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Cellular residual disease (CRD) in early breast cancer –Expanding the concept of minimal residual disease monitoring?

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ABSTRACT

Despite a significant evolution in treatment strategies for early breast cancer (EBC) patients, up to 30% of them experience recurrence due to occult micrometastasis. The minimal residual disease (minimal RD) in EBC patients after the treatment with curative intent cannot be easily detected by clinical examination and radiological imaging, as they are both burdened by limited sensitivity. A new frontier and promising approach to address this unmet need is the study of liquid biopsy (LB). The most studied tumor-derived analytes in the peripheral blood for minimal RD monitoring are currently: i) the circulating tumor DNA (ctDNA), for the detection of somatic DNA alterations, so referred to as molecular residual disease (MRD); ii) circulating tumor cells (CTCs), for the detection of cellular residual disease (CRD).

MRD detection, while reaching a high specificity, is still presenting a number of limitations. On the other hand, CRD allows a real-time disease monitoring, detecting live cells, and possess the potential to provide an enormous amount of biological information. Indeed, CTCs can provide a multi-level portrait (*i.e.*, DNA, RNA and proteins) of the tumor, longitudinally depicting its evolving landscape, and can be used for functional (*in vitro/in vivo*) characterization. Moreover, CRD goes beyond the association with the risk of recurrence: predictive biomarkers for treatment response can also be evaluated. Nevertheless, CTCs are less studied in this context, because of their need to be immediately processed and their limited detection in a small fraction of patients in the early and post-surgery setting. These limitations could however be overcome by the use of newly developed technologies that enable an increased CTC detection rate and retrospective studies.

Here, we review the strengths and limitations of using MRD and CRD for minimal RD detection, focusing on the methodologies available for LB analysis in this setting, and on the main clinical studies investigating MRD and CRD in EBC. Considering the limits and the advantages of both MRD and CRD, we propose the integration of ctDNA and CTCs as complementary tools for minimal RD assessment to achieve a synergistic and novel approach for minimal RD analysis.

1. Introduction

Breast cancer (BC) is the most commonly diagnosed female cancer worldwide and represents the second cause of cancer mortality in women in the United States, with more than 40,000 estimated deaths in 2023 [1]. Early BC (EBC) accounts for >90% of all diagnosed BC. Despite a significant evolution in treatment strategies for EBC, up to 30% of patients will experience recurrence often developing distant metastases due to occult micrometastases (i.e., minimal residual disease, minimal RD) that has not been eradicated by systemic therapy [2]. Early recurrences take place in the first 5-years after diagnosis and represents the majority of cases, often associated with more aggressive disease subtypes [3], even

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though a sizable portion of those may occur decades after the primary disease diagnosis, especially in the hormone receptor positive (HR+) disease [2,4–6]. Thus, the ability to detect and monitor minimal RD, following curative intent in the early setting, would help refining risk of relapse and guide personalized treatment intensity. Nonetheless, minimal RD detection still remains a major challenge mainly relying on clinical examination and radiological imaging, both burdened by limited sensitivity [7].

Liquid biopsy (LB) is an increasingly promising option in addressing this unmet need, due to its minimally invasive approach to detect tumor-derived analytes, including circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs) [3].

ctDNA, the most used LB biomarker for minimal RD monitoring, allows for the detection of somatic DNA alternations in the blood and can therefore be referred to as *molecular residual disease* (MRD). On the other hand, CTCs have the potential to investigate minimal RD from a different perspective, allowing to detect the *cellular residual disease* (CRD). CRD holds the promise to gain knowledge about the molecular, but also phenotypic and functional features of the RD. Thus, it would provide additional insights to i) predict tumor relapse, ii) identify biomarkers to guide treatment, and iii) understand the biology of metastasis formation.

Herein, we review the strengths and weaknesses of ctDNA and CTCs as biomarkers for minimal RD detection, with a focus on methods and on results obtained in the EBC setting. Moreover, new opportunities offered by CRD are discussed.

2. ctDNA as a biomarker for MRD

Cell free DNA (cfDNA) can be detected in several biological fluids such as blood, saliva, urine, cerebrospinal fluid, and pleural fluid. It is typically double stranded, highly fragmented, long in length (>150 base pairs) and it is released by multiple mechanisms (e.g., apoptosis, necrosis and phagocytosis) [8]. cfDNA concentration, which is physiologically low, may be increased in pathological conditions, such as cancer [9]. ctDNA is the portion of cfDNA released by tumor cells, it is characterized by a shorter fragment length (130-140 base pairs) and its concentration varies depending on several factors, such as tumor type, staging and sites of involvement [8,10]. It, moreover, encompasses a broad range of cancer-related genetic and epigenetic information, such as single nucleotide variants (SNVs), copy number variations (CNVs), chromosomal aberrations (deletion/amplification and gene fusion), and methylation patterns [9,11].

2.1. Currently available ctDNA-based methods for MRD detection

Compared to all the LB analytes, ctDNA has been among the most studied, and different assays have been developed for its analysis. Most of currently available commercial assays are designed for advanced stage tumors, where ctDNA concentration is high, and are not suitable for MRD detection due the significantly lower plasma ctDNA levels in the early and post-surgery settings [8]. Consequently, significant resources have been invested in developing new technologies with a higher sensitivity and accuracy to be implemented for MRD detection [12,13]. Based on how ctDNA is investigated, these methods can be divided into two categories: tumor-agnostic and tumor-informed. Tumor-agnostic assays operate without an *a priori* knowledge of the primary tumor's genomic profile; they can be based on the detection of common actionable targets/hotspot mutations, chromosomal aberrations, or epigenetic features. Meanwhile, tumor-informed approaches are designed after primary tumor tissue sequencing and are therefore specific for each patient. The type of ctDNA-detection method should be chosen depending on the study objective and the clinical question [14].

2.1.1. Tumor-agnostic/naïve methods

These approaches are conducted without *a priori* knowledge about the primary tumor's genomic profile. Most of these assays are based on large, targeted next-generation sequencing (NGS) panels designed around hotspot mutations. Other strategies, such as epigenetic profiling or whole exome and whole genome sequencing (WES and WGS, respectively) are starting to gain attention. These methodologies could theoretically be more suitable for the clinical practice, since (i) one technique could be used for a broad spectrum of patients, (ii) they offer the possibility to comprehensively understand and explore several genetic alterations, and (iii) they allow to detect new alterations/clones that might emerge after surgery. By contrast, they are burdened by low sensitivity, due to the reduced sequencing depth with respect to tumor-informed methods and a higher rate of false positives and non-tumor derived mutations (such as those deriving from clonal hematopoiesis of indeterminate potential, CHIP) [15].

