



# Circulating tumor cells *et al.*: towards a comprehensive liquid biopsy approach in breast cancer

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**Abstract:** Liquid biopsy has emerged as a crucial tool in managing breast cancer (BC) patients, offering a minimally invasive approach to detect circulating tumor biomarkers. Until recently, the majority of the studies in BC focused on evaluating a single liquid biopsy analyte, primarily circulating tumor DNA and circulating tumor cells (CTCs). Despite the proven prognostic and predictive value of CTCs, their low abundance when detected using enrichment methods, especially in the early stages, poses a significant challenge. It is becoming evident that combining diverse circulating biomarkers, each representing different facets of tumor biology, has the potential to enhance the management of patients with BC. This article emphasizes the importance of considering these biomarkers as complementary/synergistic rather than competitive, recognizing their ability to contribute to a comprehensive disease profile. The review provides an overview of the clinical significance of simultaneously analyzing CTCs and other biomarkers, including cell-free circulating DNA, extracellular vesicles, non-canonical CTCs, cell-free RNAs, and non-malignant cells. Such a comprehensive liquid biopsy approach holds promise not only in BC but also in other cancer types, offering opportunities for early detection, prognostication, and therapy monitoring. However, addressing associated challenges, such as refining detection methods and establishing standardized protocols, is crucial for realizing the full potential of liquid biopsy in transforming our understanding and approach to BC. As the field evolves, collaborative efforts will be instrumental in unlocking the revolutionary impact of liquid biopsy in BC research and management.

**Keywords:** Breast cancer (BC); circulating tumor cells (CTCs); circulating tumor DNA (ctDNA); tumor-derived extracellular vesicles (tdEVs); liquid biopsy

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## Introduction

Over the course of the past decade, liquid biopsy has gained increased prominence in the management of patients with breast cancer (BC) (1). This minimally invasive approach for detecting circulating tumor biomarkers has proven invaluable in unraveling key information about tumor biology. Its validity extends to prognosis, prediction, and monitoring of treatment response in patients with BC. One of the noteworthy attributes of liquid biopsy is its ability to surmount the inherent limitations associated with traditional tissue biopsy. By circumventing challenges such as the inability to capture spatial tumor heterogeneity and the impracticability of serial sampling, liquid biopsy offers a real-time lens into the evolving landscape of tumors, providing a dynamic and comprehensive assessment of tumor evolution (2).

Even though nowadays the term liquid biopsy is predominantly associated with the detection of circulating tumor DNA (ctDNA), it was originally coined to describe the presence of circulating tumor cells (CTCs) (3). CTCs have been shown to play a pivotal role in the metastatic process, intravasating into the bloodstream and disseminating at distant sites (4). Many studies in patients with BC have focused on the detection of CTCs and CTCs detected using the Food and Drug Administration (FDA)-approved CellSearch<sup>®</sup> system have emerged as an independent and strong biomarker for survival in patients with early and metastatic BC (MBC) (5). Beyond the established prognostic role, also the predictive value of CTCs has been evaluated both based on the enumeration (6) and expression of surface biomarkers of CTCs, notably the human epidermal growth factor receptor 2 (HER2) (7).

Despite advancements in the CTC field, the main challenge lies in their low abundance, especially in the early setting. In fact, CTCs are elevated in up to 50% of patients with MBC (8,9) (cutoff of 5 CTCs per 7.5 mL of blood) and 25% of early-stage BC (EBC; cutoff of 1 CTC per 7.5 mL of blood) (10,11). Moreover, although these cutoffs have been validated and used for many years, discrepancies between CTC number and outcomes for individual patients were frequently observed. Therefore, additional markers are needed to stratify patient prognosis more precisely. Recognizing this, the utility of combining various circulating biomarkers, including cell-free DNA (cfDNA), extracellular vesicles (EVs), cell-free RNAs (mRNAs, long noncoding RNAs, and microRNAs), and other non-malignant circulating cells, becomes evident. Moreover,

tumors are complex, and CTCs defined by positivity for epithelial cellular adhesion molecule (EpCAM) and cytokeratins (CKs) represent only a fraction of circulating cells involved in disease dissemination.

The simultaneous evaluation of different liquid biopsy analytes can maximize the information derived from a single peripheral blood sample and provide a more accurate picture of the complexity of BC (*Figure 1*) (12). While the clinical significance of these circulating biomarkers has been largely assessed independently, understanding their potential complementarity could enhance prognosis definition thus enabling a more precise management of BC patients.

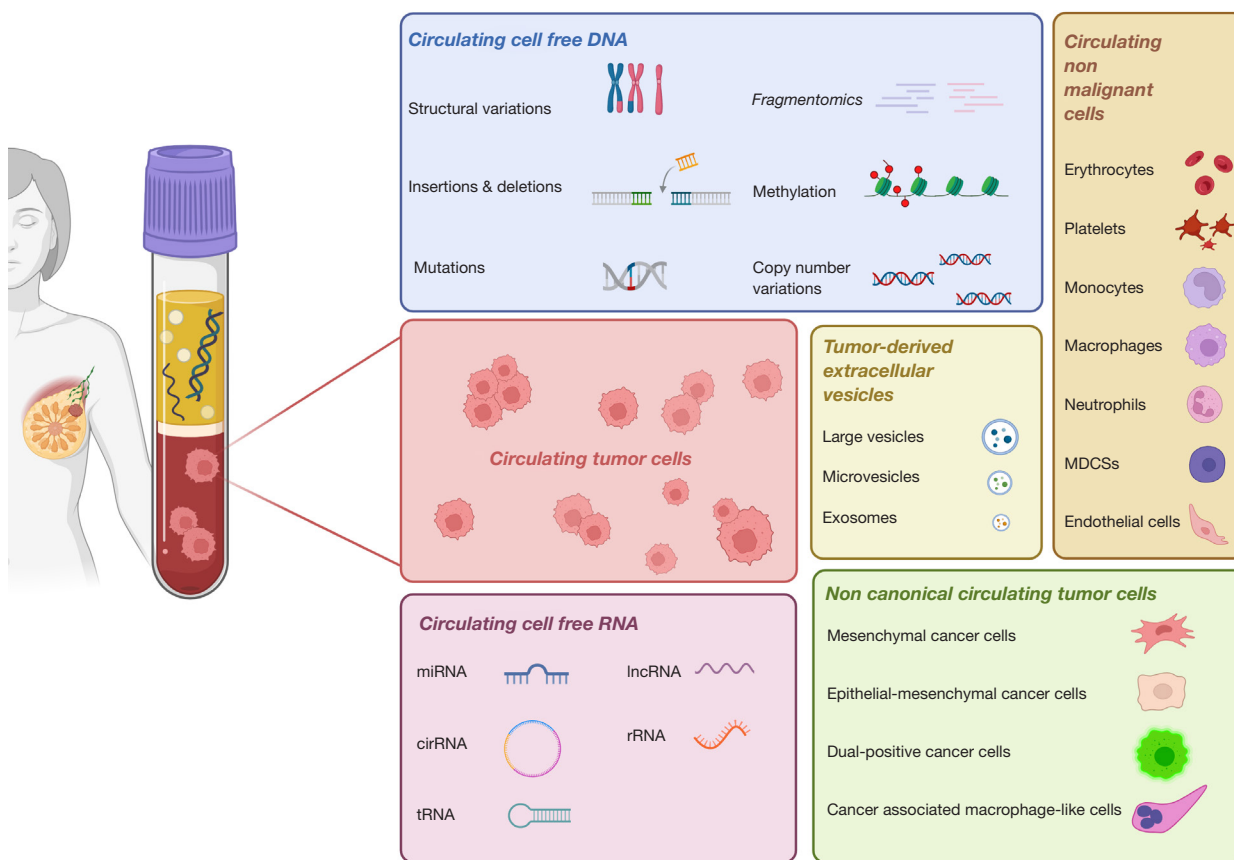
This article provides an overview of studies investigating the clinical value of concomitant analysis of CTCs and other circulating biomarkers. It delves into the opportunities offered by a comprehensive liquid biopsy approach in BC and the associated challenges.

