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Mapping breast cancer therapy with circulating tumor cells: The expert perspective

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ABSTRACT

Circulating tumor cells (CTCs) have emerged as a key prognostic biomarker for breast cancer, with their role becoming more pronounced in metastatic cases. In metastatic breast cancer, having five or more CTCs per 7.5 mL of blood is linked to poorer survival and more aggressive disease, marking it as stage $IV_{aggressive}$. Conversely, fewer than five CTCs per 7.5 mL of blood indicates a less aggressive, stage $IV_{indolent}$ disease. Additionally, molecular CTCs characterization provides a real-time snapshot of tumor biology, capturing its temporal and spatial variability and providing insights into tumor behavior.

Beyond their role in predicting outcomes, CTCs can help guide treatment intensity as shown in clinical trials like the STIC trial, offering a new way to tailor therapy alongside other liquid biopsy biomarkers such as circulating tumor DNA.

The aim of our review is to focus on both enumeration and phenotyping of CTCs and examine how CTC-guided strategies can improve treatment tailoring and patient outcomes. We also explore the potential for integrating CTCs with other biomarkers, such as circulating tumor DNA, and discuss how innovative biomarker-driven clinical trial designs could further advance personalized treatment strategies.

1. Introduction

Over the past 20 years, circulating tumor cells (CTCs) have become an established prognostic biomarker for patients with breast cancer (BC). Their role in disease monitoring has expanded, especially after the Food and Drug Administration (FDA) approved the CellSearch® system (Menarini-Silicon Biosystems, Huntingdon Valley, PA) as semi-automated platform for detecting CTCs in patients with metastatic BC (MBC). This approval was supported by multiple prospective and retrospective evidence all demonstrating that CTC count offers insights into disease behavior, distinguishing between stage $IV_{aggressive}$ ($\geq$5 CTCs/7.5 mL) and stage $IV_{indolent}$ (<5 CTCs/7.5 mL) [1–3]. These subgroups are characterized by specific outcomes, with higher CTC count associated to worse prognosis, independent of other clinic-pathological characteristics [2]. Although less frequently detected in patients with early breast cancer (EBC), CTCs still hold prognostic significance in this population, as a lower threshold of $\geq$1 CTCs/7.5 mL of blood has been proposed for prognostication [4].

Beyond their prognostic role, CTCs can be utilized to guide treatment intensity in patients with BC. Here we provide an overview, focusing on both enumeration and phenotyping, and explore how CTC-guided strategies can improve treatment tailoring and patient outcomes. We also discuss the potential for integrating CTCs with other biomarkers, primarly circulating tumor DNA (ctDNA), and how to further enhance personalized treatment strategies through new biomarker-driven clinical trial designs.

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2. CTC enumeration as a biomarker in clinical trials: from dynamic response assessment to prediction

The significance of CTC enumeration in predicting treatment response and guiding therapeutic decisions for patients with MBC patients has been investigated in prospective randomized clinical trials. The SWOG0500 trial was the first to prospectively assess the clinical utility of CTCs in treatment strategy as a dynamic marker of response [5]. The trial enrolled 595 patients with estrogen receptor (ER)-negative and endocrine therapy (ET)-refractory ER-positive MBC, evaluating CTC counts before starting first-line chemotherapy. Among them, 123 patients with ≥5 CTCs/7.5 mL at baseline whose CTC levels did not decrease after one chemotherapy cycle were randomized to either continue the same treatment or switch to an investigator's choice, unselected, non-biomarker-driven second-line chemotherapy. This early switching strategy did not improve progression-free survival (PFS) or overall survival (OS), failing to establish clinical validity for this CTC-guided strategy. However, the strong prognostic value of persistently elevated CTCs (median OS of 13 months, $p < 0.001$) suggested that failure to reduce CTCs after one cycle might indicate general resistance to chemotherapy, identifying a population that may benefit from alternative, targeted treatments [6]. Similar findings were reported in the phase III CirCe01 study, which randomized MBC patients with ≥5 CTCs/7.5 mL, on third-line chemotherapy or beyond, into standard versus CTC-driven approach (switching chemotherapy in case of no CTC response after the first cycle of chemotherapy) [7]. While OS did not differ between the two groups (hazard ratio: 0.95, $p = 0.8$), subgroup analysis showed longer survival for patients with no CTC clearance who underwent a chemotherapy change.