Targeted NGS approaches include, as an example, the Cancer Personalized Profiling by Deep Sequencing (CAPP-Seq) [16] and AVENIO assay (Roche Diagnostics, Canada). Meanwhile, methods investigating also the epigenetic information, such as Guardant Reveal (Guardant Health, Palo Alto, CA), are promising, since they might increase the assay sensitivity (*i.e.*, epigenetic patterns are less heterogeneous within the tumor and are not as polyclonal as SNVs).

2.1.2. Tumor-informed methods

Contrary to the previously described techniques, tumor-informed methodologies are based on the features obtained from the primary tumor's characterization, used as somatic reference to design a personalized ctDNA panel [17].

One of the oldest approaches of this kind was amplification-refractory mutation system (ARMS) used for the rapid analysis of specific alleles in plasma or serum [18]. The method, able to detect a specific allele at once, was used as a diagnostic tool for the analysis of single nucleotide polymorphisms or hotspot mutations. However, the limit of variant allele frequency (VAF) detection at 0.1-1% and the possibility to only query one genetic locus per assay, made it be rapidly replaced by more innovative methods.

Nowadays, the most commonly used methods are the ultrasensitive targeted approaches [e.g., droplet digital polymerase chain reaction (ddPCR) or beads, emulsion, amplification, and magnetics (BEAM)ing-PCR], based on partitioning a DNA sample into a large number of small reactions to provide an absolute quantification of each investigated mutation, improving sensitivity and limiting false detection rate [8]. Additionally, epigenetic characterization of ctDNA can be investigated using ddPCR. Meanwhile, other technologies are based on multiplex-PCR, such as the commercially available Signatera™ assay (Natera Inc), that received three Food and Drug Administration (FDA) breakthrough device designations for MRD testing.

Although the high sensitivity required for MRD detection is reached by these methods, they still present limitations, such as a high background rate [19], the significant cost [20,21] and the inability to detect newly emerged clones. Overall, the optimal method for MRD detection has yet to be defined and developed.

2.2. Clinical studies on MRD detection in EBC patients

In the last decade, there has been an exponentially growing interest into evaluating the role of ctDNA as a monitoring biomarker in patients with EBC to stratify those with complete disease eradication vs. residual disease after surgery. As a matter of fact, multiple studies have been conducted to investigate the prognostic role of MRD, considering its ability to anticipate radiological relapse (Table 1) [22], and they are herein described.

The pivotal study demonstrating the feasibility of MRD detection and tracking through ctDNA in a non-invasive way was published in 2015 by Garcia-Murillas et al. [23]. Their approach was based on a personalized ddPCR assay, developed using the primary tumor mutation profile of a relatively small cohort of patients with EBC (n = 55) who received neoadjuvant chemotherapy before surgery. While ctDNA detection at

Table 1
Clinical studies on Molecular Residual Disease (MRD) detection in EBC patients Abbreviations: circulating tumor DNA (ctDNA); confidence interval (CI); distant disease-free survival (DDFS); disease free survival (DFS); early breast cancer (EBC); hazard ratio (HR); inflammatory breast cancer (IBC); not available (NA); neo-adjuvant therapy (NAT); overall survival (OS); pathological complete response (pathCR); polymerase chain reaction (PCR); prospective (Prosp); relapse free survival (RFS); retrospective (Retrosp.); triple negative breast cancer (TNBC); unique molecular indexes (UMIs).

Author year	Setting	Patient #	Subtype	Type of study	Timepoints	ctDNA method of detection	Detection Rate	Association with outcome	Average lead time - median [range]
[23]	EBC patients treated with neoadjuvant chemotherapy	43	Various	Prosp.	1- After surgery (2 to 4 weeks after surgery) 2- Mutation tracking (after surgery and every 6 months during follow-up)	ctDNA (tumor informed) *primary tumor:* small panel targeting 14 known breast cancer driver genes *ctDNA:* personalized digital polymerase chain reaction (dPCR) assays	1- 19% (7/37) of total patients (not experiencing relapse 4%; experiencing relapse 50%) 2- 30% (13/43) of total patients (not experiencing relapse 4%; experiencing relapse: 80%)	1- DFS: HR 25.1, 95% CI 4.08 -130.5, $p < 0.0001$ 2- DFS: HR 12.0, 95% CI 3.36-43.07, $p < 0.0001$	7.9 months [0.03-13.6]
[24]	EBC patients	20	Various	Retrosp.	After surgery and during follow-up	ctDNA (tumor informed) *primary tumor: low pass* whole genome sequencing ctDNA: droplet digital PCR (ddPCR)	93% (13/14) of patients experiencing relapse; 0% (0/6) of patients not experiencing relapse	Level of ctDNA associated to the risk of metastatic disease ($p = 0.02$) and death ($p = 0.04$)	11 months [0-37]
[25]	EBC patients	49	Various	Prosp.	After surgery and during adjuvant treatment/follow-up	ctDNA (tumor informed) *primary tumor and white blood cells:* whole exome sequencing (Signatera) *ctDNA:* Signatera	89% (16/18) of patients experiencing relapse; 0% (0/31) of patients not experiencing relapse	1- ctDNA at first postsurgical sample. RFS: HR 11.8; 95% CI 4.3–32.5; $p < 0.0001$ 2- ctDNA in follow-up samples. RFS: HR 35.8; 95% CI 8.0–161.3; $p < 0.0001$	8.9 months [0.5–24]
[26]	EBC patients receiving neoadjuvant chemotherapy followed by surgery or surgery before adjuvant chemotherapy	101	Various	Prosp.	Mutation tracking (every 3 months for the first year of follow-up and subsequently every 6 months for 5 years)	ctDNA (tumor informed) *primary tumor:* small panel targeting 14 known breast cancer driver genes *ctDNA:* personalized digital polymerase chain reaction (dPCR) assays	79% (23/29) of patients experiencing relapse	RFS: HR 25.2; 95% CI 6.7-95.6, $p < 0.001$	10.7 months [8.1-19.1]
[27]	EBC who had residual disease after neoadjuvant chemotherapy	196	TNBC	Prosp.	Prior initiation of adjuvant treatment	ctDNA (tumor agnostic) hybridization-captured adaptor ligated based libraries of frequently rearranged genes in cancer	65% (37/57) in Arm A and 62% (23/85) in Arm B; 79% (23/29) experiencing relapse: 52% (43/83) not experiencing relapse	DDFS:HR 2.99, 95% CI 1.38-6.48, $p = 0.006$ DFS: HR 2.67, 95% CI 1.28-5.57, $p = 0.009$ OS: HR 4.16, 95% CI 1.66-10.42, $p = 0.002$	NA
[20]	EBC patients who received curative-intent treatment	142	Various	Retrosp.	1- After surgery (median 3.6 months) 2- 1 year follow-up (median 14.3 months)	ctDNA (tumor informed) *primary tumor:* whole exome sequencing *ctDNA:* 142 patient-specific panels	1- 70% (7/10) of patients experiencing relapse; 23% (23/101) of patients not experiencing relapse 2- 100% (6/6) of patients experiencing relapse; 22% (26/116) of patients not experiencing relapse	1- DRFS: HR 5.1; 95% CI 2.0–12.7, $p = 0.00048$ 2- DRFS: HR 20.8; 95% CI 7.3-58.9, $p < 0.0001$	18.9 months [3.4 - 39.2]