## CTCs and ctDNA

ctDNA constitutes a small fraction of the cfDNA, released into the bloodstream by cancer cells after cell death (13). The presence of ctDNA has been extensively studied, revealing its prognostic significance in both EBC and MBC (14). Furthermore, the analysis of ctDNA alterations enhances the identification of potentially actionable mutations, making it a valuable tool for guiding treatment selection.

### CTC and ctDNA levels

Dawson *et al.* pioneered a proof-of-concept study, revealing ctDNA as an informative, specific, and highly sensitive biomarker in MBC (15). This study analyzed ctDNA and CTCs in 30 patients with MBC demonstrating that ctDNA outperformed CTCs in detection frequency, dynamic range, and correlation with tumor burden. Larger studies confirmed the prognostic significance of ctDNA detection, although in contrast to CTCs a cutoff of ctDNA correlated with worse prognosis has not been validated yet (16,17). Several studies have thus investigated the complementary prognostic value of ctDNA and CTC levels in MBC demonstrating that their combination is more informative than a single analyte for the prediction of patient outcomes (18-23). Nevertheless, the clinical validity of such a multimodal liquid biopsy approach has not been established yet. Shaw *et al.* reported a significant correlation



**Figure 1** Overview of liquid biopsy analytes in blood. MDSCs, myeloid-derived suppressor cells; miRNA, microRNA; cirRNA, circular RNA; tRNA, transfer RNA; lncRNA, long non-coding RNA; rRNA, ribosomal RNA.

between CTC count and cfDNA levels, establishing both as independent indicators for overall survival (OS) in MBC patients (24). Ye *et al.* evaluating 117 patients with MBC confirmed the individual impact of CTC and cfDNA on progression-free survival (PFS) (hazard ratio =2.58 and 2.05, respectively) and OS (hazard ratio =3.63 and 3.56, respectively), independently of clinical covariates and CTC/cfDNA levels, emphasizing the non-overlapping prognostic value of CTCs and cfDNA (25). Moreover, this analysis highlighted the joint effect of CTCs and cfDNA levels: patients with high levels of both markers had a >17-fold increased death risk ( $P < 0.001$ ) compared to those with low CTC and ctDNA levels. This joint effect was less prominent on PFS than OS suggesting that both CTC and cfDNA provide more values that are prognostic but not predictive of response to specific treatments. Additionally, longitudinal analysis from 22 patients revealed that responders exhibited lower CTC and cfDNA levels at first follow-up than non-responder patients and interestingly

suggested that the inconsistency between CTC changes and treatment response in some patients could be largely explained by cfDNA level. In the prospective COMET study, an analysis of 198 HER2-negative MBC patients receiving first-line chemotherapy showed a moderate correlation between CTCs and ctDNA detection at baseline and four weeks of treatment and confirmed the validity of both blood biomarker levels as prognostic markers before and during therapy (26). Moreover, combining CTCs and ctDNA improved the prognostic accuracy of multivariate clinicopathological models, with the best models for PFS and OS incorporating ctDNA variant allele frequency (VAF) at baseline (as a continuous variable) and CTC count at four weeks (as a dichotomized variable). Remarkably, only 13% of patients had neither ctDNA nor CTCs detected at baseline, suggesting that combining both markers could enhance the number of patients assessable for marker detection and monitoring. Gerrata *et al.* evaluated the longitudinal evolution of CTCs and ctDNA and their

impact on outcome prediction (27). They observed that while mutant allele frequency (MAF) in ctDNA appeared to follow treatment response versus progression, the number of CTCs increased only at the time of clinical progression. This suggests that ctDNA might be more suitable for real-time assessment of tumor burden and treatment effectiveness, while CTC counts could be more indicative of metastatic biology, offering an overall readout of the clinical aggressiveness of the disease.

CTCs and ctDNA complementarity has been assessed also in the early setting. Compared to CTCs, which are detectable in almost a quarter of patients with EBC (10,11), ctDNA can be identified in the majority of EBC patients thus representing a broadly applicable biomarker for disease monitoring (28-31). However, CTCs may provide additional information about the presence of minimal residual disease (MRD). In a secondary analysis of the BRE12-158 phase II clinical trial, the detection of ctDNA and CTCs in patients with early-stage TNBC after neoadjuvant chemotherapy was independently associated with disease recurrence (32). Notably, there was no significant association between CTC positivity (defined as  $\geq 1$  CTC detected) and ctDNA positivity, with a proportion of patients positive for only one marker. Thus, combining both markers significantly increased sensitivity for recurrence detection, from 79% with ctDNA alone and 62% with CTC alone to 90% when combined. The combined analysis demonstrated a stepwise reduction in distant disease-free survival (DDFS), with patients positive for both ctDNA and CTCs having a median DDFS of 32.5 months, compared to those positive for one marker or negative for both [median DDFS not reached for ctDNA-/CTCs-; hazard ratio =5.29; 95% confidence interval (CI): 1.50–18.62;  $P=0.009$ ]. This association was stronger than the detection of ctDNA alone (hazard ratio =2.99), with similar trends observed for disease-free survival (DFS) and OS when comparing ctDNA+/CTCs+ to ctDNA-/CTCs-. Another recently published study offered insights into the role of comprehensive liquid biopsy analysis for the therapeutic management of EBC patients (33). Over a ten-year period, the study examined 13 EBC patients through multianalyte liquid biopsy approach, encompassing enumeration, phenotypic characterization, and molecular monitoring of CTCs along with corresponding ctDNA mutation and methylation profile. Among the ten patients negative for liquid biopsy biomarkers throughout the follow-up, no relapse was observed. In contrast, three patients who tested positive for at least one liquid biopsy

biomarker experienced relapse during the follow-up period. The comprehensive approach used in this study demonstrated the potential to identify MRD four years before clinically detectable metastatic disease manifests. These findings suggest that beyond the established role of ctDNA in the detection of MRD in the circulation after surgery (34-36), the definition of a combined liquid biopsy-MRD might provide additional information and have a higher discriminatory capacity. This might lead to a broader definition of MRD through the combination of ctDNA (molecular residual disease) and CTCs (cellular residual disease) (37).

