from first to second-line therapy. Patients who had >5 CTCs after 3 weeks of therapy were randomized to ARM 1 (continuation of same therapy) or to ARM 2 (switch to second-line therapy). Disappointingly, no difference in OS or DFS was observed in either arm. There are several ongoing interventional clinical trials that stratify patients based on CTC count for either aggressive chemotherapy or hormonal therapy [52]. Another trial is investigating the change of therapy based on CTC number at the third or subsequent lines of therapy for MBC [71]. In conclusion, the present data is insufficient to recommend the use of CTC enumeration for risk stratification and treatment response. Also, early changes in therapy based on CTC enumeration in MBC patients are not recommended at this time, although ongoing studies may yield more definitive results.

4. CTC heterogeneity

A number of studies have addressed the heterogeneous nature of CTCs with the ultimate goal of understanding what molecular signature is required for successful metastasis. The two main phenomena that orchestrate tumor heterogeneity and metastases are cancer stem cells (CSC) and EMT. CSCs are pluripotent, highly resistant to conventional chemotherapy [72–74] and contribute to the heterogeneous nature of the tumor as well as its ability for self-renewal and metastasis [75]. Notably, not all tumor cells are capable of distant organ metastasis; CSCs seem to have such metastatic potential [76]. Likewise, the process of EMT plays an essential role in invasion and metastasis. At the primary tumor site, a subpopulation of cells loses their

epithelial characteristics (such as cell polarity or adhesion to the matrix and other cells) and acquires mesenchymal features (including the ability to invade the basement membrane and surrounding tissues), which in turn supports eventual intravasation into the circulation, the first step in the metastatic cascade [77]. These two processes are interconnected, adding further complexity to our understanding of metastasis. Recent studies have shown a direct link between EMT and CSCs in breast cancer, suggesting that EMT generates cancer cells with stem cell-like traits. Mani et al. showed that the induction of EMT in immortalized human mammary epithelial cells results in *de novo* expression of stem cell markers and the acquisition of functional stem cell properties, including the ability to form mammospheres [78–80]. Both CSC and EMT markers have been identified in CTCs, and while a CTC count itself is an independent prognostic marker, the addition of functional marker expression among CTCs will likely strengthen their prognostic value. This section specifically focuses on CTC heterogeneity of CSC and EMT nature.

4.1. Stem-like CTCs

CSCs are derived both intrinsically and extrinsically [72–74]; although the mechanism for extrinsic acquisition of CSC properties is not clearly understood, several lines of evidence suggest a close link to EMT [81–84]. CSCs are pluripotent and highly resistant to conventional chemotherapy [72–74]. Currently, there is no therapeutics effective in eradicating CSCs [85]; therefore, CTCs with CSC properties are postulated to be an important subset. In 2010, Theodoropoulos et al. investigated whether bulk CTCs contain a subset of cells with CSC characteristics. The protein expression of CSC markers CD44, CD24, and ALDH1 was assessed in cytokeratin+ CTCs isolated from MBC patients using immunofluorescence microscopy. In approximately 1500 CTCs identified from 20 MBC patients, 35.2% had the stem-like phenotype (CD44$^+$/CD24$^{-/low}$), whereas 17.7% of the CTCs were ALDH1^{-high}/CD24$^{-/low}$ [86]. This is in concordance with another study that found 19% of EpCAM$^+$/Cytokeratin$^+$ CTCs are also CD44$^+$/CD24$^{-/low}$ cells [87]. Further support came from an experimental model that demonstrated a stem-like CD44$^+$ CTC subset isolated from MBC patient blood having metastatic potential. Interestingly, the six recipient NSG, immunocompromised mice in this study developed multiple bone, lung, and liver metastases within 6–12 months following injection of bulk CTCs into their bone marrow, confirming the existence of metastatic-initiating cells (MICs) among CTCs. To determine the phenotype of the MIC-CTC subpopulation, flow cytometry analyses showed that all analyzed CTCs expressed CD44 and CD47. CD47 has been implicated in facilitating cancer cell evasion of the innate immune system through its inhibitory role in phagocytosis. Around 33% of CD44$^+$/CD47$^+$ CTCs express the hepatocyte growth factor (HGF) receptor MET, a tyrosine kinase involved in the activation of the migration and putative invasion program in several cancers. To functionally assess the presence of MICs in this cell population, CD44$^+$/CD47$^+$/MET$^{+/-}$ CTCs were isolated by FACS and directly transplanted into the femoral medullar cavity of an NSG recipient mouse. After 8 months, bone metastasis developed in the mouse, demonstrating that CD44$^+$CD47$^+$MET$^{+/-}$ CTCs contain functional MICs. These markers were further examined in four patients before and after disease progression. An increased frequency of CTCs with CD44$^+$/CD47$^+$/MET$^+$ was detected after disease progression (fold increase of 1.78; p = 0.019). Additionally, in a total of eight patients,