baseline (i.e., before neoadjuvant treatment) was not associated with disease-free survival (DFS), patients with ctDNA detection after surgery (i.e., 2 to 4 weeks after surgery) (19%) had shorter DFS [6.5 months vs. median not reached for ctDNA-negative; hazard ratio (HR): 25.1; 95% confidence interval (CI) 4.08-130.5]. Moreover, ctDNA detection in serial samples after surgery (*i.e.*, mutation tracking) was predictive of early relapse (HR: 12.0; 95% CI 3.36-43.07). Of note, the single time point approach had low sensitivity since only half of relapses were detected

through ctDNA in a single postsurgical sample. Nevertheless, detection rate increased to 80% with mutation tracking. The median lead time from ctDNA detection to clinical relapse was 7.9 months. Noteworthy, 96% of patients that did not experience relapse were ctDNA negative with either a single or multiple time point approach. Nevertheless, the authors highlighted the need to improve the detection of variant alleles with low frequencies and to incorporate strategies to control sequencing errors and artifacts. Successively, Olsson et al. conducted a retrospective analysis of ctDNA in 20 patients with EBC, using a low coverage WGS combined with a ddPCR-based personalized panel [24]. Their approach was based on the profiling of both primary tumor and normal tissue to identify tumor-associated chromosomal rearrangements. Among the 14 patients with known clinical recurrence, 13 (93%) had ctDNA detected in one or more time points after surgery. Conversely, none of the patients with long DFS had detectable ctDNA at any time point. ctDNA-based detection of occult metastases preceded the clinical diagnosis of distant disease in 12/14 patients (86%), with an average lead time of 11 months (range 0-37 months). Moreover, in this study the level of ctDNA was a quantitative risk factor for developing metastases (odds ratio 2.1 for each doubling of ctDNA levels, $p = 0.02$) or death (odds ratio 1.3 per ctDNA doubling, $p = 0.04$). Similar promising results on the role of ctDNA for early recurrence detection were annotated by Coombes and colleagues in a prospective multicenter study in EBC [25]. In their study, patient-specific somatic variants were identified upfront comparing paired primary tumor and matched white blood cell through WES. Subsequently, ctDNA was analyzed from plasma samples collected after surgery and during adjuvant treatment. The authors detected ctDNA in plasma samples from 16/18 patients who experienced disease recurrence, with a lead time of up to 2 years prior to distant metastatic relapse (median lead time 8.9 months). Although, none of the patients without recurrence was ctDNA-positive at any time point. Thus, their assay showed a 100% specificity and 89% sensitivity. Notably, sensitivity differed across BC subtypes, being 82%, 100%, and 100% in HR+/human epidermal growth factor receptor 2 negative (HER2−), HR+/HER2+, and triple-negative BC (TNBC), respectively. Additionally, ctDNA detection at either first postsurgical or follow-up samples was significantly associated with poorer prognosis (HR 11.8 and 35.8, respectively). Nevertheless, an inherent limitation of the assay was the inability to detect second primary BCs being limited to the mutational profile of the original primary. Moreover, a high volume of plasma (up to 5 mL) was used to prevent low detection.

Another prospective, multicenter, study by Garcia-Murillas et al. supported the association between MRD and relapse (HR: 25.2; 95% CI 6.7-95.6; $p < 0.001$) in a cohort of 101 patients with EBC [26]. Of 29 patients experiencing recurrence, 23 (79.3%) had prior ctDNA detection (median lead time of 10.7 months), while 6 (20.7%) relapsed without ctDNA detection before or at the time of recurrence. Of note all ctDNA-negative patients who experienced recurrence had a single site of relapse. Moreover, differences were observed across sites of relapse. While extracranial relapse was detected by ctDNA in 96% of patients (22/23), only 17% of patients with brain-only metastasis were ctDNA positive (1/6), suggesting that brain-only relapses are unlikely to be detected by ctDNA. In this study a higher level of ctDNA was observed among patients with TNBC as compared to other BC subtypes.

Radovich et al. conducted an analysis of patients with early-stage, high-risk, TNBC with pathological residual disease after neoadjuvant chemotherapy ($n = 196$) enrolled in the BRE12-158 phase II study [27]. ctDNA at the time of post-neoadjuvant treatment initiation was detected in up to 65% of patients and was associated with inferior distant disease-free survival (DDFS, median 32.5 months vs not reached, HR 2.99, 95% CI 1.38-6.48, $p = 0.006$). Similar results were observed for DFS and overall survival (OS). The OS probability at 24 months was 57% for ctDNA positive patients compared to 80% for ctDNA negative patients. Of note, in this study CTCs were concomitantly evaluated with ctDNA highlighting an increased sensitivity and discriminatory capacity when the two biomarkers were combined.

Finally, Parsons et al. tested an ultrasensitive tumor-informed assay in a cohort of 142 patients with EBC treated with curative intent with available postoperative samples [20]. Detection of ctDNA immediately after surgery (median time to plasma collection was 3.53 months) was associated with distant relapse (HR: 5.1, 95% CI 2.0-12.7). However, MRD was not associated with local-only recurrence. The prediction for distant recurrence was stronger for ctDNA positivity after 1 year from surgery (HR: 20.8; 95% CI 7.3–58.9). Sensitivity was driven by the number of tumor mutations available to track in cfDNA. Remarkably, the published longest lead time to clinical recurrence was observed (18.9 months, range 3.4-39.2) in this study. This crucial finding could allow an early intervention, oriented to potentially curative approaches.

