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LOCAL-REGIONAL EVALUATION AND THERAPY (DM EUHUS, SECTION EDITOR)

Role of Liquid Biopsy in Clinical Decision-Making for Breast Cancer

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Abstract

Purpose of Review Liquid biopsies are easily obtainable, non-invasive, longitudinal snapshots that can be used to measure micrometastatic disease burden, monitor disease progression, and provide genomic assessments of primary tumor/metastatic lesions. To date, most published studies have focused on circulating tumor cells (CTCs) and cell-free circulating tumor DNA (ctDNA); however, the liquid biopsy field is expanding exponentially and new blood components are currently under investigation.

Recent Findings CTCs and ctDNA remain the most extensively studied liquid biopsy components to date. Several additional blood-based components are the basis of active, ongoing investigations. Some on the horizon include serum/plasma exosomes, platelet-mRNA, miRNA characterization, and global proteomic studies.

Summary In the era of individualized medicine, liquid biopsy has potential to improve upon current breast cancer management by offering dynamic monitoring possibilities as well as novel targets for therapy.

Keywords Breast cancer · Liquid biopsy · Circulating tumor cells · Circulating tumor DNA

Introduction

Breast cancer is the most commonly diagnosed cancer among women. Metastasis, a complex, multi-step process, remains the primary cause of death for these patients. Primary tumor biopsy, primarily the assessment of cellular morphology, Ki-67 staining, estrogen and progesterone receptor expression, and human epidermal growth factor receptor 2 (HER2), remains the gold standard for breast cancer treatment decision-making. However, breast tumors are highly heterogeneous; a single tumor analysis is not likely to detect aggressive subclones within the tumor. Tumor biopsy has limitations; the biopsy procedure is invasive, and tumor tissue is not always available to monitor dynamic genomic alterations/changes that are acquired during treatment pressure and/or disease progression. In the era of individualized medicine, longitudinally investigating inter/intra-tumor heterogeneity is crucial for understanding the biology of breast cancer and providing molecular targets for effective patient management. The "liquid biopsy" approach is based on the analysis of tumor-derived cells (circulating tumor cells, CTCs), circulating cell-free tumor DNA (ctDNA), mircoRNA, and proteins isolated from peripheral blood samples. Liquid biopsies are easily obtained, non-invasive, longitudinal snapshots that can be used to measure micrometastatic disease burden, monitor disease progression, and provide genomic assessments of primary tumor/metastatic lesions. Incorporating information obtained from liquid biopsy analyses into clinical practice has the potential to improve upon current breast cancer management.

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Liquid Biopsy Component: Circulating Tumor Cells

Although the mechanisms involved in tumor cell invasion into the bloodstream are unknown, we know that circulating tumor cells (CTCs) are rare, occurring in as few as 1 CTC/10^{7-8} hematopoietic cells [1]. Circulating tumor cells are heterogeneous populations of cells with varying viability, dormancy, biomarker expression, and metastatic capabilities. This heterogeneity makes detecting CTCs and determining their clinical significance challenging. To be clinically useful, CTC assays

must be sensitive enough to enable the isolation and detection of rare, heterogeneous CTCs within exponentially higher numbers of white blood cells. Numerous methodologies have been developed for CTC enrichment and identification (reviewed previously [2–4]). An enrichment step is typically employed to separate CTCs from leukocytes and red blood cells within the blood sample, thus improving the efficiency of subsequent CTC detection. Some methodologies utilize the unique physical characteristics of CTCs to enrich them from hematopoietic cells (density, size, and membrane surface charge). CTCs can also be enriched based upon their biological properties, primarily membrane protein expression, by using antibodies against either epithelial-associated proteins (positive selection), or by using antibodies against antigens expressed by white blood cells (WBCs) (negative selection). All of the methodologies developed thus far have advantages and limitations with regard to sensitivity, reproducibility, and standardization. To date, the only CTC detection assay approved by the US FDA is CellSearch® (Menarini Silicon Biosystems), and the vast majority of clinically relevant publications over the last 15 years have utilized CellSearch®. Therefore, we will focus on CTC findings using this methodology in this review.

CTCs as Prognostic Biomarkers

Many studies have demonstrated that CTCs are prognostic in metastatic breast cancer patients [5–8]. The largest multi-institutional study to date, authored by Bidard et al. in 2014, included 1944 metastatic patients from 20 different studies. This study was sufficiently powered to demonstrate the prognostic significance of CTC detection within various subtypes of patients beyond that provided by standard prognostic parameters routinely used in the clinic. In this study, 911/1944 (46.9%) of patients had ≥ 5 CTCs/7.5 mL blood at baseline, which was associated with decreased progression-free survival (PFS) and overall survival (OS) compared with patients with ≤ 5 CTCs/7.5 mL blood at baseline. Increased CTC counts 3–5 weeks after start of treatment, adjusted for CTC count at baseline, were associated with shortened PFS and OS as were increased CTC counts after 6–8 weeks. Interestingly, ≥ 5 CTC/7.5 mL at baseline and at 3–5 weeks remained prognostically significant irrespective of breast cancer subtype and type of treatment administered [9].

Circulating tumor cells can disseminate early in breast cancer; ≥ 1 CTC/7.5 mL blood can be identified in 30% of patients with T1/T2 tumors [10]. The reported positivity rate (≥ 1 CTC) using CellSearch® ranges between 19 and 30% in non-metastatic locally advanced breast cancer (LABC) patients [10, 11, 12••, 13–19]. The CTC detection rate seems to be higher in non-metastatic inflammatory breast cancer patients, ranging from 27 to 40% [20, 21]. In all of these studies, the detection of one or more CTCs was associated with poor relapse-free and/or overall survival.