The randomized non-inferiority phase III STIC-CTC trial was the first to truly investigate and demonstrate that CTC enumeration could reliably inform treatment decisions and predict outcome in ER-positive MBC [8]. In this study, 778 patients with ER-positive/Human Epidermal Growth Factor Receptor 2 (HER2)-negative, untreated MBC were assigned to a first-line treatment regimen based on either CTC count or clinician's choice, including chemotherapy or endocrine monotherapy. In the experimental arm, patients with a high CTC count (≥5 CTCs/7.5 mL) were treated with chemotherapy, while those with low CTC counts received ET. Discrepancies between clinical assessment and CTC enumeration occurred in 38 % of cases. Improved PFS and OS were observed in patients with clinical low-risk but stage $IV_{aggressive}$ (as determined by CTC count) who received chemotherapy, suggesting that single-agent ET may not be effective for this subgroup [9]. Although STIC-CTC trial's primary limitation was its design before cyclin-dependent kinase 4/6(CDK4/6) inhibitors became the first-line standard for ER-positive/HER2negative MBC, it paved the way to a concept where CTC enumeration's prognostic impact could be leveraged for escalation or de-escalation strategies.

Treatment escalation in patients with Stage $IV_{aggressive}$ is also supported by an exploratory analysis of the phase II PACE trial. The trial enrolled patients with ER-positive/HER2-negative MBC previously treated with a CDK4/6 inhibitor-based first line endocrine therapy with aromatase inhibitors (AI). Patients were randomized to either receive fulvestrant alone, fulvestrant plus palbociclib, or fulvestrant plus palbociclib and avelumab [10]. While the primary endpoint of improved PFS was not met, patients in the Stage $IV_{aggressive}$ subgroup showed a better prognosis in the combination therapy arms, further suggesting the value of CTC enumeration in treatment tailoring [11].

On the other hand, in EBC, notwithstanding the strong evidence supporting the prognostic role of CTC count, clinical utility remains untested. Studies like NeoALTTO [12] and pooled analyses from the BEVERLY 1 and 2 trials [13] suggested that, while CTCs hold prognostic value, CTC enumeration is not associated with pathological complete response following neoadjuvant therapy in HER2-positive EBC, suggesting that CTCs might be an independent prognostic biomarker and not a mere surrogate for pathological complete response. Nevertheless, CTC-positive patients (≥1 CTCs/7.5 mL) treated with radiotherapy after breast-conserving surgery demonstrated longer OS in the SUCCESS trial (time ratio (TR), 4.37; 95 % CI, 2.71–7.05; $p < 0.001$), that was not achieved in patients without detectable CTCs (TR, 0.87; 95 % CI, 0.47–1.62; $p = 0.77$), suggesting a potential role for CTC-guided adjuvant radiotherapy [14].

Beyond predictive role, CTC enumeration still faces several critical limitations. The variability in detection techniques poses a concern; different methodologies, antigen dependent such as the CellSearch® system, or antigen independent (such as microfluidic devices as Parsortix™ Cell Separation System, imaging based or physical based methods), have unique strengths and weaknesses [15]. The major challenge of CTC enumerations is achieving reliable sensitivity and specificity, as various detection methods may miss low numbers of CTCs or misidentify non-cancerous cells as CTCs, resulting in false positives. Another significant limitation is the lack of standardized external quality assessments for various CTC enumeration techniques. This absence of validation can lead to inconsistencies across laboratories and studies, complicating comparisons and hindering the establishment of universal protocols. Approved in January 2004, the CELLSEARCH® System remains the sole CTC test authorized by the FDA for clinical use, demonstrating verified analytical accuracy, reproducibility, and system linearity [16]. However, it is an epithelial EPCAM based method, which may overlook other CTC subtypes (i.e. mesenchymal CTCs).

3. CTC phenotype characterization: potential targets for a personalized approach

Additional insights from CTC profiling have emerged by moving beyond simple enumeration to analyzing their phenotype. The characterization of CTCs offers a *real-time* view of tumor biology, addressing temporal and spatial heterogeneity [17]. Several biomarkers, such as HER2 and ER, which play key roles in BC treatment, can be evaluated on CTCs. Growing evidence supports their role in guiding treatment strategies in patients with BC.