those with >12 CD44$^+$/CD47$^+$/MET$^+$ (triple positive) CTCs per 7.5 ml of blood had significantly more metastasis sites than those with <12 triple positive CTCs (mean: 3.25 sites vs. 2.25 sites; p = 0.03), and the presence of CD44$^+$/CD47$^+$/MET$^+$ CTCs was associated with shorter OS (HR: 7.4; p = 0.0246) [88]. The association of CSC-CTCs with advanced disease was further supported by the work of Papadaki *et al.* who found that MBC patients have a higher percentage of ALDH1$^+$ CTCs than those with early breast cancer. ALDH1$^+$ CTCs were observed in 38.7% of CTCs from early-breast cancer patients compared to 83.5% from MBC patients [89]. Together, these data suggest that CTCs with CSC characteristics have more biological relevance for disease development, progression, and outcomes than bulk CTC data. However, the value of CSC-like CTCs in prognosis and therapy-response prediction requires further confirmation in a large prospective clinical study.

4.2. Mesenchymal CTCs

EMT contributes to the acquisition of invasiveness in cancer cells, and therefore it is believed that CTCs with mesenchymal features may contribute to metastasis. However, this question has not been extensively addressed due to the use of affinity-based CTC enrichment methods that rely on EpCAM and/or Cytokeratin markers, lacking mesenchymal cell surface marker selection. EMT is a gradual process that yields epithelial cells which have not gone through EMT, intermediate mesenchymal (cells that have partially completed EMT), and exclusively mesenchymal cells (ones that have completed EMT). Yu et al. characterized the EMT status of CTCs captured on the microfluidic herringbone chip with an antibody cocktail directed against EpCAM, EGFR, and HER2. These researchers established a quantifiable, dual-colorimetric RNA-in situ hybridization (ISH) assay to examine tumor cells for expression of seven pooled epithelial transcripts (Cytokeratin 5, 7, 8, 18, and 19, EpCAM, and Cadherin 1) and three mesenchymal transcripts (Fibronectin 1, Cadherin 2, and Serpin peptidase inhibitor/clade E [SERPINE1/PAI1]). Five categories of cells ranging from exclusively epithelial, intermediate (more epithelial, equal, and more mesenchymal), and exclusively mesenchymal were determined [90]. Similarly, a study by Polioudaki et al. used the ratio of Cytokeratin to Vimentin protein expression (measured by immunofluorescence) to study on a single cell basis the EMT status of 110 CTCs detected in 5 MBC patients. This study identified that 46% of CTCs were "epithelial," 5.4% were "mesenchymal," and 48.2% were "intermediate" [91]. The existence of CTCs across the EMT spectrum was further confirmed by another single cell level study. Using DEPArray to select viable CTCs from 56 MBC patients, Bulfoni et al. determined the EMT status of single CTCs by staining with an antibody cocktail that recognized both epithelial (EpCAM, E-Cad) and mesenchymal (CD44, CD146, and N-Cadherin) markers. This study also reported the presence of diverse CTC phenotypes based on their EMT statuses [92]. CTC heterogeneity was further investigated on a genetic level using single CTCs. Powell et al. were the first to perform microfluidic-based single cell transcriptional profiling of 87 cancer-associated and reference genes in CTCs. Their study found that CTCs are heterogeneous and can be separated into two major subgroups based on 31 highly expressed genes including mesenchymal and metastatic associated genes (*VIMENTIN, TGFß1, ZEB2, FOXC1, CXCR4, NPTN, S100A4, and S100A9*) [93].