Two recent pooled analysis reports have validated these earlier findings. A 2016 report by Janni et al. included information from 3173 stage I breast cancer patients from four European institutions and our data from the USA. One or more CTCs were detected in 20.2% of these patients, and CTC detection was an independent predictor of poor disease-free (HR 1.82), overall (HR 1.97), breast cancer-specific (HR 2.04), and distant disease-free survival (HR 1.89) [14]. A 2018 study by Bidard et al. investigated the prognostic value of CTCs in non-metastatic patients treated with neoadjuvant chemotherapy (NAC). This multi-institutional analysis included information from 21 studies and more than 1500 patients. Circulating tumor cells were identified in 25% of patients pre-NAC. While post-NAC CTC detection was prognostically significant, pre-NAC CTC detection adding pre-NAC CTC information increased the prognostic power of multivariable models for overall survival ($P < .001$), distant disease-free survival ($P < .001$), and even loco-regional relapse-free interval ($P = .008$) [12••]. The prognostic significance of CTC detection in non-metastatic are summarized in Table 1.

The looming question is should CTC enumeration guide treatment decisions in breast cancer? Recent trials for metastatic breast cancer based on CTC information are summarized in Table 2.The first trial to address this question was the SWOG S0500 trial, which evaluated whether changing first-line chemotherapy in metastatic patients with ≥ 5 CTCs/7.5 mL blood 3 weeks after initiation of chemotherapy could improve outcome [30]. Patients with ≥ 5 CTCs/7.5 mL blood at 3 weeks post treatment initiation were randomized to early change of treatment versus continuation of the same therapy as standard of care. With the inclusion of 595 patients, 120 patients were randomized; no significant improvement in PFS or OS was identified in the patients randomized to the CTC-based early change of treatment arm. Some criticisms of the SWOG S0500 trial include the likely selection of highly chemo-resistant patients who usually are not responsive to additional chemotherapy and the fact this study was underpowered [32]. A preliminary report describing the observational phase of the CirCe01 trial, which combines CTC monitoring with other prognostic parameters (serum albumin level, lymphocyte level, LDH level, prognostic inflammatory and nutritional index (PINI) and Barbot's score), has recently been published. Prognostic parameters used in the multivariable analysis were low serum albumin, poor performance status, ≥ 5 CTC/7.5 ml, and triple negative subtype (HER2+ and hormone positive vs triple negative breast cancer). Patients with ≥ 5 CTC/7.5 ml at baseline who had a ≥ 70% decrease of their baseline CTC count (also those who had a decrease to ≤ 5 CTCs) after first-line therapy had a significantly improved progression-free survival (> 4 months) [31]. The second

Table 1 CTC detection techniques and outcomes in non-metastatic breast cancer patients

Study	Detection method	Positive/total patients (%)	Findings	Correlation between CTC positivity and clinicopathological characteristics	CTC analysis performed
Bidard [12]	CellSearch®	398//1574 (25%) 181/1200 (15%)	↓DFS ↓OS	[a]	Pre-NAC Pre-operative Pooled analysis
Janni [14]	CellSearch®	640/3173 (20.2%)	↓DFS ↓OS	[a]	Pooled analysis
Rack [16]	CellSearch®	435/2026 (21.5%) 330/1493 (22%)	↓DFS ↓OS	[a], significant for lymph node status	Pre adjuvant chemotherapy Post adjuvant chemotherapy
Van Dalum [17]	CellSearch®	75/403 (19%) 40/263 (15%) 30/235 (12%) 18/144 (11%)	↓DFS ↓OS		Before surgery After adjuvant chemotherapy One Year Two Years
Hall [18]	CellSearch®	124/509 (24%)	↓DFS ↓OS	[a]	Post-NAC
Riethdorf [19]	CellSearch®	65/213 (30%) 35/17%)	↓DFS ↓OS Pre-NAC only	[a]	Pre-NAC Post-NAC
Hall [20]	CellSearch®	17/63 (27%)	↓DFS	[a]	After NAC in inflammatory
Pierga [21]	CellSearch®	55/141 (39%) 11/127 (9%) 10/106 (9%) 6/100 (6%)	↓DFS ↓OS	[a]	Pre-NAC After four cycles of NAC Post-NAC After surgery in inflammatory

CTC circulating tumor cell, *DFS* disease-free survival, *NAC* neoadjuvant chemotherapy, *OS* overall survival

[a] Not significant for clinicopathological characteristics

(interventional) phase of this study is ongoing. Three hundred four metastatic patients with ≥ 5 CTCs before the start of the third line of chemotherapy will be randomized between a CTC-driven arm and the standard arm. The medical primary endpoint of the trial is the overall survival. In the CTC-driven arm, CTC counts will be performed after each first cycle of every new chemotherapy and will indicate whether or not this regimen is continued. Patients with ≤ 70% decrease in CTCs will be switched from this chemotherapy line and be offered another treatment, which will be, again, evaluated by early CTC changes (and so on). Patients with a ≥ 70% CTC decrease before the second chemotherapy cycle will continue their treatment and then managed by standard clinical/radiological tools. The subsequent chemotherapy lines will be managed using CTC counts. In the ongoing STIC CTC METABREAST trial, 1000 hormone receptor-positive metastatic breast cancer patients are randomized between the clinician choice and CTC count-driven choice. In the CTC arm, patients with ≥ 5 CTC/7.5 mL receive chemotherapy whereas patients with < 5 CTC/7.5 mL receive endocrine therapy as first-line treatment. Within each treatment category (hormone or chemotherapy), the treatment type is the clinician's choice. To date, no results from this trial have been reported on the clinicaltrials.gov website.