In the meantime, the clinical utility of MRD-related assay has been for the first time investigated by Turner and colleagues in order to select patients for adjuvant systemic therapy [28]. Several trials with the same objective are currently ongoing. Among them, we can mention: i) LEADER (NCT03285412), evaluating therapy escalation for early-stage estrogen receptor (ER) positive BC, ii) DARE (NCT04567420), investigating the impact of fulvestrant and palbociclib in ER+/HER2-stage II/III BC patients with MRD, iii) TREAT-ctDNA trial (NCT05512364), a phase III trial evaluating the efficacy of the newly approved elacestrant compared to SoC (standard of care) in patients with HR+/HER2-localized BC, positive to MRD after curative therapy. Nonetheless, this approach shows significant challenges as demonstrated by the early discontinuation of ZEST (NCT04915755), a phase III study aimed at assessing the potential for ctDNA to guide therapy in TNBC and *BRCA1/2* (BReast CAncer gene 1/2) mutated BC. Specifically, fewer than 5% of the 800 patients have been randomized so far, rendering the study unfeasible.

Some limitations in the use of ctDNA alone for minimal RD detection should be addressed. For example, interval from blood collection to plasma isolation, centrifugation protocol, purification methods and plasma storage are all parameters that influence the results and still need to be standardized [29]. The large availability of storage and extraction methods and sequencing techniques make the reproducibility tricky. Additionally, the choice of which tumor variants should be included in the tumor-personalized panel may be arbitrary; there is no standardization regarding the optimal time point for ctDNA assessment; consensus cut-off value that should be used to discriminate high and low ctDNA concentrations is still not defined. Comparison of clinical sensitivity and lead time among studies is challenging, due to numerous potential confounders such as differences in patient populations, treatments, timing of sampling, and duration of follow-up. Lastly, ctDNA can only provide limited information about the tumor, mainly related to its genomic features, without allowing for a characterization of phenotypic features such as expression of markers that might be treatment targets, or the activation of pathways related to resistance mechanisms at the RNA level.

3. CRD: CTCs as a biomarker for minimal RD

CTCs are defined as cancer cells that have detached from the primary tumor and have been released into the circulatory system [30]. They are being investigated as a LB biomarker for *real-time* disease monitoring, as they can be detected since the early stages of disease even before clinical evidence of metastasis [31]. Even though most studies on CTCs have investigated their role in the metastatic setting [32,33], CTCs can also be detected in EBC [34] and, when detected at diagnosis or at the time of surgery in a cohort of 3173 patients with stage I-III BC, have shown to be an independent negative prognostic factor for DFS, BC specific survival, and OS (W. J. [35]). A similar prognostic value in EBC has been subsequently reported in other studies [36–38]. These studies support the possibility to use CTCs also for the detection of minimal RD after curative treatment (*i.e.*, CRD).

3.1. Methods for CTCs detection

The study of CTCs is challenging due to their short half-life in the blood (estimated to be around 1 h)[39] and because CTCs represent rare cells as compared to other cells found in the bloodstream. Therefore, the enrichment of CTCs is a key step for their isolation and identification. Many enrichment methods have been developed to this aim, based on different strategies, among which the most common is the detection of epithelial markers such as cytokeratin and epithelial cell adhesion molecule (EPCAM) [40]. The most used technology, especially in the context of clinical studies, is the CellSearch® system (Menarini Silicon Biosystems, Florence, Italy), which was approved in 2004 by the FDA for CTCs enumeration in metastatic BC. CellSearch® is a platform for the enumeration of CTCs based on the selection of EPCAM-expressing cells through antibody-labelled magnetic ferrofluids and the detection of cells positive for cytokeratin and 4′,6-diamidino-2-phenylindole (DAPI) and negative for CD45 [41].

3.2. Clinical studies on CRD detection in EBC patients

Various studies have investigated CTCs for minimal RD detection in patients with EBC who underwent surgery (Table 2). In the SUCCESS-A study, blood samples from more than 2000 patients with EBC were collected after surgery and after completion of adjuvant chemotherapy [42]. The study showed that CTC detection (≥1 CTC per 30 mL of blood) before adjuvant chemotherapy was an independent prognostic factor, associated to shorter DFS (HR 2.257; 95% CI 1.595-3.195; $p < 0.0001$) and OS (HR, 2.447; 95% CI 1.491- 4.015; $p = 0.0004$). Moreover, the persistence of CTCs after adjuvant chemotherapy also showed a negative impact on outcome with a significantly reduced DFS for those patients persistently positive for CTCs (both before and after adjuvant chemotherapy) compared with all other subgroups. A subsequent analysis from the SUCCESS-A trial after 5 years of completion of adjuvant chemotherapy, confirmed the importance of CTCs evaluation during follow-up as CTC detection after 5 years from chemotherapy was associated with decreased relapse-free survival (RFS), suggesting that persisting CTCs during long term follow-up independently predict late recurrences in patients with HR + BC (W. [43]). Results from the ECOG-ACRIN-E5103 study further supported the role of CTCs to stratify the risk of late recurrence in HR + EBC patients [44]. The CTC-positivity after five years was indeed the strongest predictor of late disease recurrence in patients with HR + BC, with a median time to recurrence of 2.8 years. In a longitudinal study performed by van Dalum et al., in 2014, the detection of ≥1 CTC before surgery, after adjuvant therapy, and one and two years after surgery, was associated with poorer RFS and OS, however no significant association was reported for CTC detection immediately after surgery [45]. Goodman et al., in 2018 in their retrospective analysis reported results regarding the interactions between adjuvant radiotherapy, CTC detection and clinical outcome in patients with EBC [46]. Interestingly, radiotherapy was associated with longer OS in patients with CTCs detected before adjuvant therapy, but not in those without detectable CTCs. These data suggest that the detection of CTCs after surgery could serve as a predictive marker of the benefit to this treatment. Beyond the evidence supporting the role of CRD alone to predict risk of recurrence, initial data is supporting the combination of CTCs and ctDNA to stratify the prognosis of patients with EBC. In the study by Radovich and colleagues [27], patients with TNBC who had pathological residual disease after neoadjuvant chemotherapy were evaluated for both ctDNA and CTCs. At this time point CTCs were detected in up to 43% of patients and increasing CTC count was significantly associated with inferior DDFS (HR 1.07; 95% CI 1.01-1.13; $p = 0.02$), DFS (HR 1.11; 95% CI 1.03-1.19; $p = 0.004$), and OS (HR 1.09; 95% CI 1.02-1.17; $p = 0.01$), suggesting an association between the quantitative burden of CTCs and outcomes. Interestingly, in this study no significant association between CTC positivity (defined as ≥ 1 CTC detected) and ctDNA positivity was found ($p = 0.19$). A proportion of patients were positive for one marker and not the other, such that the sensitivity to detect recurrences went from 79% (23 of 29) with ctDNA alone and 62% (18 of 29) with CTC alone to 90% (26 of 29) when combined. The combination of ctDNA and CTCs was associated with increased discriminatory capacity: patients who were positive for both ctDNA and CTCs had inferior DDFS compared with those who were positive for ctDNA alone or CTC alone, while patients who were negative for both ctDNA and CTCs had the best outcomes. The latter group might be a subgroup in which patients do not benefit from additional therapy, and this may be an ideal place to study novel de-escalation strategies. Therefore, study aimed at evaluating the combined prognostic value of MRD and CRD are hugely needed.