### CTC and ctDNA alterations

Various studies have explored the association between CTC count and ctDNA alterations, aiming to uncover the biological mechanisms that underlie their presence. Utilizing the PredicinePLUS™ platform for ctDNA sequencing, a study revealed a significant association between ctDNA alterations in *ESR1* and *GATA3* and an increased number of CTCs (38). Furthermore, alterations in *CDH1* were associated with a higher occurrence of CTC clusters, defined as aggregates of two or more CTCs. A report by Bortolini Silveira *et al.* demonstrated an association between specific ctDNA alterations and CTC counts: lysine (K)-specific methyltransferase 2 C gene (*KMT2C/MLL3*) variants were significantly associated with reduced CTC count, while the opposite trend was seen with *GATA3* alterations (26). Although the latter association did not reach statistical significance ( $P=0.07$ ), it supports the finding of Davis *et al.* (38) and may be related to the supposed role of *GATA3* in the migration and dissemination of BC cells (39). Regarding *KMT2C*, its inactivation in a gastric model was shown to promote epithelial-to-mesenchymal transition (EMT) and the acquisition of stem cell-like phenotypes (40), potentially explaining the lower CTC detection rate using the EpCAM-based CellSearch® system. Notably, *KMT2C* is frequently mutated in hormone receptor (HR)-positive BC and associated with resistance to endocrine therapies (41).

Moving beyond the mere enumeration of CTCs, researchers have explored the simultaneous molecular characterization of both CTCs and ctDNA. Some studies reported an advantage in analyzing emerging resistance mutations in CTCs compared to cfDNA (42,43). Paolillo *et al.* analyzed single CTC DNA and matched ctDNA in patients with HR-positive MBC, confirming all *ESR1*

mutations in ctDNA within the endocrine receptor-positive (ER+) CTC population, establishing a mechanistic link between CTCs and ctDNA (44). A subsequent study comparing *ESR1* mutations in BC patients demonstrated higher sensitivity in ctDNA compared to CTCs: at progression after endocrine therapy the *ESR1* mutation detection rate based on ctDNA and CTCs was 42% and 8%, respectively (45). This suggests that analyzing a large number of CTCs may be necessary to identify the full range of mutations in MBC. Another study comparing *PIK3CA* hotspot mutations in CTCs and paired ctDNA reported higher concordance in the metastatic setting (66.6%) than in the early setting (48.2%) (46), emphasizing the importance of CTC levels.

Recent research revealed the complementary nature of cfDNA and CTCs in identifying mutations in MBC, with a 28% overlap of mutations between the two fractions, while the majority of variants were unique in either cfDNA or CTCs (47). Interestingly, *ERBB2* variants were exclusively detected in CTCs, while *BRCA1/2* variants were more frequently found in CTC than in cfDNA, and *PIK3CA* and *ESR1* mutations were more common in cfDNA compared to CTCs. This may suggest that the choice between different analytes may depend on the specific clinical question. Moreover, a small percentage of patients (6%) showed no variants in either cfDNA or CTCs, highlighting the potential of multiparametric mutational analysis to optimize the identification of actionable targets, maximizing the benefit of targeted therapies.

Moreover, information residing in ctDNA that can be coupled with CTCs, extends beyond variants detection. Methylation of cfDNA (48), analysis of cfDNA fragments length (49), and end motifs (50,51) can provide additional layers of information. Studies comparing methylation profiles in matched cfDNA and CTCs revealed a high correlation in *SOX17* promoter methylation in both early and MBC, associated with outcomes in the latter setting (52,53). Additionally, a high concordance (98.3%) in *ESR1* methylation in cfDNA and CTCs was reported in BC patients, with *ESR1* methylation in CTCs associated with a lack of response to everolimus-exemestane (54).

## CTCs and EVs

EVs are membrane-enclosed structures of different size and origin released from cells, which play a crucial role in intercellular communication (55). Different subclasses of EVs play various roles in disease progression and metastatic

processes. Small EVs, especially exosomes, have been the main focus of BC research. However, the complex and time-consuming techniques needed for their isolation, enumeration, and phenotype posed scalability issues for clinical use and alternative approaches are currently being investigated (56-58). Large tumor-derived EVs (tdEVs), though much less abundant than small EVs, offer potential advantages. With a diameter exceeding 1,000 nm, tdEVs can be isolated alongside CTCs using the CellSearch® system. Initially identified through the ACCEPT software in castration-resistant prostate cancer (CRPC) patients (59), tdEVs are defined as 1–12 µm particles coexpressing CK and EpCAM but lacking the leukocyte marker CD45 and DNA stain. Although, because of the isolation method, tdEVs have not been fully characterized, their size is consistent with large oncosomes. Notably, tdEVs were around twenty times more frequent than CTCs in these patients, significantly correlating with survival. Subsequent studies by Nanou *et al.* confirmed the high frequency and prognostic value of tdEVs in MBC (60). A normal range of tdEVs (<20 per 7.5 mL of blood) was established in healthy controls, while in MBC patients, a cutoff of ≥80 tdEVs led to a significant dichotomization of patients with <5 CTCs, indicating a 4.9-fold higher risk of death in those with unfavorable tdEV counts. This finding suggested the value of combining CTCs and tdEVs for a more accurate assessment of patient outcomes. An analysis of the SWOG S0500 clinical trial affirmed the independent and complementary prognostic significance of tdEVs hormone-insensitive MBC receiving first-line chemotherapy (61). In the overall population, the median OS significantly varied based on tdEV counts: 43.3, 29, and 17.1 months for low [0–19], intermediate [20–79], and high [≥80] tdEVs, respectively. Of note, among patients with 0–4 CTC (favorable prognosis, median OS =34.2 months), those with high/intermediate tdEVs had significantly worse OS (23.2 and 30.9 months, respectively) than patients with low tdEVs (42.5 months). This suggests that among patients considered to have a more indolent disease based on low CTCs, there is a higher-risk subpopulation that might be identified using tdEVs. Even in patients with ≥5 CTCs (poor prognosis), tdEVs provided additional prognostic insights, with a median OS of 26.5 and 16.0 months for patients with intermediate and high tdEVs, respectively. Intriguingly, four patients with high CTCs and low tdEVs reported an exceptionally long median OS of 83.7 months. Further research should be aimed at unraveling the composition and exact origin of these abundant subcellular objects

to elucidate their biophysical properties and contribute to better understand the mechanisms underlying their prognostic significance.