Characterization of CTCs and HER2-Directed Clinical Trials

There is ample evidence regarding prognostic importance of CTCs in both the metastatic and non-metastatic setting. Many clinical research groups are now focusing on CTC characterization to better understand the metastatic process and identify potentially useful, CTC-directed therapeutic targets to improve patient outcomes. For more than a decade, evaluation of primary tumor estrogen receptor (ER)/progesterone receptor (PR), and HER2 hormone receptor status has been the standard practice employed to identify candidates for endocrine or HER2-targeted therapy. While targeted therapies are less toxic and more effective (in many cases) than systemic therapies, discordant ER and/or HER2 expression can exist between primary tumor and metastases. ER expression (assessed using immunocytochemical, or PCR-based methods) is absent in the metastatic lesions of up to half of patients with ER-positive primary tumors, while HER2 amplification (assessed using immunocytochemical, PCR-based, or fluorescent in situ hybridization (FISH) methods) is identified in the metastases of up to one third of patients with HER2-negative primary tumors [33]. This discordance is also reflected in discordant ER expression rates between ER-

Table 2 CTC detection techniques and outcomes in metastatic breast cancer patients

Author/trial name	Trial objective	CTC detection method	Positive/total patients (%)	Findings
Phase II				
Agelaki [22]	Evaluate the efficacy of lapatinib in therapy-resistant MBC patients with HER2-positive CTCs	Immunofluorescence microscopy using PBMC cytospins stained for HER2 or EGFR and cytokeratin	16/21 (76.2%)	Lapatinib decreased HER2-positive CTCs in MBC patients
Stebbing [23]	Evaluate the efficacy of lapatinib in HER2-negative MBC patients with EGFR-positive CTCs	CellSearch®	16/43 (37%)	No clinical response observed. All patients progressed.
Kalykaki [24]	Evaluate the efficacy of gefitinib in MBC patients with EGFR-positive CTCs	Immunofluorescence microscopy using PBMC cytospins stained with A45-B/B3 and anti-CD45 or anti-EGFR	Post-cycle 1 11/17 (64.7%) decreased Post-cycle 2 12/17 (70.6%) decreased, 5/17 (29.4%) increased 9/17 (52.9%) EGFR-positive	Decrease in CTCs and EGFR-positive CTCs treated by gefitinib
Pestrin [25]	Evaluate the efficacy of lapatinib in HER2-negative MBC patients with HER2-positive CTCs	CellSearch®	7/96 (7.3%)	No objective response observed (0/7).
Georgoulias [26]	Evaluate the efficacy of trastuzumab in therapy-resistant MBC patients with CTCs	RT-PCR (CK 19) and immunofluorescence microscopy stained for CK and HER2	51/57 (89%) HER2-positive CTCs Post-trastuzumab 27/36 (75%) CK19 mRNA-negative Observational 7/39 (17.9%) CK19 mRNA-negative	Trastuzumab may eliminate treatment-resistant CK19 mRNA-positive CTCs. ↓Recurrence ↑DFS
Hainsworth [27]	Evaluate the efficacy of trastuzumab plus pertuzumab in HER2-negative MBC patients with HER2-positive CTCs	PRO Onc assay	24/226 (11%) 14/24 (58%) treated with trastuzumab & pertuzumab	12/14 patients with early progression, 1 partial response, 1 stable disease
Ignatiadis [28] Treat CTC (trastuzumab in HER2-negative early breast cancer as adjuvant treatment for circulating tumor cells)	Compare trastuzumab vs. observation for HER2-negative, non-MBC patients who are CTC-positive	CellSearch®	63/1317 (4.8%) Week 18: Trastuzumab 5/58 (8.6%) Observational 4/58 (6.9%)	Trastuzumab does not decrease CTC detection in HER2-negative non-MBC
Phase III				
Jaeger [29] DETECT III (a multicenter, phase III study to compare standard therapy +/- lapatinib in HER2-ve MBC-patients with HER2+ve CTCs)	Compare standard therapy to standard therapy plus lapatinib in HER2-negative MBC patients with HER2-positive CTCs	CellSearch®		Ongoing
Smerage [30] SWOG S0500 (treatment decision-making based on blood levels of tumor cells for metastatic breast cancer treated with chemo)	Evaluate effects of changing chemotherapy after one-cycle of first-line chemotherapy in MBC patients with persistent CTC detection	CellSearch®	Baseline: 276/595 (46%), no increase of CTCs 319/595 (54%), increased CTCs Follow-up: 165/288 (57%), no increase of CTCs 123/288 (43%), persistent increase	OS and PFS did not improve when chemotherapy regimen was changed in MBC with persistent CTCs.
Helissey [31]		CellSearch®		Ongoing

Table 2 (continued)

Author/trial name	Trial objective	CTC detection method	Positive/total patients (%)	Findings
CirCe01 (circulating tumor cells to guide chemotherapy for metastatic breast cancer)	Determine the clinical utility of CTC assessment in MBC patients before first cycle of third line chemotherapy			Ongoing
STIC-CTC (medico-economic interest of taking into account circulating tumor cells to determine the kind of first-line treatment for metastatic, hormone receptors positive, breast cancers)	Determine first-line treatment in hormone receptor-positive MBC patients based on CTC baseline value (hormone therapy if <5 CTCs, chemotherapy ≤5 CTCs).	CellSearch®		

A45-B/B3 anticytokeratin monoclonal antibody, *CD45* lymphocyte common antigen, *CK* cytokeratin, *CK19* cytokeratin-19, *CTC* circulating tumor cell, *DFS* disease-free survival, *EGFR* epidermal growth factor receptor, *HER2* human epidermal growth factor receptor 2, *MBC* metastatic breast cancer, *mRNA* messenger ribonucleic acid, *OS* overall survival, *PBMC* peripheral blood mononuclear cell, *PFS* progression-free survival, *RT-PCR* reverse transcription-polymerase chain reaction

positive primary tumor and ER-negative CTCs in metastatic patients (discordance range 38–71%) [34–38]. Our group and others have demonstrated that non-metastatic patients with HER2-negative tumors often times harbor HER2-positive CTCs (discordance range 6–49%) [39–43]. Similarly, HER2 primary tumor and CTC discordance rates in metastatic patients range from 7 to 35% [36, 38, 44–46]. It is unclear if the broad ranges of discordance reported for both ER and HER2 are due to the diverse methodologies used to measure ER and HER2, and/or the heterogeneous nature of CTCs.