3.3. Challenges and new opportunities for CRD monitoring

To date, despite the evidence on the prognostic role of CTCs and CRD detection in EBC, their clinical use for minimal RD monitoring is limited. This is mainly due to several hurdles, especially: i) CTC detection requires specialized instruments and personnel, ii) they need to be processed immediately or within a few days of blood collection, making them not suitable for retrospective studies, iii) CTCs are detected only in a small fraction of patients in the early and post-surgery setting. In fact, in the early setting, about to 20% of patients have CTCs detected after surgery, before adjuvant therapy using the CellSearch® system (Table 2). However, several different label-free methods have been developed for CTC enrichment mainly based on physical properties such as density, size, and deformability [47], which might increase the detection rate. In this regard, in the study of Radovich et al. [27], by using a technology combining microfluidics, immunomagnetics and size-based filtration [48], CTCs were detected in about 40% of samples collected after surgery from patients with EBC. Detection methods that do not rely on the expression of epithelial markers could further increase CTC detection, since CTCs have a heterogeneous and transitional nature concerning epithelial-to-mesenchymal transition (EMT) [49]. In a study conducted in 2021 by Reduzzi et al. [50], CellSearch® was compared to two size-based technologies (CellSieve™ and ScreenCell® filters) for identifying CTC-clusters in early and metastatic BC. In samples spiked-in with epithelial breast cancer cell line cells, the methods had similar recovery capability, suggesting that, when the tumor cells express epithelial markers, the methodologies have a similar efficiency in cluster detection. However, in 19 samples from patients with metastatic BC processed in parallel with CellSearch® and CellSieve™ filters, the latter allowed CTC-cluster detection in a higher proportion of cases: 80% and 53%, respectively, suggesting that CTCs in patients might have a heterogeneous epithelial/mesenchymal phenotype, reducing the efficiency of methods that are based on EPCAM expression. Interestingly, in the EBC cohort, the filtration enrichment allowed the detection of CTC-clusters at baseline in 26/37 (70%) cases and after surgery in 7/18 (39%) samples. Marker-independent approaches might, therefore, improve the detection of single CTCs and CTC-clusters. Another strategy for improving CTC detection is avoiding the enrichment altogether. In recent years, 2 platforms (Epic Sciences platform, Epic Sciences, San Diego, CA, USA); See.d, Tethis S.p.A., Milan, Italy) have been developed that produce a set of glass slides on which all nucleated blood cells are placed and stabilized as a monolayer. The slides can be used for different kind of staining and can be scanned and analyzed with algorithms to automatically identify putative CTCs. Krol et al. [51] used the See.d technology for CTC detection in a cohort of 28 EBC patients and 30 healthy donors and were able to detect CTCs in a large percentage of BC patients with a sensitivity of 75% and a specificity of 96.7%. Interestingly, this method also reported the presence of CTC-clusters in EBC, detected in 5/28 patients. Another major advantage of these platforms is the possibility to store the slides for subsequent analysis, opening the way for retrospective studies in the CRD setting.

Table 2
Clinical studies on Cellular Residual Disease (CRD) detection in EBC patients
Abbreviations: breast cancer specific survival (BCSS); circulating tumor cells (CTCs) confidence interval (CI); distant disease-free survival (DDFS); disease free survival (DFS); distant metastasis free survival (DMFS); hazard ratio (HR); hormone receptor positive (HR+) local recurrence-free survival (LRFS); median time to recurrence (mTTR); not available (NA); prospective (Prosp); relapse free survival (RFS); retrospective (Retrosp.); time ratio (TR); triple negative breast cancer (TNBC).

Author, year	Stage	Patient #	Subtype	Type of study	Timepoints	Detection method	Detection rate	Association with outcome	Observed association/lead time
SUCCESS A trial: - [42] - [43]	I-III	2026	Various	Prosp.	**1)** before adjuvant chemotherapy **2)** after adjuvant chemotherapy (1492 patients) **3)** 5 years after adjuvant chemotherapy (206 patients)	CellSearch®	**1)** 21.5% (435/2026); 19.6% of node-negative and 22.4% of node-positive Overall CTCs detected in 19% and 30% of patients with locoregional or distant relapse, respectively **2)** 22.1% (330/1493) **3)** 7.8% (16/206)	**1)** DFS: HR,2.257; 95% CI 1.595-3.195; $p < 0.0001$ OS: HR, 2.447; 95% CI 1.491- 4.015; $p = 0.0004$ **2)** DFS HR, 1.124; 95% CI 1.02-1.25; $p = 0.02$ OS HR, 1.162; 95% CI 0.99- 1.37; $p = 0.06$ **3)** RFS in HR + BC: HR 5.14, 95% CI 1.47-18.03, $p = 0.011$	Patients with ≥5 CTCs: at 36 months, 28.1% of patients presented with recurrent disease and 14.3% had died
[44]	II-III	547	Various	Prosp.	Approximately 5 years after diagnosis	CellSearch®	4,8% (26/547)	Risk of recurrence overall: HR 12.7, 95% CI 4.7-34.7, $p < 0.001$ Risk of recurrence HR+: HR 10.82, 95% CI 4.42-26.47, $p < 0.001$	mTTR: 2.8 years (range, 0.1-2.8 years)
[46]	I-II	1697 NCDB and 1516 SUCCESS	Various	Prosp.	Before adjuvant therapy	CellSearch®	NCBD: 23.5% (399/1697) SUCCESS: 19.4% (294/1516)	NCDB: CTC-negative patients had longer OS compared with CTC-positive patients (TR, 1.78; 95% CI, 1.34-2.36; $p < 0.001$) SUCCESS: CTC-negative status was independently associated with significantly longer DFS (TR, 2.18; 95% CI, 1.42-3.35; $p < 0.001$), LRFS (TR, 2.12; 95% CI, 1.21-3.72; $p = 0.008$), and OS (TR, 1.97; 95% CI, 1.20-3.23; $p = 0.007$)	NA
van Dalum, 2014	I-III	403	Various	Prosp.	**1**) before surgery **2**) 1 week after surgery **3**) After completion of adjuvant chemo- and/or radiotherapy or before start endocrine therapy **4**) 1-year post surgery **5**) 2-years post surgery **6**) 3-years after surgery	CellSearch®	**1)** 19% (75/403) **2)** 18% (66/367) **3)** 15% (40/263) **4)** 12% (30/235) **5)** 11% (18/144) **6)** 13% (11/83)	**1)** RFS $p = 0.022$; OS $p = 0.006$ **2)** RFS $p = 0.852$; OS $p = 0.182$ **3)** RFS $p < 0.001$; OS $p = 0.018$ **4)** RFS $p = 0.006$; OS $p = 0.013$ **5)** RFS $p < 0.001$; OS $p = 0.045$ **6)** RFS $p = 0.439$; OS $p = 0.056$	NA
[27]	I-III	196	TNBC	Prosp.	After surgery	Microchip system (combining immunomagnetics, high-flow rate fluidics, and size-based separation)	Arm A: 43% (21/49) Arm B: 39% (29/74)	DDFS (HR, 1.07; 95% CI, 1.01-1.13; $p = 0.02$), DFS (HR, 1.11; 95% CI, 1.03-1.19; $p = 0.004$), OS (HR, 1.09; 95% CI, 1.02-1.17; $p = 0.01$)	NA