Several studies have investigated the clinical relevance of CTCs based on their EMT status. Yu et al. reported the CTCs isolated from ER$^+$/PR$^+$ breast cancer patients were predominantly epithelial, whereas those from the TN and HER2$^+$ subtypes were predominantly mesenchymal in a sample of 41 MBC patients [90]. Similarly, Polioudaki et al. retrospectively analyzed 1000 CTCs isolated from 61 MBC patients at baseline using CellSearch® and investigated the correlation between the level of cytokeratin expression and tumor subtype. Interestingly, CTCs from TN patients showed a lower average cytokeratin expression level compared to those from the remaining patients (122 vs. 175; $p < 0.001$) [91]. Moreover, Kallergi et al. found that the proportion of CTCs coexpressing cytokeratins 7, 8, or 18 together with the mesenchymal marker Twist (measured by Immunofluorescence) is lower in patients with non-MBC than in patients with MBC (53% vs. 97%, respectively; $p < 0.001$). Similarly, the proportion of CTCs coexpressing cytokeratins and Vimentin was lower in patients with non-MBC than in those with MBC (56% vs. 74%; $p = 0.005$) [94]. Likewise, Papadaki et al. found that nuclear Twist localization was detected in the CTCs of 70.3% of MBC patients, whereas it was detected in only 32.3% of CTCs from early breast cancer patients [89]. Moreover, Markiewicz et al. found that CTCs isolated from lymph node-positive breast cancer patients are more frequently Vimentin and Snail mRNA expression positive compared to those from lymph node-negative patients [95]. Polioudaki et al. reported that 1-year OS of patients with high cytokeratin$^+$ CTCs was 73.3%, whereas 1-year OS declined by 46.2% in patients with low cytokeratin$^+$ CTCs ($p = 0.038$) [91].

4.3. CTC with CSC and EMT characteristics

Given the implications of both CSCs and EMT in metastasis, the existence of CTCs displaying both traits has been investigated. Aktas et al. tested the mRNA expression of three EMT markers (Twist1, Akt2, PI3Kα) and the CSC marker ALDH1 in CTCs from 39 MBC patients and found CTCs expressing at least one EMT marker, ALDH1, or both in 21 patients (81% of CTC-positive patients) [96]. The presence of CTCs coexpressing one of the EMT markers and ALDH1 was further confirmed by Raimondi et al. The mRNA expression of ALDH1 in bulk CTCs is correlated with the mRNA expression of Vimentin and Fibronectin ($p < 0.001$) [97]. Papadaki et al. further investigated the coexpression of ALDH1 and Twist in individual CTCs from both early and MBC patients and found that the prevalence of an ALDH1$^+$/Twist$^+$ subpopulation was significantly higher in MBC patients compared to those with early disease (76% vs. 15.4%, $p = 0.001$) [89]. Overall, these results indicate that the identification of a subpopulation of CTCs bearing mesenchymal properties, cancer stem cell characteristics, or both may help in discerning which patients are at higher risk for disease progression.