Primary tumor versus CTC HER2 and/or EGFR discordance (measured by CellSearch®) has been the basis of some recent phase 2 and 3 clinical trials for metastatic patients. Some trials reported only changes in CTC numbers following CTC-directed therapies [22–24], while some trials are also including outcomes data (summarized in Table 2). Pestrin et al. reported the results of a multicenter phase II trial designed to evaluate the activity of lapatinib, a dual epithelial growth factor receptor (EGFR) and HER2 tyrosine kinase inhibitor, in metastatic breast cancer patients with HER2-negative primary tumors and HER2-positive CTCs. Lapatinib was administered to patients with HER2-negative primary tumors with HER2-positive CTCs who had been previously treated with at least a first-line therapy for metastatic disease. Unfortunately, only 7/96 patients screened had HER2-positive CTCs; no objective tumor responses occurred in any of the seven patients. One of the seven patients experienced disease stabilization lasting 8.5 months [25]. The ongoing DETECT III trial is also investigating the use of lapatinib in metastatic patients with HER2-negative primary tumors and HER2-positive CTCs. DETECT III is a two-arm study for patients with HER2-positve CTCs, randomized to physician's choice therapy (chemotherapy or endocrine treatment) with or without additional HER2-targeted treatment with lapatinib [29]. The ongoing CirCe T-DM1 trial will test the validity of HER2-amplified CTCs to select metastatic breast cancer patients who would normally be considered HER2-negative for Trastuzumab-emtansine (T-DM1) treatment. No results appear to have been published to date for this study. Hainsworth et al. used a multiplex immunoassay, PRO Onc (Prometheus Laboratories), to assess CTCs and CTC HER2 positivity in 226 metastatic patients with HER2-negative primary tumors. Twenty-four of 226 patients (11%) had HER2-positive CTCs. Fourteen patients were treated with trastuzumab and pertuzumab; 12/14 patients (86%) progressed within 6 weeks, 1 patient had a brief (12 weeks) partial response, and 1 patient was stable for 12 weeks [27]. The EORTC 90091-10093 Treat-CTC randomized phase II trial was the first, multicenter international trial assessing CTC detection in non-metastatic breast patients to test a new treatment strategy: using trastuzumab in patients with HER2-negative primary tumors who have ≥ 1 CTC (irrespective of CTC HER2 status) identified after NAC. The rationale for this

study comes from subset analyses of the NASBP B-31 and the NCCTG N9831 trials that demonstrated the benefit of adjuvant trastuzumab in patients with HER2-negative tumors [47], and a single-center study by Georgoulias et al., which showed a decrease in cytokeratin-19-positive CTCs following the addition of trastuzumab to chemotherapy in patients with HER2-negative primary tumors [26]. As of October 2016, 1317 patients were screened; 95 (7.2%) had detectable CTC(s), and 63 (4.8%) were randomized to trastuzumab ($n = 31$) or observation ($n = 32$). Fifty-eight patients (29 in each arm) were assessable for the primary end-point (18 weeks), In 9 of the 58 patients, CTC(s) were still detected at week 18, 5 in the trastuzumab, and 4 in the observation arm. This trial has been terminated [28].

Characterization of CTCs and Potential Utility

Single-cell CTC characterization has the potential to provide valuable information regarding CTC heterogeneity, identifying genetic mutations that are acquired during therapy, and facilitating the development of novel, individualized therapies to eradicate minimal residual disease. However, these technologies need to be reliable, reproducible, sensitive, standardized, and cost-effective to be useful in the clinical setting. Pizon et al. showed that CTCs with insulin-like growth factor 1 receptor (IGF-1R) and vascular endothelial growth factor receptor 2 (VEGFR-2) protein expression can be identified in a significant number of non-metastatic patients [48]. Circulating tumor cell androgen receptor mRNA (AR) can be identified in 30% of metastatic patients; anti-AR therapies might benefit patients with AR-positive CTCs [49]. Single-cell CTC isolation is tedious and a single CTC contains only a few picograms of DNA so reliable whole-genome amplification of each cell is required for subsequent mutational analysis. CTC populations are heterogeneous, and each single cell can harbor unique mutations, making clinical decision-making difficult. Given the level of technical expertise required to perform CTC mutational analyses and the costliness of many sequencing technologies, only a limited number of reports on CTC mutations have been published thus far. These studies have focused on the metastatic setting as typically only one CTC is identified in operable patients. The most common CTC mutations identified are tumor protein 53 (TP53) [50–52], phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA) [50, 52], cyclin D1 (CCND1) [50], and Kirsten rat sarcoma (KRAS) [52] genes. Although PIK3CA, CCND1, and KRAS are all potentially druggable targets, no clinical studies have been reported to date. Several groups have detected and monitored estrogen receptor 1 (*ESR1*) gene mutations at the single CTC level in ER-positive metastatic patients; serial CTC ESR1 characterizations could be used to add valuable information with respect to anti-estrogen therapy resistance [51–54]. A specific 13-gene CTC microRNA (miR) signature is associated with trastuzumab resistance in HER2-positive metastatic patients; thus, investigating miRs in CTCs might provide mechanistic information for other types of drug resistance.

Liquid Biopsy Component: Cell-Free ctDNA

A PubMed search using terms “circulating tumor DNA and breast cancer” resulted in more than 300 reports published since 2015. This enormous number of published studies reflects the overall interest in ctDNA in the age of precision medicine. ctDNA is easily obtained from blood samples, and it provides “real-time” global methylation and somatic mutation information (even those acquired from clonal selection during therapy) as it is released by all tumor cells within the cancer patient’s primary tumor, CTCs, and metastatic lesions. As documented in the paragraphs below, ctDNA is showing great promise for improving upon current strategies used for clinical decision-making in breast cancer. Several comprehensive ctDNA reviews have been published; they describe in detail various ctDNA extraction and detection methodologies, and the potential clinical utility of ctDNA analysis in breast cancer [55–58]. For this review, we will focus on recently published studies that have demonstrated associations between ctDNA characterization and breast cancer detection, prognosis, and/or response to therapy.