Incorporating tdEVs into the management of patients with MBC could offer additional benefits. TdEVs, being more abundant than CTCs, might better reflect tumor heterogeneity and increase the number of patients' samples evaluable for therapeutic targets, since only a small number of patients have undetectable tdEVs. Studies have reported the expression of tumor-associated antigens such as HER2 and programmed death-ligand 1 (PD-L1) on tdEVs. As for CTCs, these biomarkers can be identified and quantified on tdEVs using CellSearch<sup>®</sup>. Investigations from Terstappen and his group showed that HER2 staining of tdEVs outperformed CTC staining in predicting the HER2 status of BC: a threshold of  $\geq 7\%$  of HER2-positive tdEVs predicted tissue HER2 expression with 74% specificity and sensitivity (62). However, a limitation was the use of HER2 amplification status from primary tissue, often diagnosed years before CTC and tdEV analysis. Given HER2's dynamic expression changes between primary and metastatic disease, further validation is essential to confirm concordance. Moreover, as compared to CTCs, the higher tdEV levels might make the latter a better indicator of tumor burden and possibly a more sensitive biomarker for treatment monitoring (63). Finally, the increased stability of EVs in the circulation may facilitate the evaluation of the mutational status, transcriptome, and proteome profile of the tumor. The characterization of tdEVs could unravel the presence of therapeutic targets and contribute to the comprehension of mechanisms that promote resistance and progression in BC. Previous studies identified androgen receptor (*AR*) or epidermal growth factor receptor (*EGFR*) mutations in CellSearch<sup>®</sup>-enriched blood samples of CRPC (64) and non-small cell lung cancer patients (65), respectively, that did not have any CellSearch<sup>®</sup>-defined CTCs. Though these gene mutations could also derive from CK- CTCs (66), it is possible that these are encapsulated within tdEVs, as supported by the presence of most tumor DNA in large CK+ tdEVs found in the plasma of prostate cancer patients (67).

Of note, these large tdEVs isolated with CTCs from the blood cell pellet represent only a small subset of probably <1% of the total EVs present in the blood before centrifugation. A larger fraction of smaller EVs, including exosomes and microvesicles, reside in the plasma fraction and their association with CTCs remains less explored. In 2018 a study compared messenger RNA (mRNA) profiles of

matched CTCs and EVs (from plasma fraction) in patients with HR-positive/HER2-negative MBC for the first time (68). The analysis revealed only a 5% overlap in signals between CTCs and EVs, emphasizing significant differences in mRNA profiles and the complementary nature of these analytes. Interestingly, divergent clinical outcomes were observed for mTOR transcript overexpression in CTCs versus EVs. Moreover, EV levels from plasma fraction pre- and post-neoadjuvant therapy have been associated with outcomes in patients with EBC suggesting that EVs may represent a complementary marker for providing information on MRD status (69). In this study an inverse association between stem cell-like and/or resistant CTCs and EV concentrations after therapy was observed, hypothesizing that these cells may internalize EVs.

### Beyond canonical CTCs

Most clinical studies on CTCs in BC have been conducted using the CellSearch<sup>®</sup> system, defining CTCs as nucleated cells expressing EpCAM and CK but lacking CD45. Despite the unquestionable value of such definition (9), emerging evidence suggests the potential role of other circulating cells not clearly reported by the CellSearch<sup>®</sup> that could aid in the management of patients with BC.

### Mesenchymal and epithelial-mesenchymal CTCs

EMT is an important phenomenon linked to increased invasiveness and resistance in tumor cells (70). Epithelial markers like EpCAM are lost during EMT, thus CTCs expressing only mesenchymal markers are missed using EpCAM-based enrichment methods. Studies using the CellSearch<sup>®</sup> system revealed the presence of cells expressing CK and lacking CD45 in the EpCAM-depleted fraction of roughly 70% blood samples and confirmed the malignant nature of these cells (71,72). Mesenchymal CTCs were reported also in samples with undetectable canonical CTCs, highlighting the potential additional information that the analysis of this subpopulation can provide. Additionally, CTCs can exhibit a mixed expression of epithelial and mesenchymal markers, indicating increased plasticity, a central feature in tumor progression and metastasis formation (73-76). A significant proportion (74-97%) of CK-positive CTCs in MBC patients also express mesenchymal markers, with lower prevalence in EBC, supporting the role of EMT in the metastatic potential of CTCs (77). Moreover, epithelial, mesenchymal and

mixed CTCs may stem different biological processes. Yu *et al.* suggested an association of mesenchymal CTCs with disease progression (78). Bulfoni and colleagues observed that whereas epithelial and mixed epithelial-mesenchymal CTCs were associated with worse OS among patients with MBC, mesenchymal CTCs were associated with a better outcome (79). Other studies, supported the association of mixed CTCs with chemoresistance and poor outcomes in patients with MBC (80-82). In a study of MBC patients receiving eribulin-based treatment (N=22), total CTCs and mesenchymal CTCs were associated with PFS, while no difference in PFS was observed according to the epithelial CTC count (83). However, an updated analysis suggested that epithelial CTCs may be more important determinants of OS than EMT-CTCs (84). Whether the observed PFS difference is linked to eribulin's effect on EMT suppression is yet unknown (85,86).

As for the early setting, Mego and colleagues found that EMT-CTCs were associated with poor prognosis in 427 patients with EBC, emphasizing their prognostic significance (87). A similar study based on gene expression analysis evaluated 83 EBC patients and showed that mesenchymal CTCs were characterized by the most aggressive phenotype, presence of lymph node metastases, larger tumor size, and seven-fold higher risk of death in the multivariable analysis (P=0.04) (88). In contrast, mixed epithelial-mesenchymal CTCs, believed to correspond to a highly plastic and aggressive state, did not show a significant impact on survival. A recent meta-analysis suggested that the prognostic value of EMT-CTCs might be more significant in patients with EBC compared to those with MBC (89).

Altogether these findings underscore the importance of evaluating mixed epithelial-mesenchymal and mesenchymal CTCs, as relying solely on epithelial markers might overlook a subset of patients at high risk for disease progression. Many CTC-detection approaches based on physical properties, including the FDA-approved Parsortix system (90), or on CD45-depletion have been developed to detect mesenchymal CTCs, providing additional tools for clinical assessment (91).

### ***Cancer associated macrophage-like cells***

Another population of circulating cells detected in BC patients is represented by cancer-associated macrophage-like cells (CAMLs) (92). CAMLs are defined as highly differentiated giant phagocytic cells (up to 300  $\mu\text{m}$ ) of

myeloid lineage (CD14+/CD11c+) expressing EpCAM, CK but also CD45, characterized by large-atypical/multiple nuclei and by an oblong or amorphous shape. Using microfilters, CAMLs were isolated in 93% of BC patients, either non-metastatic or metastatic (average 19.4 cells per sample); of note, no CAMLs were detected in healthy subjects (93). Mu and colleagues evaluated the prognostic significance of CAMLs in MBC patients (N=127) using the CellSearch<sup>®</sup> System (94). At least one CAML was detected in 21 patients (16.5%) and associated with increased risk of disease progression (hazard ratio =1.75; 95% CI: 1.03–2.98; P=0.037) and death (hazard ratio =3.75; 95% CI: 1.52–9.26; P=0.004) in the multivariable analysis. Interestingly, when combining the presence of CAMLs and CTCs, patients with <5 CTCs and with CAMLs, with  $\geq 5$  CTCs but without CAMLs, or with  $\geq 5$  CTCs and with CAMLs, had an increasing trend of risk of disease progression and death as compared to patients with <5 CTCs and without CAMLs, (PFS: hazard ratio =0.84, 3.42, and 4.04 respectively, P<0.0001; OS: hazard ratio =2.66, 6.14, and 9.13, respectively, P<0.0001). Thus, the presence of CAMLs in peripheral blood might provide a potential biomarker with additional prognostic values over CTC enumeration alone in MBC patients.