With the enhanced efficacy of anti-HER2 treatments, a critical question is whether it is feasible to identify and target HER2 expression on CTCs. Results from randomized trials have been inconsistent, and definitive conclusions have been limited mainly by the variability in methods used to assess HER2 status on CTCs in different studies [18]. The CirCe T-DM1 phase II trial, for instance, did not show significant predictive value for HER2-positive CTCs in HER2-negative BC patients [19]. Eligibility for T-DM1 monotherapy was determined by the presence of ≥1 CTC with HER2 amplification (HER2/CEP17 ratio ≥2.2) by fluorescence in situ hybridization. In contrast, the DETECT III trial demonstrated that CTC phenotyping is valuable for stratifying patients with MBC for anti-HER2 therapy [20]. This study showed that patients with HER2-negative MBC and HER2-positive CTCs who received lapatinib plus standard treatment, had a significantly improved OS compared to those receiving only standard treatment. Patients with CTC clearance at first follow-up and at the end of treatment had a better OS benefit (HR 0.33; 95 % CI, 0.16–0.68; $p = 0.002$). HER2 CTC positivity in this trial was defined by either strong immunofluorescence (IF 3+) or moderate (IF 2+) HER2 expression in at least one or two CTCs, respectively. The ongoing NCT04993014 trial will evaluate if HER2 expression on CTCs in patients with an EBC which achieved pathological complete response can predict outcomes with pertuzumab-trastuzumab in the adjuvant setting and aid in treatment de/escalation strategies. The possibility to target HER2 expression on CTCs warrants further investigation and future studies should include novel anti-HER2 agents like antibody-drug conjugates (ADCs).

CTC phenotyping can also provide insights into the mechanisms of resistance to endocrine therapy. Research showed heterogeneity in ER expression among CTCs in patients with ER+/HER2- MBC, potentially predicting endocrine resistance [21–23]. A loss of ER expression is often linked to an unfavorable prognosis, suggesting that either phenotype

switching, or selection of ER-negative clones may be driving resistance [24]. Consistently, a preliminary sub-analysis of the PACE trial showed low ER expression on CTCs at disease progression [25]. To address these complexities, the CTC-Endocrine Therapy Index (CTC-ETI) was developed. This multi-parameter scoring system evaluates ER, BCL-2, ErbB2, and Ki-67 expression in CTCs, demonstrating predictive value for endocrine therapy resistance in patients with ER-positive/HER2-negative MBC enrolled in the COMETI trial [26–28]. Another mechanism related to ET resistance is the presence of *ESR1* mutations. Currently, approved methods for detecting these mutations include ctDNA analysis and tissue genomic evaluation. Studies demonstrated the feasibility of detecting *ESR1* mutations on CTC, however its predictive value remains unproven [29,30].

Another biomarker that can be assessed in CTCs is programmed death-ligand 1 (PD-L1), which provides a valuable tool particularly for patients undergoing immunotherapy [31]. PD-L1 expression on CTCs has shown heterogeneity compared to tissue and serves as a prognostic biomarker for poorer survival in various cancers, including breast cancer [32–35]. An analysis of 82 patients from the ALICE and ICON trials who received chemotherapy with or without immune checkpoint inhibitors showed inferior OS in those with PD-L1-positive CTCs, though the small sample size limits the predictive value [36]. With increasing use of immunotherapy in breast cancer, exploring PD-L1 expression on CTCs as a predictive biomarker in both neoadjuvant/adjuvant and advanced settings with larger cohorts is crucial [37–39].

The list of biomarkers that can be evaluated on CTCs is set to expand, especially considering the growing number of ADCs that are entering clinical practice, whose target expression levels can be evaluated in *real-time* on CTCs [40]. Although there is increasing evidence supporting the role of CTC phenotype in guiding treatment for BC patients, a standardized method for defining positive CTC samples is required. Advancing analytical and technical validation to establish predictive biomarkers for CTC phenotypes is essential for confirming their clinical validity and utility.

4. Innovative composite biomarkers: when CTC enumeration meets DNA and RNA profiling

In view of the rise of targeted therapies in MBC, ctDNA profiling has become increasingly widespread in the clinical management of this setting. In this perspective, it is crucial to design new pathways that embed both CTCs and ctDNA, given their complementary role in describing tumor biology and progression.

An emerging perspective is the integration of ctDNA mutational profiling with CTC-derived DNA sequencing. This approach could significantly enhance the overall sensitivity of liquid biopsy and give further details about clonal evolution and dynamics [30]. As a matter of fact, ctDNA often shows co-occurent mutations with different variant allele frequency (VAF) [41]. Single-cell analysis of CTCs can identify intercellular heterogeneity suggesting the presence of independent tumor subpopulations and therefore the need for a granular clonal profiling [42–44]. This additional layer of genetic information could refine the understanding of tumor evolution and provide deeper insights into mechanisms of resistance, particularly in cases where ctDNA may not fully capture the tumor's genetic diversity.

Moreover, while VAF dynamics detected through ctDNA have shown promise as a potential marker of early treatment response, the lack of a unified threshold for response interpretation remains a challenge [43,44]. On the other hand, CTC enumeration, backed by a more substantial body of evidence, serves as a dynamic proxy of tumor biology, providing valuable insights into metastatic disease evolution and potential treatment intensification strategies [45,46].