Cell-free DNA (cfDNA) release into the bloodstream is a normal physiologic process, resulting from routine cell turnover. Tumor-derived, cell-free circulating tumor DNA (ctDNA) is released by primary and metastatic tumors, and CTCs, through apoptosis, necrosis, autophagy, necroptosis, and cellular stress [59]. Circulating DNA is just a small fraction of the total cfDNA (germline) found in blood. However, since healthy individuals have low cell turnover rates and more efficient phagocytic removal of defective cells from the circulation, the ctDNA/cfDNA fraction is increased in patients with cancer. Plasma samples are typically used for cfDNA isolation, as serum contains a higher background of germline DNA caused by blood clotting [60, 61]. In the past, the low ratio of ctDNA/cfDNA was a challenge for ctDNA characterization, but digital droplet polymerase chain reaction (ddPCR) and next-generation sequencing (NGS) methodologies now allow for both targeted and non-targeted ctDNA somatic mutation analysis (reviewed in [55, 56, 58]). Many studies have demonstrated a high concordance rate between genomic mutations found in primary tumor and corresponding ctDNA samples [58, 62], as well as metastatic lesions and ctDNA obtained at the time of resection [63]. Technical issues associated with ctDNA isolation include serum vs. plasma issues; the various anti-coagulants used for plasma collection, fast degradation rate of ctDNA once it is collected, and volume of plasma required for sufficient ctDNA isolation in early

breast cancer [60]. In addition, there has been no standardization of ctDNA characterization with respect to isolation, quantification, and mutational analyses used in published studies. All of the ctDNA reports published thus far have included small numbers of patients and/or heterogeneous patient cohorts. Many of these variables should be standardized so the results can be compared and interpreted. Finally, ctDNA mutation analyses are either costly (NGS) or labor-intensive and require significant technical expertise (ddPCR), which makes the incorporation of ctDNA information into routine practice challenging.

ctDNA Concentration

Plasma ctDNA concentrations are reported to be threefold higher in patients with breast cancer as compared to healthy control values [64–67]. Although there is no established baseline concentration range for ctDNA in breast cancer patients, changes in ctDNA concentration over time do reflect disease burden [64, 66]. Recent results from the I-SPY-2 trial, presented at the 2018 San Antonio Breast Cancer Symposium, show that ctDNA concentrations correlated with non-response to NAC (measured as having no residual tumor in breast or nodes, or pathologic complete response, "pCR") in non-metastatic patients [68]; this data is in congruence with the increase in specific ctDNA copy number variations (CNVs) and pCR reported recently from the Neo-ALTTO trial [69]. Circulating tumor DNA concentration at surgery is also associated with progression in non-metastatic patients [70, 71]. Similar findings have been reported for metastatic patients. Low-coverage genome-wide sequencing and ichorCNA software was used in a study by Stover et al. to quantify tumor content in cfDNA in 164 triple-negative metastatic patients who had received prior NAC or first-line chemotherapy. In this blinded study, a cfDNA tumor fraction threshold of ≥ 10% was independently associated with significantly worse OS [72].

ctDNA Characterization

Beyond the concentration of ctDNA, ctDNA characterization also has clinical utility for non-metastatic patients (summarized in Table 3). Epigenetic alterations, such as methylation of promoter/enhancer regions of tumor-suppressor DNA that results in gene silencing, are important steps in the metastatic cascade. Methylation-specific PCR assessment of ctDNA is a promising tool in non-metastatic breast cancer. Numerous studies have shown that ctDNA methylation of ras association domain family protein 1A (RASSF1A) is associated with response to NAC, [73] and outcome [73, 74, 89]. Fujita et al. assessed the methylation statuses of RASSF1A, glutathione S-transferase P1 (GSTP1), and retinoic acid receptor β2 (RARβ2) and demonstrated that methylation of these genes was associated with decreased OS [75]. Breast cancer 1 (BRCA1) and GSTP1 methylation are also associated with disease recurrence [76]. Oshiro et al. reported that 25/110 (23%) of stage I-III breast cancer patients with PIK3CA mutated tumors had PIK3CA ctDNA mutations identified at surgery; high levels of serum PIK3CA mutations were associated with worse recurrence-free and OS [77]. TP53 ctDNA mutations following NAC in triple-negative patients were associated with relapse in one study [78].

ctDNA Characterization in Metastatic Patients

ESR1 and PIK3CA ctDNA mutations are the most commonly reported to date (summarized in Table 3). Several clinical trials have investigated whether or not ctDNA analyses add predictive value using various treatment regimens. Results from the BOLERO-2 (exemestane + placebo vs. exemestane + everolimus) [79], SoFEA ((fulvestrant vs. exemestane), and PALOMA-3 (fulvestrant + palbociclib vs. fulvestrant + placebo) [80, 81, 82•] trials, as well as results reported by Clatot et al. (various treatments) [83] and Schiavon et al. (various treatments) [84], all demonstrated that ESR1 mutations were associated with agent-associated PFS and/or OS. However, the FERGI (pictilisib + fulvestrant vs. placebo + fulvestrant) trial did not identify any associations [85]. Similarly, PIK3CA ctDNA mutations were associated with PFS and/or OS in the PALOMA-3 (serial PIK3CA measurements) [82•], and BELLE-2 (buparlisib + fulvestrant) [86] trials, but this association was not observed in the BOLERO-2 [87], FERGI [85], and MONALEESA-2 (ribociclib + letrozole vs. placebo + letrozole) trials [88]. Disparate findings between these studies may be a result of the heterogeneous nature of the patients included in each particular trial (various prior treatments, number(s) of previous treatments, time points used to assess ctDNA), treatments used within each trial, and the varied methodologies used to analyze the ctDNA. Preliminary data suggest that ctDNA dynamics could potentially be used to predict treatment outcome before tumor response can be assessed by a change in clinical symptoms or imaging. Therefore, many of the above studies incorporate serial ctDNA assessments into the trials. Dynamic ctDNA information, as well as results from ongoing trials, such as the PAlbociclib and Circulating Tumor DNA for ESR1 Mutation Detection (PADA-1), and large observational Aiming to Understand the Molecular Aberrations in Metastatic Breast Cancer. (AURORA) trials, will add further information regarding the clinical utility of ctDNA monitoring for patient management.