3.4. Advantages offered by the assessment of CRD

CTCs as living cells have the potential to provide an enormous quantity of biological information compared to MRD. Recent studies have reported the potential of CTC analysis for in-depth characterization at various levels (DNA, RNA, and protein level) and at the functional level (*in vitro/in vivo*) [52–54].

Thus, the utility of evaluating CRD goes beyond its mere association with the risk of recurrence and the possibility to escalate/de-escalate treatment based on this risk. Biomarkers associated with response/resistance in BC can be evaluated on CTCs to guide treatment choices. A proof-of-concept study in patients with early TNBC showed that CTCs detected after neoadjuvant treatment shared more genomic alterations with pathological residual disease than primary tumor, suggesting that

these cells represent resistant clones that, if targeted, may reduce the risk of recurrence [55]. Also, failure of systemic treatment in EBC may be due to the presence of intratumor heterogeneity of the primary tumor. This heterogeneity can be addressed by profiling CTCs to provide clinicians with a more accurate molecular picture driving personalized treatment algorithms. A study conducted in 75 patients with HER2-negative EBC with detectable CTCs before and after adjuvant chemotherapy showed that the administration of trastuzumab after adjuvant chemotherapy reduced the risk of recurrence as compared to observation [56]. This could be explained by the fact that almost 90% of the evaluated patients had HER2-expressing CTCs, although the actionability of HER2 expression on CTCs is still an open question. Beyond HER2, other biomarkers can be evaluated on CTCs such as programmed cell death ligand 1 (PD-L1) and ER [57] and the assessment of these therapeutic targets on persistent CTCs after surgery might enable an individualized and optimized treatment to prevent relapse, increasing the cure rate of patients with EBC. Moreover, there is a growing amount of evidence that ER and HER2 status change over time, especially during relapse or disease progression in patients with BC [58]. Thus, the evaluation of membrane protein expression in CTCs can be used to guide a tailored approach to emerging resistant clones that can be different from the primary tumor [59]. Finally, the characterization of CTCs in EBC can help in understanding the biology of metastasis formation. In particular, the possibility to detect and study CTC-clusters in the EBC setting that can be attained by using marker-independent technologies offers an unprecedented opportunity. In fact, CTC-clusters have a much higher metastatic potential than single CTCs [60] and are considered the true seeds of metastasis but their analysis have been so far limited to the metastatic setting because their detection in EBC with the CellSearch® is extremely rare. Research efforts in the field of CTCs in EBC are needed considering the new opportunities offered by the evaluation of CRD.

4. Conclusions

The application of LB in the early and post-surgery settings requires a technological and clinical paradigm shift. The central hypothesis of this transition is the consideration that the inherent strengths and limitations of ctDNA and CTCs should be considered as complementary tools for minimal RD assessment. Indeed, ctDNA, though invaluable in monitoring minimal RD, confronts specific constraints. Tumor-informed approaches are hampered by their limited capacity to track the acquisition of new genetic alterations, rendering them less suitable for assessing evolving tumor landscapes and the tumor clonal evolution. On the other hand, tumor-agnostic assays, while potentially more dynamic, may grapple with sensitivity pitfalls. Moreover, MRD offers insights into genomic alterations, but overlooks the complex phenotypic characteristics of tumors. In contrast, CTCs, as dynamic living entities, not only provide molecular information but also offer a holistic view of tumor biology. The ability to explore additional biomarkers such as HER2, ER and PD-L1 enhances the potential clinical utility of CRD. Moreover, CTCs permit high-throughput characterizations like RNA sequencing, enabling a comprehensive assessment of the genetic and transcriptomic profile of the tumor (Fig. 1).

Nevertheless, in the context of CRD, there is a pivotal shift from the commonly used epithelial-based enrichment approach to an epithelial-agnostic one. This transition is predicated on the recognition that CRD may encompass CTC subpopulations with varying phenotypic characteristics, and an epithelial-agnostic approach better captures this heterogeneity.