### ***Dual-positive cells (DPcells)***

Another subpopulation of circulating cells worth further investigation is represented by DPcells which express both CD45 and epithelial markers (95). Studies in MBC patients have reported a wide range of DPcell detection (5–100%), mainly affected by the CTC-enrichment methods used (72,96,97). This subpopulation has been widely understudied so far since DPcells were detected also in healthy individuals, although at significantly lower levels compared to BC patients (96), and considered as false positive staining events (98). The origin and clinical meaning of these cells are still under investigation and in 2018, for the first time, a study on patients with pancreatic cancer suggested that DPcells are hybrid cells derived from the fusion of cancer cells with macrophages (99). Such fusion hybrids acquire macrophage-associated features endowing them with accelerated growth, increased motility, enhanced invasion activity, and thus a higher efficiency in metastasis formation. In the mentioned study, the presence of DPcells was also significantly associated with shorter OS, albeit the small cohort of patients (N=20) prevented definitive conclusions. By performing single-cell low-pass

whole genome sequencing of DPcells collected from the blood of cancer patients (including BC) was demonstrated that a portion of DPcells have aberrant copy number aberration profiles (95), thus confirming their malignancy and reinforcing the heterotypic-cell fusion hypothesis. The fusion hypothesis is further supported by the observation that the majority of DPcells also express the macrophage marker CD68 (96). The association of circulating DPcells and OS was recently reported in a cohort of 341 BC patients supporting their tumor origin, with a prognostic impact observed primarily in patients with <5 CTCs and triple-negative BC (100). The fact that DPcells were present also in patients who do not present canonical CTCs (DPcells detected in 45% of samples of which 43% and 57% had  $\geq 5$  and <5 CTCs, respectively), raises new questions about their clinical relevance which should be further addressed, independently from their origin.

In summary, these studies highlight that beyond canonical CTCs other subpopulations of circulating rare cells, either tumor or stroma-derived cells, can be detected in the bloodstream and the notion of increased clinical utility through the utilization of pathological insights accompanying the identification of multiple rare circulating cell types simultaneously has been suggested (101). This multitype cell-based liquid biopsy approach would present a mosaic-like depiction of the underlying disease, thus offering a valuable tool for the management of patients with BC. Nevertheless, studies on these non-canonical cells are currently limited, and their clinical validity is not yet well-established. The primary limitation stems from the diverse technologies employed across studies, emphasizing the need for more standardized approaches to study this subpopulation and gain a clearer understanding of the mechanisms governing their presence and biological significance.

### Circulating tumor RNAs (ctRNAs)

Recently, there has been increased research interest surrounding circulating noncoding RNAs (ncRNAs), which do not evolve into protein synthesis, as novel biomarkers in cancer (102). ncRNAs include a vast group of circulating oligonucleotides, such as long noncoding RNAs (lncRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and small RNAs, such as microRNAs (miRNAs; short single-stranded noncoding RNAs of 20–25 nucleotides) and circular RNAs (circRNA), among many others. These molecules serve as regulators of gene transcription, exhibiting resistance to enzymatic cleavage in the blood (103).

Among various types of ncRNA, miRNAs have shown a major role in BC, acting as oncogenes (oncomiRNAs) or gene suppressors and playing an important role in cancer progression and oncogenic transformation (104,105). In the bloodstream, tumor-released miRNAs are protected from ubiquitous ribonuclease (RNase) enzyme-mediated degradation by embedding in exosomes or larger microvesicles shed from the plasma membrane. They also coexist as free-form ctRNA associated with a ribonucleoprotein complex or an argonaute-2 protein (106,107).

These non-exosomal free oligonucleotides have been shown to be involved in driving the progression of CTCs. From secondary analyses done on a cohort of 48 post-operative patients recruited in the frame of the SUCCESS A trial, miR-127 correlated with the presence of CTCs (108). Some miRNAs, particularly miR-199a-5p, have been shown to predict CTC clearance during treatment in patients with locally advanced BC exposed to neoadjuvant treatment, providing a molecular response assessment (109).

In a study conducted on an extended cohort of 269 individuals, a panel of circulating miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801) predicted the CTC status of patients with MBC and was able to differentiate cancer patients from healthy donors (110). In this research miR-200b was identified as the best marker to discriminate CTC-positive from negative patients.

Statistical evaluation of a model for OS and PFS prediction supported this finding showing that adding miR-200b to CTCs, compared with CTCs alone, significantly improved the model's adherence to the data (likelihood-ratio test,  $P=0.002$  for PFS and  $P<0.001$  for OS) (111,112). In the study by Fischer *et al.*, the circulating miR-200 family confirmed its role in BC and EMT, showing how increased levels of miR-200s and elevated CTC count correlated independently with poorer OS and PFS (112). However, combined data are not shown. The main limitation of this study is the small sample size and the use of the CellSearch<sup>®</sup> system, which is not sensitive in measuring CTCs that have undergone EMT.

Even though data regarding the prognostic role of miRNA are strong and rapidly expanding, data on the combined predictive and prognostic role of miRNA and CTCs are still limited and underpowered.

### CTCs and circulating non-malignant cells

Considering the rising awareness about the key role of the



tumor microenvironment and peripheral immunological processes, the role of blood analytes, though not directly derived from tumor cells, has gained importance. The various circulating blood cell ratios [neutrophil-to-lymphocyte ratio (NLR); monocyte-to-lymphocyte ratio (MLR); platelet-to-lymphocyte ratio (PLR)] could potentially serve as indicators to assess the immunological status, prognosis, and treatment outcomes (113,114). Monocytes and neutrophils, especially when considering the MLR and NLR index, have been associated with CTCs as prognostic indicators for OS in metastatic settings (115). In particular, MLR correlated with CTCs in triple-negative MBC, remaining significant also at the multivariable analysis.

Moreover, CTCs during the hematogenous dissemination can interact with different normal blood components such as platelets, neutrophils, monocytes, and endothelial cells to enhance their survival. Heterotypic clusters consist of not only CTCs but also immune cells, blood, and stromal cells associated with the tumor cells. Although these associated cells lack carcinogenic mutations, their phenotype undergoes changes that contribute to promoting tumor progression (116). It has been recently reported that red blood cells (RBCs) that accompany CTCs have prognostic value in MBC patients (117) and acquire modifications in their proteomic composition that were not observed among healthy donors (118). The lysosome-associated membrane glycoprotein 2 (LAMP2) was overexpressed in RBCs and high levels were associated with worse outcomes in MBC patients, suggesting that alterations in non-malignant components may provide additional information next to CTCs, though these are preliminary results and further studies are needed. The interaction between tumor cells and platelets has been demonstrated to modify the signaling pathways of platelets, referred to as tumor-educated platelets (TEP) (119). In research including various cancer types, TEP-derived RNA profiles, were used to successfully identify BC patients with an average accuracy of 62%, distinguishing also molecular subtypes (HER2+, PIK3CA, and triple-negative) (120). Data regarding the potential association between CTCs and TEP are lacking with wide room for further studies in the field.

Immunosuppression and smoldering-inflammation is a hallmark of BC with inflammatory circulating cells demonstrating to be involved in cancer progression and CTCs chaperoning (121). Macrophages and neutrophils can polarize into the pro-tumoral population.