Table 3 ctDNA characterization and clinical utility

Study	Type of study	ctDNA target		Findings
		ctDNA in non-metastatic patients		
Garcia-Murillas [70]	Prospective	NAC	ctDNA concentration	$N = 55$ patients. ctDNA detection predicted metastatic relapse with high accuracy [hazard ratio, 25.1 (confidence interval, 4.08 to 130.5; log-rank $P < 0.0001$]
Riva [71]	Prospective	NAC in non-metastatic TNBC	ctDNA	$N = 38$ TNBC patients. ctDNA detection was associated with tumor grade ($P = 0.003$), and stage ($P = 0.03$). During treatment, we observed a drop of ctDNA levels in all patients but 1. ctDNA detection after 1 cycle of NAC was associated with shorter disease-free (P 0.001) and overall survival (P 0.006).
Takahashi [73]	Prospective	NAC	RASSF1A ctDNA methylation	$N = 87$ patients.RASSF1A ctDNA methylation significantly associated with NAC responders ($P = 0.006$), and residual tumor burden ($P = 0.008$) 43% of patients who showed increased RASSF1A ctDNA methylation at 1 year after surgery developed recurrence
Fiegl [74]	Prospective	Adjuvant	RASSF1A ctDNA methylation	$N = 148$ patients.RASSF1A ctDNA methylation was identified in 33/148 patients (22%) 1-year post surgery with tamoxifen treatment and independently predicted poor outcome. With a relative risk (95% confidence interval) for relapse of 5.1 (1.3–19.8) and for death of 6.9 (1.9–25.9).
Gobel (75]	Prospective	At surgery	RASSF1A ctDNA methylation	RASSF1A ctDNA methylation ($n = 428$ patients analyzed) at surgery independently predicted distant disease-free ($P = 0.002$) and OS ($P = 0.021$). PITX2 was independently associated with OS ($P = 0.02$)
Fujita [75]	Prospective	At surgery	RASSF1A, GSTP1, RARβ2 ctDNA methylation	33/336 stage I/II patients (10%) were positive for this 3 gene ctDNA methylation panel and showed a significantly worse overall survival (OS) rate at 100 months (78 vs. 95%; $P = 0.002$) than those with negative findings ($n = 303$). Patients with high total DNA in serum ($n = 112$) also showed a significantly worse OS rate at 100 months (86 vs. 97%; $P = 0.001$) than those with low total DNA in serum ($n = 224$).
Sharma [76]	Prospective	NAC	BRCA1,GSTP1ctDNA methylation	$N = 100$ patients. GSTP1 and BRCA1 hypermethylation were found to be independent of other prognostic factors in predicting disease recurrence ($P = 0.02$, HR = 7.6, 95% C.I. = 1.4–44.1; $P = 0.04$, HR = 6.2, 95% C.I. = 1.1–35.7).
Oshiro [77]	Prospective	At surgery	PIK3CA mutations	25/110 (23%) of stage I-III breast cancer patients with PIK3CA mutated tumors had PIK3CA ctDNA mutations identified at surgery; high levels of serum PIK3CA mutations were associated with RFS ($P = 0.0002$) and OS ($P = 0.005$).
Chen [78]	Retrospective analysis from BRE09–146 trial	NAC	ctDNA mutations	ctDNA mutations were detected in 4/33 patients (12%) with mutation identified in their primary tumor. All four patients had a rapid disease recurrence (100% specificity), but sensitivity was limited to detecting only 4 of 13 patients who clinically relapsed (31% sensitivity). Patients with detectable ctDNA had an inferior disease-free survival ($P < 0.0001$; median disease-free survival 4.6 mos. vs. not reached; hazard ratio = 12.6, 95% confidence interval 3.06–52.2).
ctDNA in metastatic patients				
Stover [72]	Retrospective	Metastatic TNBC	cfDNA tumor fraction threshold	$N = 164$ patients. ≥ 10% ctDNA threshold was independently associated with OS (median, 6.4 v 15.9 months) (hazard ratio, 2.14; $P < .001$).
Chandarlapaty [79]	Retrospective from the BOLERO-2 trial	Metastatic ER+	ESR1 mutations	156/541 (28.8%) had ctDNA ESR1 mutations. Patients who failed on previous aromatase inhibition were randomized to treatment with exemestane or exemestane + everolimus.