5. Summary

CTCs have received significant attention as a liquid biopsy to facilitate longitudinal disease monitoring. The current consensus based on large clinical studies is that CTC count is an independent prognostic marker in MBC, yet it is still controversial whether CTC count is predictive

of prognosis in non-MBC or could be used for monitoring of therapy response. Several clinical trials are currently ongoing to determine the utility of CTCs in making an early decision to change the course of therapy and spare toxicity. The isolation of CTCs has been a challenging task due to their rarity in the blood; however, a number of new isolation and detection strategies have emerged in the past 10 years, making CTC detection in relatively small amounts of blood feasible. The current challenge in this era is tackling the heterogeneous nature of CTCs and understanding which subpopulations drive metastasis. The count of CTCs with mesenchymal features was shown to be more sensitive in the prediction of prognosis than the number of bulk CTCs. Similarly, CTCs with both stem-like and mesenchymal features sensitively predicted prognosis. Currently, the detection of mesenchymal CTCs that have lost their epithelial markers requires laborious work necessitating either the detection of intracellular markers or mRNA expression. Therefore, discovery of a new CTC-specific functional surface marker that is relevant to metastasis would greatly advance the realistic clinical utility of CTCs. Additionally, in-depth understanding of CTC's heterogeneity utilizing single cell level analysis will improve our knowledge of hematogenous metastasis.

Abbreviations

AKT2	RAC-beta serine/threonine-protein kinase
ALDH1	Aldehyde dehydrogenase 1 family
AURKA	Aurora kinase A
BEVERLY-2	Single-arm phase II trial assessed the efficacy and safety of combining neoadjuvant chemotherapy with bevacizumab and trastuzumab for the treatment of HER2-positive breast cancer
CAM	Cell Adhesion Matrix
CCD	Charge-Coupled Devices
CD24	Signal transducer CD24 (Modulates B-cell activation responses)
CD44	CD44 antigen (receptor for hyaluronic acid)
CD45	Receptor-type tyrosine-protein phosphatase C
CD47	Leukocyte surface antigen CD47
CK19	Cytokeratin 19
CSC	Cancer Stem Cells
CT	Computerized tomography
CTCs	Circulating Tumor Cells
CXCR4	C-X-C chemokine receptor type 4

DAPI	4',6-Diamidino-2-phenylindole
DEP	DielEctroPhoretic
DFS	Disease-Free Survival
DNA	Deoxyribonucleic acid
EGFR	Epithelial Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
EpCAM	Epithelial cell adhesion molecule
EPISPOT	EPithelial ImmunoSPOT
ER	Estrogen receptor
ERBB2	Receptor tyrosine-protein kinase erbB-2 (Gene)
ERBB3	Receptor tyrosine-protein kinase erbB-3 (Gene)
ERCC1	DNA excision repair protein ERCC-1
FDA	Food and Drug Administration
FOXC1	Forkhead box protein C1
HB	Heringbone
HER2	Receptor tyrosine-protein kinase erbB-2 (protein)
HGF	Hepatocyte growth factor
HL	Hematopoietic lineage
HR	Hazard ratio
ISET	Isolation by size of epithelial tumor cells test
ISH	In situ hybridization
MBC	Metastatic breast cancer
MET	The hepatocyte growth factor receptor
MGB1	Mammaglobin
MICs	Metastatic-initiating cells
mRNA	Messenger ribonucleic acid
MUC1	Mucin-1
NPTN	Neuroplastin
NSG	NOD Scid Gamma
OS	Overall Survival

PBS	Phosphate-Buffered Saline
PFS	Progression-Free Survival
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
PR	Progesterone receptor
REMAGUS02	Phase II clinical study: Standard Neoadjuvant Chemotherapy Versus Genomic Driven Chemotherapy in Patients With Breast Cancer
RNA	Ribonucleic acid
RR	Relative risk
RT-PCR	Reverse transcription polymerase chain reaction
S0500 SWOG	Treatment decision making based on blood levels of tumor cells in women with metastatic breast cancer receiving chemotherapy
S100A4	Gene encodes Protein S100-A4
S100A9	Gene encodes Protein S100-A9
SERPINE1/PAI1	Plasminogen activator inhibitor 1
SIM	Single-cell isolation microfluidic
SUCCESS	Simultaneous Study of Gemcitabine-Docetaxel Combination adjuvant treatment
TGFβ1	Transforming growth factor beta-1
TN	Triple negative
ZEB2	Zinc finger E-box-binding homeobox 2

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