Additionally, CRD may offer a cost-effective tumor-agnostic applicability and provide a higher degree of certainty that the detected multi-omics alterations originate from the tumor itself, as opposed to ctDNA, which can be confounded by external factors like CHIP. Remarkably,

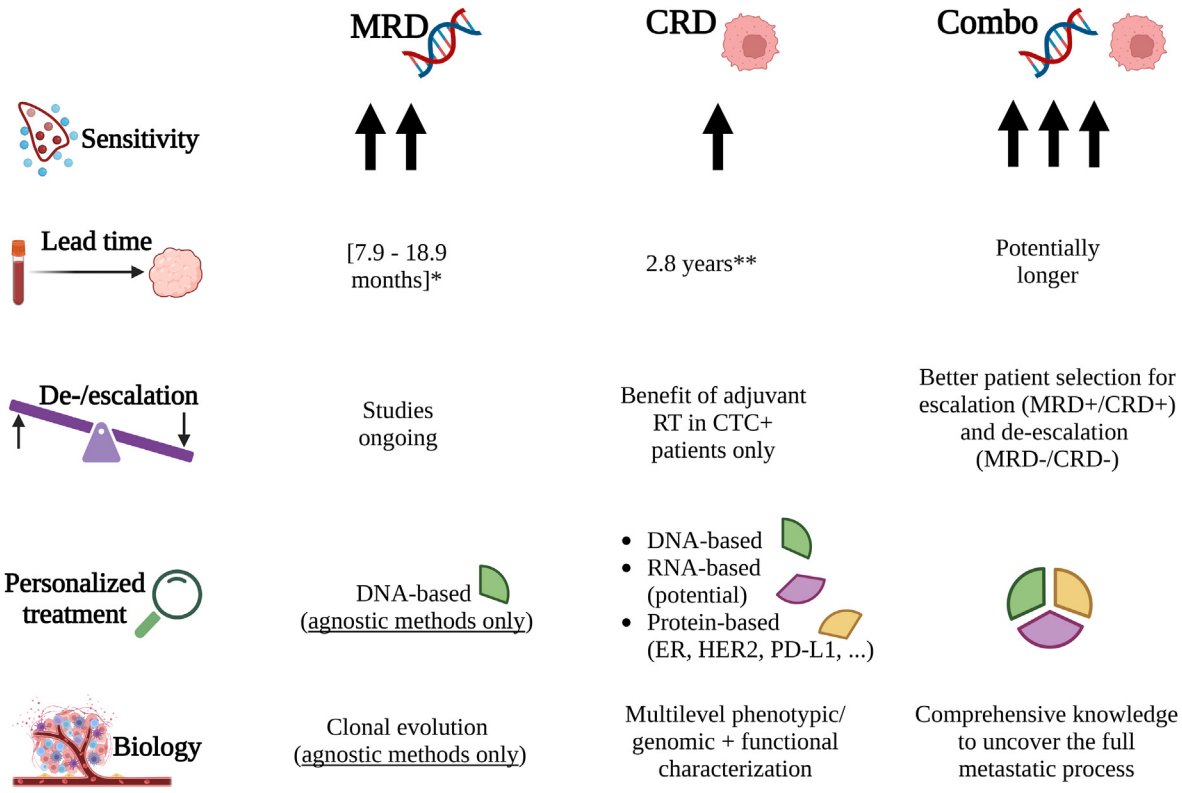

Fig. 1. Summary of main advantages of combining molecular with cellular residual disease (MRD and CRD, respectively) detection. The combination would increase sensitivity and lead-time, improve the selection of patients eligible for adjuvant treatment de-/escalation, provide more targets for personalized treatment and give comprehensive information on the biology of the metastatic process. Created with BioRender.com *range of median lead time observed in studies from Table 1 **median lead time observed in 1 study (Table 2) RT, radiotherapy; CTC, circulating tumor cell; MRD, molecular residual disease; CRD, cellular residual disease; ER, estrogen receptor; HER2, human epidermal growth factor receptor; PD-L1, programmed death-ligand 1.

CTCs have already been included into the *5*th *edition of the WHO Classification of Tumors: Breast Tumours* [61] and *7*th *edition of the AJCC Cancer Staging manual* [62], thus, their importance has already been highlighted. Nevertheless, they are not frequently monitored.

In conclusion, considering the limits and advantages of both MRD and CRD, a synergistic approach that leverages the strengths of ctDNA to enhance sensitivity while capitalizing on CTCs' capacity to provide comprehensive insights into tumor biology should be considered. The integration of these methodologies holds the promise of revolutionizing minimal RD monitoring, providing a more nuanced and accurate understanding of early-stage treatments, and ultimately impacting on survival and quality of life of patients with BC.

Authors' contribution

Conception and Design: M.C., C.R., L.G. Literature research, data accumulation and interpretation: M.S.S, E.M. Manuscript writing and original draft preparation: M.S.S, and E.M. Manuscript Supervision: M.C., C.R. Figure preparation: C.R., M.S.S., E.M. Tables preparation: M.S.S., E.M., E.N. Critical Review and Editing: All the authors. All authors have read and approved to the submitted version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: MC reports personal fees from Lilly, Sermonix, Data Genomics, Foundation Medicine, Guardant Health, Celcuity, Iylon, and Ellipses and grants and personal fees from Pfizer, AZ and Menarini, all outside the submitted work. Given his role as Editorial Board Member, MC had no involvement in the peer review of this article and has no access to information regarding its peer review. No other potential conflicts of interest are reported.

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References

[1] Siegel Rebecca L, Miller Kimberly D. Nikita Sandeep Wagle, and Ahmedin Jemal. "Cancer Statistics, 2023." CA: A Cancer Journal for Clinicians 2023;73(1):17–48. https://doi.org/10.3322/caac.21763.

[2] Colleoni Marco, Sun Zhuoxin, Price Karen N, Karlsson Per, Forbes John F, Thürlimann Beat, Gianni Lorenzo, et al. Annual hazard rates of recurrence for breast cancer during 24 Years of follow-up: results from the international breast cancer study group trials I to V. J Clin Oncol 2016;34(9):927–35. https://doi.org/10.1200/JCO.2015.62.3504.

[3] Stergiopoulou Dimitra, Markou Athina, Strati Areti, Zavridou Martha, Tzanikou Eleni, Mastoraki Sophia, Kallergi Galatea, Georgoulias Vassilis, Lianidou Evi. Comprehensive liquid biopsy analysis as a tool for the early detection of minimal residual disease in breast cancer. Sci Rep 2023;13(1):1258. https://doi.org/10.1038/s41598-022-25400-1.

[4] Pedersen Rikke Nørgaard, Esen Buket Öztürk, Mellemkjær Lene, Christiansen Peer, Ejlertsen Bent, Lee Lash Timothy, Nørgaard Mette, Cronin-Fenton Deirdre. The incidence of breast cancer recurrence 10-32 Years after primary diagnosis. J Natl Cancer Inst: J Natl Cancer Inst 2022;114(3):391–9. https://doi.org/10.1093/jnci/djab202.