In the work of the Aceto group, CTC-neutrophil clusters were associated with a more unfavorable prognosis in patients with MBC, as opposed to singular CTCs or CTC clusters (122). In the study, using single-cell RNA-sequencing (scRNA-seq) analysis, the researchers uncovered that CTC-myeloid clusters were predominantly composed of lymphocyte antigen 6 complex, locus G (LY6G) + neutrophils exhibiting a protumor 'N2-like' signature, indicative of a polarized state linked to immune suppression.

Findings concerning circulating cancer-associated cells exhibiting both epithelial and macrophage/myeloid characteristics provided backing for the hypothesis that fusion events between CTCs and pro-tumoral macrophages could play a crucial role in tumor progression. As previously said, CAMLs have been detected in the peripheral blood of patients with BC, showing to be able to provide prognostic information along with CTC detection (94). Circulating myeloid-derived suppressor cells (MDSCs) may be another heterogeneous immunosuppressive cell population correlating with CTCs and inducing worse survival outcomes in BC (123). High levels of monocytic-MDSCs tend to be related to a higher number of CTCs in patients with aggressive disease and *de novo* metastatic disease suggesting their role in the immunoregulatory switch responsible for cancer dissemination (124).

Few data regarding circulating endothelial cells (CECs) and CTCs suggested a potential correlation between them. Two studies, in the metastatic setting, showed CECs and CTCs to be independently correlated to a significantly worse outcome, however, the two counts did not result to be correlated with each other (125,126). A study conducted including EBC patients demonstrated a significantly poorer DFS in patients who experienced a rise in CEC/CTC counts ratio during adjuvant radiotherapy ( $P=0.004$ ) (127).

### Challenges of a multiparametric approach

All these data emphasize that the information depicted by a single blood analyte is limited and a multiparametric approach integrating information on different levels might be more informative and more comprehensively reflect the characteristics of the disease (*Tables 1,2*). However, along with opportunities, it is crucial to acknowledge and address the challenges that come with the implementation of such a holistic approach.

First, pre-analytical issues related to the collection, storage, and preparation of blood samples require shared validated protocols (128). Indeed analysis of different

**Table 1** Multimodal liquid biopsy studies including CTCs and non-cellular components

Reference	Clinical purpose	Clinical setting	Analytes				Conclusions
			CTCs	cfDNA/ctDNA	tdEVs	miRNA	
Dawon <i>et al.</i> (15)	Monitoring Prognosis	MBC	Count	Levels			Greater dynamic range and correlation with changes in tumor burden of ctDNA than CA 15-3 or CTC
Shaw <i>et al.</i> (24)	Prognosis	MBC	Count	Levels			High CTC count and total cfDNA level were significantly associated with poorer OS
Ye <i>et al.</i> (25)	Prognosis	MBC	Count	Levels			CTC and total cfDNA levels were individually and jointly associated with PFS and OS
Bortolini Silveira <i>et al.</i> (26)	Prognosis	HER2– MBC	Count	Level mutations			Both CTC and ctDNA levels were correlated with survival. KMT2C/MLL3 variants by ctDNA significantly associated with a lower CTC count; opposite trend was seen with GATA3 alterations
Fernandez-Garcia <i>et al.</i> (20)	Prognosis Therapy monitoring	MBC	Count	Level			Both cfDNA and CTCs predictors of OS, only cfDNA predictor for PFS and disease response
Gerratana <i>et al.</i> (27)	Longitudinal evolution Prognosis	MBC	Count	Level (MAF) NOA			MAF trends reflected the treatment response. NOA steadily increased across time points. CTCs enumeration significantly increased only between first evaluation and progression
Radovich <i>et al.</i> (32)	Relapse prediction	TN EBC	Count	Detection			The positivity of both CTC and ctDNA had a negative impact on DDFS, DFS and OS
Stergiopoulou <i>et al.</i> (33)	Relapse prediction	EBC	Count Phenotypic analysis Gene expression Methylation	Mutations			Positivity for at least one LB marker predictive of relapse. The molecular characteristics of CTCs were highly different at different time points, and always increased before the clinical relapse
Davis <i>et al.</i> (38)	Analytic validity	MBC	Count	SNVs, CNVs, and gene fusions			CDKN2A correlated with lower number of CTC; while ESR1, GATA3, CDH1 and CCND1 correlated with higher number of CTC. CTC clusters were significantly associated with somatic genomic alterations in CDH1, CCND1, and BRCA1
Paolillo <i>et al.</i> (44)	Technical feasibility	HR+ MBC	ESR1	ESR1			Concordance of ESR1 mutations between ER+ CTC and cfDNA
Beije <i>et al.</i> (45)	Therapy monitoring	HR+ MBC	ESR1 (hotspot mutations, splice variants)	ESR1			The ESR1 mutations' detection rate after ET therapy based on ctDNA and CTCs was 42% and 8%, respectively

Table 1 (continued)

Table 1 (continued)

Reference	Clinical purpose	Clinical setting	Analytes				Conclusions
			CTCs	cfDNA/ctDNA	tdEVs	miRNA	
Tzanikou <i>et al.</i> (46)	Molecular characterization	EBC and MBC	PIK3CA (hotspot mutations)	PIK3CA			The detection and concordance of PIK3CA hotspot mutations between plasma-ctDNA and CTCs are higher in the metastatic setting
Keup <i>et al.</i> (47)	Molecular characterization	HR+/HER2–MBC	Gene expression	Mutations			Most variants were unique in either ctDNA or CTCs; only 28% overlapped. PIK3CA and ESR1 variants were less common in CTC gDNA, while ERBB2 variants were only detected in CTC gDNA
Chimonidou <i>et al.</i> (52)	Molecular characterization and Prognosis	EBC and MBC	Methylation	Methylation			High correlation in SOX17 promoter methylation in both early and MBC, with a negative prognostic impact on OS
Mastoraki <i>et al.</i> (54)	Therapy monitoring	HR+/HER2–MBC	Methylation	Methylation			Highly concordant ESR1 methylation in CTCs and corresponding ctDNA. ESR1 methylation in CTCs associated with a lack of response to everolimus-exemestane
Nanou <i>et al.</i> (60)	Prognosis	MBC	Count		Count		Presence of unfavorable CTCs (cut-off >5) and tdEVs (cut-off ≥20) is predictive of OS. TdEVs can further stratify prognosis of patients with favorable CTC count
Nanou <i>et al.</i> (61)	Prognosis	MBC	Count		Count		Elevated tdEV levels were independently associated with poorer OS. Complementary prognostic significance of tdEVs and CTCs
Nanou <i>et al.</i> (62)	HER2 assessment	EBC and MBC	Count		Count		Inter- and intra-patient heterogeneity for the CTC/tdEV phenotypes associated with worse prognosis. The use of ≥7% HER2+CK+ tdEVs can predict HER2 expression of the tissue with 74% sensitivity and specificity using the HER2 amplification status of the primary tumor as a classification variable
	Prognosis		CK and HER2 phenotype		CK and HER2 phenotype		
Keup <i>et al.</i> (68)	Therapy management	HR+/HER2–MBC	mRNA		mRNA		Concordance 5%. Divergent clinical outcomes were observed for mTOR transcript overexpression in CTCs versus EVs