In addition to DNA-based analyses, the integration of an RNA-based characterization is gaining momentum due to the development of new CTC preservation and enrichment technologies together with the enhanced sensitivity of RNA sequencing. RNA analysis of multiple CTCs allows for the evaluation of gene expression and molecular characteristics across a population of CTCs, providing insights into tumor heterogeneity and treatment response, while single-cell RNA analysis focuses on the detailed characterization of individual CTCs, uncovering unique genetic alterations and signaling pathways that may drive metastasis, disease tropism and therapeutic resistance [47,48]. Galardi et al. proposed a possible application of digital droplet PCR RNA analysis within the cTREnd study, showing how ER-positive MBC patients with detectable CTC expression of *RB1* at any timepoint showed a numerically favorable outcome [49]. Coupled with other ctDNA-based biomarkers, such as *RB1* and *FGFR1* mutations and copy number alterations, this might better inform currently debated strategies such as CDK4/6 inhibition beyond progression [50].

A ctDNA-only workflow may, furthermore, overestimate the targetability of detected gene alterations, due to the potential epigenetic decoupling between DNA sequence and actual expression of the mutated isoform. Notably, *ESR1* promoter methylation is being suggested as a complementary resistant mechanism which hinders the expression of ER, including its mutated isoforms. This may both affect the response to oral Selective estrogen receptor degraders (SERDs) and decrease overall ER expression, both aspects that may be dynamically assessed through clinical-grade CTCs characterization [51,52].

5. The next step: biomarker-driven treatment sequencing and drug development

As the therapeutic landscape of MBC becomes increasingly complex, especially for ER-positive MBC, there is a growing need to tailor its decisional algorithm based on molecular features and disease clinical characteristics [53–57]. As a matter of fact, endocrine resistance is still not granularly understood due to its complex interplay of latent genetic, epigenetic, and transcriptomic alterations, a combination namely "swarm resistance." CTCs enumeration represents a composite biomarker of endocrine resistance able to grasp such complexity and identify patients who are less likely to benefit from endocrine therapy, potentially informing whether further endocrine lines, either monotherapy or in combination with targeted agents, might be a viable treatment option or if ADCs or chemotherapy regimens should be considered as an earlier treatment choice [58–61].

Based on this premise we propose an umbrella trial that leverages the combined potential of ctDNA and CTCs to guide the selection of targeted therapies in patients with HR positive, HER2 negative MBC after progression to first-line CDK4/6 inhibitor (PFS1) with a PFS1 $>$ 6 months (Fig. 1).

The trial design incorporates a biomarker-driven approach with specific arms based on two stratification steps. The first step will be based solely on ctDNA and will define arms A, B and C. In arm A, patients with detectable alterations in the AKT/PIK3CA/PTEN pathway, defined as loss of *PTEN*, amplification of *PIK3CA*, or SNVs in *AKT1*, *PIK3CA* and *PTEN*, will be allocated to receive capivasertib [62]. In arm B, patients with detectable Homologous Recombination Deficiency, defined as either germline or somatic alteration of the *BRCA1*, *BRCA2* and *PALB2* genes will be allocated to receiving receive olaparib [63]. In Arm C, patients with detectable ESR1 mutations who have experienced a progression-free survival (PFS1) exceeding 12 months during prior endocrine therapy combined with CDK4/6 inhibition (indicative of sustained endocrine sensitivity as observed in the EMERALD trial) will receive elacestrant [64].

Although arms A, B, and C align with standard-of-care therapies, they are designed to investigate resistance mechanisms, including rare PIK3CA alterations, ESR1 non-hotspot mutations, and emerging targetable mutations relevant to subsequent lines of treatment [65]. Furthermore, epigenetic modifications, such as DNA methylation alterations, implicated in resistance to the AKT/PIK3CA/PTEN pathway and endocrine therapy, will be examined. In the presence of co-mutations, an interdisciplinary molecular tumor board will implement a prioritization