Table 3 (continued)

Study	Type of study	ctDNA target		Findings
		ctDNA in non-metastatic patients		
				ctDNA ESR1 mutations were associated with OS; (wild-type 32.1 months vs 20.7 months in patients with one mutation, and 15.2 months in patients with 2 mutations, $P < 0.001$).Clinical benefit with everolimus: in wild-type patients 8.48 vs 3.94 months, in ESR1 mutated patients 5.42 vs. 276 months.
Fribbens [80, 81]	Retrospective from the SoFEA and PALOMA 3 trials	Metastatic ER+	ESR1 mutations	$N = 83$ patients for SoFEA (fulvestrant vs. exemestane) first report: Of the 39 patients who progressed on the first-line aromatase inhibition, 56.4% (22/39) had ctDNA ESR1 mutations detectable median 6.7 months before clinical progression. KRAS mutations were detected in 21.2% (24/113) patients although there was no evidence that KRAS mutation status was prognostic for progression-free or overall survival. $N = 161$ patients for SoFEA (fulvestrant vs. exemestane) second report. ctDNA ESR1 mutations were found in 63/161 (39%) of patients. Patients with ctDNA ESR1 mutations had improved PFS after taking fulvestrant ($n = 45$) compared with exemestane ($n = 18$); (hazard ratio 0.52, $P = 0.02$), whereas patients with wild-type ESR1 had similar PFS fulvestrant vs. exemestane ($P = 0.77$). $N = 360$ patients. PALOMA3 (fulvestrant/palbociclib vs. fulvestrant/placebo). ctDNA ESR1 mutations were found in 91/360 (25%) of patients. Fulvestrant plus palbociclib improved PFS compared with fulvestrant plus placebo in both ctDNA ESR1 mutant (HR, 0.43, $P = .002$) and ctDNA ESR1 wild-type patients (HR, 0.49, $P < .001$).
O'Leary [82•]	Retrospective from the PALOMA-3 trial	Metastatic ER+	PIK3CA vs. ESR1 ctDNA mutations after 15 days treatment	The PALOMA-3 trial assesses the utility of serial ctDNA changes in predicting outcome on CDK4/6 inhibitor (palbociclib). $N = 100/455$ baseline (22%) of patients had a PIK3CA ctDNA mutation. 73/100 (73%) had a matched day 15 (D15) sample. High D15 PIK3CA/baseline ratio predicted poor PFS on palbociclib and fulvestrant (HR 3.94,$P = 0.0013$) vs. elevated D15 ESR1 ctDNA mutations/baseline (HR 1.68, $P = 0.21$).
Clatot [83]	Retrospective	Metastatic ER+	ESR1 ctDNA mutations	$N = 44/144$ patients with ESR1 mutated ctDNA.ESR1mutated ctDNA associated with PFS ($P = 0.002$) and OS ($P = 0.0006$). mutations were detectable in 75% of patients prior to aromatase inhibition progression, but 3 months after progression ESR1 ctDNA mutations did not correlate with outcome.
Schiavon [84]	Observational	Metastatic ER+	ESR1 mutations	$N = 171$ patients. ESR1mutated ctDNA associated with shorter PFS on subsequent aromatase inhibitor based therapy (HR 3.1, $P = 0.0041$).
Spoerke [85]	Retrospective from the FERGI study	Metastatic ER+	PIK3CA and ESR1 mutations	The FERGI trial compared the pan-PI3K inhibitor pictilisib plus fulvestrant vs. placebo plus fulvestrant in patients with who had received prior aromatase inhibitor and progressed. $N = 156$ patients. PIK3CA and ESR1 ctDNA mutation allele frequencies for individual patients were dynamic over the treatment periods. Patients with a ctDNA PIK3CA mutation at baseline had a PFS hazard ratio of 0.994 compared with ctDNAPIK3CA wild-type patients in the fulvestrant control arm, and showed a PFS hazard ratio of 0.991 comparing ctDNAPIK3CAwildtype patients in the fulvestrant plus pictilisib arm. Neither the presence of multiple ctDNA ESR1 mutations nor higher ctDNA ESR1 allele frequencies were associated with a clear difference in risk of progression on fulvestrant.
Baselga [86]		Metastatic	PIK3CA mutations	The BELLE-2 trial assessed the efficacy of the pan-PI3K inhibitor buparlisib plus fulvestrant in patients who had progressed on or after aromatase inhibitor treatment and had received

Table 3 (continued)

Study	Type of study	ctDNA target		Findings
		ctDNA in non-metastatic patients		
	Retrospective from the BELLE-2 phase III trial			up to one previous line of chemotherapy PFS: Buparlisib + fulvestrant vs. Placebo + fulvestrant: PIK3CAmutated 7.0 vs. 3.2 months (HR 0.58, p = 0.001). PIK3CA wild type showed no difference in PFS ± buparlisib (HR 1.028, *P* = 0.56).
Moynahan [87]	BOLERO-2 trial	Metastatic	PIK3CA mutations	*N* = 550 patients. 238/550 (43%) had PIK3CActDNA mutations. PFS for wild type vs. PIK3CA mutated was 2.96 months vs. 2.69 months (HR 1.26), and OS was 29.67 moths vs. 22.7 months (HR 1.32) Clinical benefit with everolimus vs placebo (exemestane) arms was similar in patients with wild-type or mutant PIK3CA ctDNA(hazard ratio (HR), 0.43 and 0.37, respectively).
Hortobagyi [88]	Retrospective from the MONALEESA-2 phase III trial	Metastatic	PIK3CA or TP53 mutations	*N* = 494 patients. This trial studied ribociclib plus letrozole vs. placebo plus letrozole. 427/494 (86%) had ctDNA mutations. Clinical benefit with ribocicib: No statistically significant difference in PFS detected for PIK3CA or TP53 mutations.
PADA-1 trial	III	Metastatic	ESR1 mutations	Ongoing with randomization to therapy according to ESR1 ctDNA levels

BELLE-2 = Phase III study of BKM120/placebo with fulvestrant in postmenopausal patients with hormone receptor-positive HER2-negative locally advanced or metastatic breast cancer refractory to aromatase inhibitor. BOLERO-2 = everolimus in combination with exemestane in the treatment of postmenopausal women with estrogen receptor-positive locally advanced or metastatic breast cancer who are refractory to letrozole or anastrozole. FERGI = study of GDC-0941 or GDC-0980 with fulvestrant versus fulvestrant in advanced or metastatic breast cancer in participants resistant to aromatase inhibitor therapy. MONALEESA-2 = study of efficacy and safety of LEE011 in postmenopausal women with advanced breast cancer trial. BRE09-146 = poly ADP ribose polymerase(PARP) inhibition for triple negative breast cancer with BRCA1/2 mutations. PALOMA-3 = palbociclib combined with fulvestrant in hormone receptor-positive HER2-negative metastatic breast cancer after endocrine failure trial. SoFEA = study of faslodex versus exemestane with or without arimidex trial