Table 1 (continued)

Table 1 (continued)

Reference	Clinical purpose	Clinical setting	Analytes				Conclusions
			CTCs	cfDNA/ctDNA	tdEVs	miRNA	
Keup <i>et al.</i> (131)	Longitudinal monitoring	HR+/HER2–MBC	mRNA	Mutations	mRNA		The presence of either ERBB3 signals in CTCs or EVs or cfDNA variants in ERBB3 showed a significant association with progressive MBC. The three analytes, each with their own unique features for disease monitoring, were shown to be complementary, underlining the usefulness of the longitudinal multi-parametric liquid biopsy approach
Keup <i>et al.</i> (130)	Clinical relevance	HR+/HER2–MBC	mRNA genomic DNA	Mutations	mRNA		A combination of two/three/four LBAs increased the prevalence of patients with actionable signals. Aggregating the results of hierarchical clustering of individual LBAs into the ELIMA score resulted in a highly significant correlation with OS
Alunni-Fabroni <i>et al.</i> (108)	Associations of miRNA with CTC Prognosis	EBC	Count			Concentration and characterization	Significant correlation between miR-200b and lymph node status and between miR-20a and tumor type. miR-127 correlated with the presence of CTCs. Borderline significant association between PFS and miR-19a levels
Akkiprik <i>et al.</i> (109)	Prognosis	EBC	Isolation and characterization for EMT, drug resistance and stemness markers			Isolation and characterization	miR-199a-5p predicts CTC clearance during treatment in patients with locally advanced BC exposed to neoadjuvant treatment, providing a molecular response assessment
Madhavan <i>et al.</i> (110)	Prediction of the CTC status	MBC	Count			Profiliation	A panel of circulating miRNAs (miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-210, miR-375, and miR-801) predicted the CTC status of patients with MBC and was able to differentiate cancer patients from healthy donors
Fischer <i>et al.</i> (111)	Prognosis	MBC	Count			Expression level- miR-200 family	The levels of miR-200s were elevated in CTC-positive versus CTC-negative pts. Increased levels of miR-200s and elevated CTC count correlated independently with poorer OS and PFS

Studies including circulating tumor cells and other non-cellular analytes (i.e., ctDNA, tdEVs, and miRNA) are described in the table, categorized for clinical purpose and setting. CTCs, circulating tumor cells; cfDNA, circulating free DNA; ctDNA, circulating tumor DNA; tdEVs, tumor-derived extracellular vesicles; miRNA, microRNAs; MBC, metastatic breast cancer; OS, overall survival; HER2, human epidermal growth factor receptor 2; MAF, mutant allele frequency; NOA, number of alterations; TN, triple-negative; EBC, early-stage breast cancer; DDFS, distant disease-free survival; DFS, disease-free survival; LB, liquid biopsy; SNVs, single-nucleotide variations; CNVs, copy number variations; HR+, hormone receptor positive; ER, estrogen receptor; mTOR, mammalian target of rapamycin; EVs, extracellular vesicles; LBAs, liquid biopsy analytes; PFS, progression-free survival; EMT, epithelial-to-mesenchymal transition.

**Table 2** Multimodal liquid biopsy studies including canonical CTCs and other cellular components

Reference	Clinical purpose	Clinical setting	Analytes	Analysis	Conclusions
Yu <i>et al.</i> (78)	Prognosis	MBC	Mesenchymal CTC	Count	Association of mesenchymal CTCs with disease progression
Bulfony <i>et al.</i> (79)	Clinicopathological features Prognosis	MBC	E-CTC, EM-CTC, MES, NEG	Detection by DEPAArray-based strategy	Significant association between specific CD45 neg subpopulations and tumor subtypes, proliferation, and sites of metastatic spread. EM-CTC was significantly associated with poorer PFS and OS
Giordano <i>et al.</i> (80)	Characterization Prognosis	HER2+ MBC	EM-CTC	Gene transcripts of EMT-TF and CSC features	HER2 + MBCs had EMT-CTCs. An enrichment of CSCs was found in CD326- and CD45- cells. EM-CTC related to lower PFS and OS
Gradilone <i>et al.</i> (81)	Prognosis	MBC	Mesenchymal CTC	Cytokeratin and markers of epithelial mesenchymal transition expression	The presence of mesenchymal markers on CTC more accurately predicted worse prognosis than the expression of cytokeratin alone
Papadaki <i>et al.</i> (82)	Prognosis	MBC	CSC <sup>+</sup> /partial-EMT <sup>+</sup> CTCs	Cytokeratin expression	CSC <sup>+</sup> /partial-EMT <sup>+</sup> CTCs correlated to lung metastases and decreased PFS. In HER2- patients related also to decreased OS and chemoresistance
Horimoto <i>et al.</i> (83); Ito <i>et al.</i> (84)	Prognosis	MBC	EM-CTC	Count and characterization	Higher CTCs number and mesenchymal CTC associated with negative prognostic impact on eribulin's PFS. Total CTCs number related to worse OS
Mego <i>et al.</i> (87)	Prognosis	EBC	EM-CTC	Detection	EM-CTC have a negative prognostic value in primary BC
Markiewicz <i>et al.</i> (88)	Clinical significance	EBC	CTCs with different EMT status	Isolation and molecular characterization	MES characterized by the most aggressive phenotype, lymph nodes metastases, larger tumor size and higher risk of death. EM-CTC no impact on survival
Mu <i>et al.</i> (94)	Prognosis	MBC	CAMLs	Detection and count	CAMLs negative prognostic impact on PFS and OS. Combining with CTCs number, the presence of CAMLs with either <5 CTCs or >5 CTS had a negative prognostic impact on both PFS and OS, while only >5 CTCs and CAMLs—retained the statistical significance
Reduzzi <i>et al.</i> (100)	Prognosis	EBC and MBC	DPcells	Detection and count	Prognostic impact observed primarily in patients with <5 CTCs and triple-negative BC
De Giorgi <i>et al.</i> (115)	Correlation between CTC and inflammation based scores	MBC	Neutrophils, lymphocytes, platelets, monocytes	MLR, NLR, PLR, CTCs count	CTC and MLR are predictors of OS in MBC. CTC correlated with monocytes, in particular in triple-negative tumors
Carmona-Ule <i>et al.</i> (117)	Prognosis	MBC	RBCs	CTC culture	The presence of RBCs in the culture was linked to a worse patient outcome

Table 2 (continued)

Table 2 (continued)