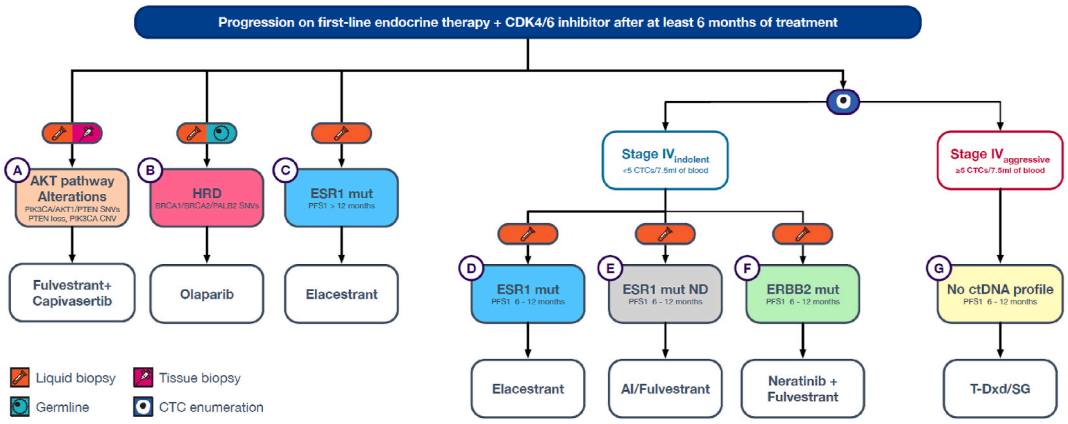

Fig. 1. Proposal for an umbrella trial using a CTC-driven approach.

algorithm to inform therapeutic decision-making, integrating mutation actionability and the potential for drug synergy or resistance.

In subgroups where a comprehensive endocrine resistance profile is lacking, a second stratification step will be applied to leverage the prognostic significance of CTCs in conjunction with the predictive value of ctDNA. Arm D, E and F will be focused on patients with an unclear residual endocrine sensitivity, defined as having experienced a PFS1 between 6 and 12 months [64,66]. Patients with Stage $IV_{indolent}$ MBC, will receive either elacestrant or AI/fulvestrant according to the ctDNA *ESR1* status. Similarly, arm F will enroll patients based on the *ERBB2* mutations, allocating to a combined neratinib and oral SERD strategy.

In Arm G, patients with stage IV aggressive MBC and a PFS1 of 6–12 months will be enrolled. An ADC-based regimen will be assigned according to the IHC score, irrespective of ESR1 or ERBB2 status. Patients with HER2-low disease will receive T-Dxd, whereas those with HER2-null disease will be treated with sacituzumab govitecan.

A retrospective synthetic control arm will then be designed by leveraging multi-institutional real-world datasets such as the Precision Medicine Academic Consortium (PMAC) database, providing a fast, feasible and flexible solution to investigate biomarker-driven algorithms notwithstanding the rapid evolution of treatment options in this population.

Developing robust, multi-modal, biomarker-driven studies, such as the proposed trial design, is essential for identifying the most promising treatment strategies for patients with MBC and addressing current limitations in drug development. However, several challenges must be overcome to ensure the robustness of the results. The development of synthetic control arms requires a strong bias mitigation strategy, incorporating stringent data harmonization and cross-validation with prospective data. Additionally, logistical challenges in patient recruitment, particularly for rare alterations such as ERBB2 mutations, necessitate a multi-institutional enrollment strategy with centralized biomarker confirmation. Furthermore, integrating activity-focused measures, such as PFS and OS, with patient-reported outcomes will be crucial to achieving a more comprehensive assessment.

CRediT authorship contribution statement

Lorenzo Gerratana: Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Caterina Gianni:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Eleonora Nicolò:** Writing – original draft, Data curation, Conceptualization. **Letizia Pontolillo:** Writing – review & editing, Formal analysis. **Francois-Clement Bidard:** Writing – review & editing, Supervision. **Carolina Reduzzi:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Massimo Cristofanilli:** Writing – review & editing, Supervision, Conceptualization.

Ethical approval

Ethical approval was not required.

Declaration of competing interest

Cristofanilli M reports personal fees from Lilly, Sermonix, Data Genomics, Foundation Medicine, Guardant Health, Celcuity, Iylon, and Ellipses and grants and personal fees from Pfizer, AZ and Menarini. Gerratana L reports consulting or advisory roles from AstraZeneca, Daiichi Sankyo, Eli Lilly, GlaxoSmithKline, Incyte, Novartis, Pfizer, Merck Sharp & Dohme, Menarini Stemline, AbbVie; Research Funding from Menarini Silicon Biosystems; Travel Expenses: Menarini Stemline. Bidard FC reports research support from Menarini Silicon Biosystems, Roche, AstraZeneca, SAGA Diagnostics, Personalis, and honoraria from Menarini Silicon Biosystems, Roche, AstraZeneca, SAGA Diagnostics. Reduzzi C reports research funding from Menarini Silicon Biosystems. Pontolillo L reports travel support from Pfizer. No disclosures were reported by the other authors.

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