BRCA1 breast cancer 1 gene, *ctDNA* cell-free circulating tumor DNA, *D15* day 15, *ESR1* estrogen receptor-1 gene, *GSTP1* glutathione S-transferase P1 gene, *KRAS* Kirsten rat sarcoma gene, *OS* overall survival, *PFS* progression-free survival, *PIK3CA* phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α gene, *RASSF1A* Ras association domain family protein 1A gene, *RARβ2* retinoic acid receptor β2 gene, *TNBC* triple negative breast cancer, *TP53* tumor protein 53 gene

Comparing/Combining Information Obtained from Liquid Biopsy

Dawson, et al., published one of the first studies to compare three blood-based biomarkers, ctDNA, cancer antigen 15.3 (CA15–3), and CTCs, in 30 metastatic breast cancer patients with known tumorPIK3CA and TP53 mutations. The concentration of ctDNA was the most sensitive of the three biomarkers. Detectable ctDNA was found in 97% of patients; CTCs were identified in 87%. Increasing levels of ctDNA were apparent in 89% of patients with disease progression, while 78% of patients had an increase in CTC number, and 50% of patients showed increases in CA15–3 levels at progression [90]. Since neither ctDNA or CTCs are identified in all patients 100% of the time, many groups are now combining ctDNA and CTC assessments to provide a more comprehensive liquid biopsy profile. Rossi et al. recently published a retrospective study using CTC and ctDNA assessments to predict prognosis in advanced (7 patients) and metastatic (84 patients) breast cancer. The authors showed that baseline < 5CTC/7.5 mL vs. ≥ 5 CTCs/7.5 mL blood, < 0.5 %ctDNA vs. ≥ 5 %ctDNA, and the number of ctDNA mutations identified < 2 ctDNA mutations vs. ≥ 2 ctDNA mutations, were associated with better PFS and OS [91]. These results are difficult to fully interpret however, because this study included both locally advanced and metastatic patients, 58/91 (64%) had inflammatory breast cancer, and most of the metastatic patients (24%) had already received 5 or more lines of treatment prior to this study. All of these variables likely contributed to the findings. Paoletti et al. measured serial changes in CTCs and ctDNA ESR1 mutation to assess pharmacodynamics and early efficacy of the anti-estrogen therapy, SERD AZD9496. Baseline < 5 CTC/7.5 mL vs. ≥ 5 CTCs/7.5 mL blood was associated with improved PFS, but baseline ctDNA ESR1 mutation was associated with PFS. Interestingly, patients with persistently elevated CTC and/or ctDNA ESR1 mutation had worse PFS than patients who did not [92••]. Upcoming results from additional ongoing studies will provide more conclusive evidence regarding the clinical utility of combining these two powerful prognostic tools.

The CancerSEEK test is a multi-analyte blood test that combines proteomic and ctDNA analyses. This novel platform was designed for the early detection of solid tumors. One thousand five patients with non-metastatic cancers of the ovary, liver, stomach, pancreas, esophagus, colorectum, lung, or breast were assessed using CancerSEEK. CancerSEEK tests were positive in a median of 70% of the eight cancer types. The sensitivities ranged from 69 to 98% for the detection of five cancer types (ovary, liver, stomach, pancreas, and esophagus). While the CancerSEEK test was highly sensitive for detecting certain types of cancers, the sensitivity levels were 33% for stage I breast cancer [93••]. This low sensitivity might have been influenced by the small number of stage I breast patients included in the study (32 patients). The impressive results obtained with CancerSEEK testing will undoubtedly be assessed in much larger patient cohorts to validate detection rates and fully establish the parameters of the test for early breast cancer detection [93••].

Conclusions

The blood-based, liquid biopsy research field is growing exponentially, and it has profound potential to improve upon current breast cancer management by offering dynamic monitoring possibilities as well as novel targets for therapy. The prognostic importance of CTCs is well documented, but a lack of predictive value hinders clinical utilization. As soon as prospective studies can be completed that show that CTCs and/or ctDNA can predict which patients are likely to respond to a particular therapy (just as HER2 predicts response to trastuzumab), these agents can find acceptance in the clinical management of patients with breast cancer. It is also feasible that CTCs and or ctDNA presence predates imaging findings, so studies are needed to determine if liquid biopsy information could potentially guide imaging in patients suspected of recurrence. Finally, early identification of novel targets (possibly discordant from primary tumor markers) could open up new avenues for treatment, especially in the adjuvant setting of patients with residual CTCs or ctDNA after completion of NAC, who have no other targeted options available (such as triple-negative breast cancer patients who have completed NAC).

Finally, while CTCs and ctDNA remain the most extensively studied liquid biopsy components to date, several additional blood-based components are the basis of active, ongoing investigations. Some on the horizon include serum/plasma exosomes, platelet-mRNA, miRNA characterization, and global proteomic studies. Once the utility of the various liquid biopsy components is determined, standardization of the methodologies used to analyze them must also be standardized and validated for use in clinical laboratories worldwide.

Compliance with Ethical Standards

Conflict of Interest Anthony Lucci reports involvement in the Speakers Bureau of Genomic Health, Inc., and being a consultant/advisor for MOREHealth, outside of the submitted work. Carolyn Hall, Vanessa Sarli, and Salyna Meas declare no conflicts of interest relevant to this manuscript.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors. All reported studies with human subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national committee standards, and international/national/institutional guidelines.

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- • Of importance
- •• Of major importance

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