Reference	Clinical purpose	Clinical setting	Analytes	Analysis	Conclusions
Pereira-Veiga <i>et al.</i> (118)	Prognosis	EBC and MBC	RBCs	Proteomic composition	Different proteomic profile compared with healthy donors and between different tumor stages. Lysosome-associated membrane glycoprotein 2 emerge as a new RBCs marker with diagnostic and prognostic potential for MBC patients
Best <i>et al.</i> (120)	Diagnostic potential	BC	TEPs	RNA-profile	TEP-derived RNA profiles, were used to successfully identify BC patients with an average accuracy of 62%, distinguishing also molecular subtypes (HER2+, PIK3CA, and triple-negative)
Mego <i>et al.</i> (121)	Correlation between CTC and PB-T cell	IBC	PB T-cell	CTC enumeration, flow cytometry for T cell phenotype and function	IBC patients with CTCs in PB had abnormalities in adaptive immunity that could potentially impact tumor cell dissemination and initiation of the metastatic cascade
Szczerba <i>et al.</i> (122)	Identification and function	BC	CTC-associated WBCs	Isolation and characterization	CTC-neutrophil clusters were associated with a more unfavorable prognosis in patients with MBC, as opposed to singular CTCs or CTC clusters
Cole <i>et al.</i> (123)	Correlation with CTCs and prognosis	MBC	MDSCs	Enumeration	MDSCs correlated with CTCs and were associated with overall survival
Bergenzfelz <i>et al.</i> (124)	Clinical significance	MBC	Monocytic-MDSCs	Flow cytometric analysis	High levels of monocytic-MDSCs tend to be related to a higher number of CTCs in patients with aggressive disease and <i>de novo</i> metastatic disease suggesting their role in the immunoregulatory switch responsible for cancer dissemination
Bidard <i>et al.</i> (125)	Prognosis	MBC	CECs	Count	CTC and CECs independent prognostic factor. Increase in CEC count was associated with improved time to progression, at the threshold of 20 CECs/4 mL
Codes <i>et al.</i> (126)	Therapy monitoring	HER2- MBC	CECs	Count	CTC and CECs independent prognostic factor. Baseline CECs >200 was associated with lower PFS
Mäurer <i>et al.</i> (127)	Disease relapse	EBC	CECs/CTC	Count	Patients with an increase in CETC/CTC numbers over the course of adjuvant RT had a significantly worse disease-free survival than patients with stable or decreasing CETC/CTC numbers

Studies including circulating tumor cells and other cellular analytes are described in the table, categorized for clinical purpose and setting. CTCs, circulating tumor cells; MBC, metastatic breast cancer; E-CTC, cells expressing only epithelial markers; EM-CTC, epithelial-to-mesenchymal transition circulating tumor cell; MES, cells expressing only mesenchymal markers; NEG, cells negative for every tested marker; PFS, progression-free survival; OS, overall survival; HER2, human epidermal growth factor receptor 2; EMT-TF, epithelial-to-mesenchymal transition-inducing transcription factors; CSC, cancer stem cell; EBC, early-stage breast cancer; BC, breast cancer; EMT, epithelial-to-mesenchymal transition; CAMLs, cancer-associated macrophage-like cells; DPcells, dual-positive cells; MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; RBCs, red blood cells; TEPs, tumor-educated platelets; IBC, inflammatory breast cancer; PB, peripheral blood; WBCs, white blood cells; MDSCs, myeloid-derived suppressor cells; CECs, circulating endothelial cells; CETC, circulating epithelial tumor cell.

circulating biomarkers is based on different isolation protocols and can require different preservative blood collection tubes for a reliable analysis (129). Thus, blood volume needed for multiple analyses is additive and can hamper patients' compliance. Workflows have been implemented to enable the parallel isolation and analysis of multiple biomarkers minimizing the blood volume. The ELIMA (Evaluation of multiple Liquid biopsy analytes In Metastatic breast cancer patients All from one blood sample) multiparametric approach included the analysis of EV mRNA, CTC mRNA, CTC genomic DNA, and cfDNA from CTC-depleted blood from 18 mL of EDTA blood (130). The integration of these four analytes enabled to simultaneously analyze the transcriptional and genomic complexity of BC and revealed improved sensitivity for actionable markers and prognostication. Another work from the same group showed the usefulness of a longitudinal multiparametric approach, combining CTC mRNA, EV mRNA and ctDNA, for disease monitoring in patients with HR-positive/HER2-negative MBC (131). A study in prostate cancer patients showed the feasibility of a combinational analysis of transcripts in CTCs, the same transcripts in whole blood, and focal amplifications in cfDNA from 12.5 mL of blood (132). This approach led to an increase in the probability of identifying resistance mechanisms as compared to the use of a single analyte (89% vs. 50%).

Furthermore, the standardization of liquid biopsy methodologies and the establishment of universally accepted protocols pose significant challenges. The variability in techniques and platforms across different studies hinders the seamless comparison of results and the translation of findings into clinical practice. Achieving a consensus on the standardization of liquid biopsy procedures is imperative to ensure the reproducibility and reliability of results across diverse clinical settings. The necessity of detecting a broader range of circulating tumor-derived material has led to the development of next generation systems. Among these, the third-generation high-definition single-cell assay (HDSCA3.0) workflow provides the ability to identify and characterize epithelial, mesenchymal, endothelial, and hematopoietic cells, as well as large EVs. A study demonstrated that, beyond CTCs, assessing a wider range of circulating events enhanced the ability to categorize normal donors, EBC, and MBC patients into distinct groups (133).

Another significant challenge pertains to the complexity of interpreting and integrating the vast array of information

gathered from different circulating biomarkers. Each biomarker contributes unique insights into tumor biology, and navigating the interplay among these various components demands sophisticated analytical approaches. The challenge lies in harmonizing these diverse data streams to derive meaningful and actionable conclusions that can guide clinical decision-making. In this context, there is increasing interest in using combinatorial approaches driven by machine learning to increase sensitivity and accuracy. A multianalyte panel consisting of tumor-associated EV miRNAs and mRNAs, cfDNA, and CA19-9, demonstrated utility for early diagnosis and staging of pancreatic cancer in 204 subjects (134). Interestingly, in this study the investigators developed an initial classification model using 14 biomarker candidates and then trained a machine-learning model achieving a sensitivity of 88% and a specificity of 95%.

Finally, prospective, multicenter clinical trials including large cohorts of patients are needed to provide strong evidence of the clinical utility of multiparametric testing.

## Conclusions

In conclusion, it appears evident that the diversity of analytes that can be obtained from the blood of patients with BC is remarkable, each presenting limitations and advantages. So far, CTC and ctDNA have been the most commonly utilized liquid biopsy biomarkers. A combined approach leveraging the strength of each, such as the heightened sensitivity of ctDNA and the ability of CTCs to offer insight into tumor biology, hold promise for improving early detection, prognostication, and treatment. Furthermore, integrating information from various circulating component, beyond CTCs and ctDNA, could yield an even more comprehensive disease profile. While these diverse analytes may be partially linked to CTCs, they mostly do not overlap, representing distinct biological processes, such as intercellular communication for EVs, or interaction with immune cells for CAMLs and DPcells. The fact that different circulating biomarker should be viewed as complementary/synergistic rather than competitive is becoming increasingly evident, not only in BC but also in other cancer types (135). While the comprehensive liquid biopsy approach opens up new opportunities in BC research and management, it is essential to address the associated challenges. Overcoming these hurdles will necessitate concerted efforts in refining detection methods, establishing standardized protocols, and developing analytical

frameworks for data integration. As the field continues to evolve, these endeavors will be instrumental for unlocking the full potential of liquid biopsy in revolutionizing our understanding and approach to BC.

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