[5] Pan Hongchao, Gray Richard, Braybrooke Jeremy, Davies Christina, Taylor Carolyn, Paul McGale, Peto Richard, et al. 20-Year risks of breast-cancer recurrence after stopping endocrine therapy at 5 years. N Engl J Med 2017;377(19):1836–46. https://doi.org/10.1056/NEJMoa1701830.

[6] Wilson Sheridan, Speers Caroline, Scott Tyldesley, Chia Stephen, Kennecke Hagen, Ellard Susan, Lohrisch Caroline. Risk of recurrence or contralateral breast cancer more than 5 Years after diagnosis of hormone receptor-positive early-stage breast cancer. Clin Breast Cancer 2016;16(4):284–90. https://doi.org/10.1016/j.clbc.2015.11.002.

[7] Tachtsidis A, McInnes LM, Jacobsen N, Thompson EW, Saunders CM. Minimal residual disease in breast cancer: an overview of circulating and disseminated tumour cells. Clin Exp Metastasis 2016;33(6):521–50. https://doi.org/10.1007/s10585-016-9796-8.

[8] Chen H, Zhou Q. Detecting liquid remnants of solid tumors treated with curative intent: Circulating tumor DNA as a biomarker of minimal residual disease (Review). Oncol Rep 2023 May;49(5):106. https://doi.org/10.3892/or.2023.8543.

[9] Sant Marta, Bernat-Peguera Adrià, Felip Eudald, Margelí Mireia. Role of CtDNA in breast cancer. Cancers 2022;14(2):310. https://doi.org/10.3390/cancers14020310.

[10] Davis Andrew A, Jacob Saya, Gerratana Lorenzo, Shah Ami N, Wehbe Firas, Katam Neelima, Zhang Qiang, et al. Landscape of circulating tumour DNA in metastatic breast cancer. EBioMedicine 2020;58(August). https://doi.org/10.1016/j.ebiom.2020.102914.

[11] Moding Everett J, Barzin Y Nabet, Alizadeh Ash A, Diehn Maximilian. Detecting liquid remnants of solid tumors: circulating tumor DNA minimal residual disease. Cancer Discov 2021;11(12):2968–86. https://doi.org/10.1158/2159-8290.CD-21-0634.

[12] Kasi Pashtoon M, Gordon Fehringer, Taniguchi Hiroya, Starling Naureen, Nakamura Yoshiaki, Kotani Daisuke, Powles Thomas, et al. Impact of circulating tumor DNA–based detection of molecular residual disease on the conduct and design of clinical trials for solid tumors. JCO Precision Oncology 2022;6(March): e2100181. https://doi.org/10.1200/PO.21.00181.

[13] Hasenleithner Samantha O, Speicher Michael R. A clinician's handbook for using CtDNA throughout the patient journey. Mol Cancer 2022;21(1):81. https://doi.org/10.1186/s12943-022-01551-7.

[14] Werner Stefan, Heidrich Isabel, Pantel Klaus. Clinical management and biology of tumor dormancy in breast cancer. Seminars in Cancer Biology, Cancer dormancy and recurrence 2022;78(January):49–62. https://doi.org/10.1016/j.semcancer.2021.02.001.

[15] Chin Re-I, Chen Kevin, Abul Usmani, Chua Chanelle, Harris Peter K, Binkley Michael S, Azad Tej D, Dudley Jonathan C, Chaudhuri Aadel A. Detection of solid tumor molecular residual disease (MRD) using circulating tumor DNA (CtDNA). Mol Diagn Ther 2019;23(3):311–31. https://doi.org/10.1007/s40291-019-00390-5.

[16] Newman Aaron M, Bratman Scott V, To Jacqueline, Wynne Jacob F, Eclov Neville CW, Modlin Leslie A, Liu Chih Long, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20(5):548–54. https://doi.org/10.1038/nm.3519.

[17] Alimirzaie Sahar, Bagherzadeh Maryam, Akbari Mohammad R. Liquid biopsy in breast cancer: a comprehensive review. Clin Genet 2019;95(6):643–60. https://doi.org/10.1111/cge.13514.

[18] Little Stephen. Amplification-refractory mutation system (ARMS) analysis of point mutations. Current Protocols in Human Genetics 1995;7(1). https://doi.org/10.1002/0471142905.hg0908s07. 9.8.1-9.8.12.

[19] Larribère Lionel, Martens Uwe M. Advantages and challenges of using CtDNA NGS to assess the presence of minimal residual disease (MRD) in solid tumors. Cancers 2021;13(22):5698. https://doi.org/10.3390/cancers13225698.

[20] Parsons Heather A, Rhoades Justin, Sarah C Reed, Gydush Gregory, Ram Priyanka, Pedro Exman, Xiong Kan, et al. Sensitive detection of minimal residual disease in patients treated for early-stage breast cancer. Clin Cancer Res 2020;26(11): 2556–64. https://doi.org/10.1158/1078-0432.CCR-19-3005.

[21] Pascual J, Attard G, Bidard F-C, Curigliano G, De Mattos-Arruda L, Diehn M, Italiano A, et al. ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO precision medicine working group. Ann Oncol 2022;33(8):750–68. https://doi.org/10.1016/j.annonc.2022.05.520.

[22] Cohen Stacey A, Liu Minetta C, Aleshin Alexey. Practical recommendations for using CtDNA in clinical decision making. Nature 2023;619(7969):259–68. https://doi.org/10.1038/s41586-023-06225-y.

[23] Garcia-Murillas Isaac, Schiavon Gaia, Weigelt Britta, Ng Charlotte, Hrebien Sarah, Cutts Rosalind J, Cheang Maggie, et al. Mutation tracking in circulating tumor DNA predicts relapse in early breast cancer. Sci Transl Med 2015;7(302). https://doi.org/10.1126/scitranslmed.aab0021. 302ra133-302ra133.

[24] Olsson Eleonor, Winter Christof, George Anthony, Chen Yilun, Howlin Jillian, Man-Hung Eric Tang, Dahlgren Malin, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. EMBO Mol Med 2015;7(8):1034–47. https://doi.org/10.15252/emmm.201404913.

[25] Coombes Raoul Charles, Page Karen, Salari Raheleh, Hastings Robert K, Armstrong Anne, Ahmed Samreen, Ali Simak, et al. Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence. Clin Cancer Res 2019;25(14):4255–63. https://doi.org/10.1158/1078-0432.CCR-18-3663.

[26] Garcia-Murillas Isaac, Chopra Neha, Comino-Méndez Iñaki, Beaney Matthew, Tovey Holly, Cutts Rosalind J, Swift Claire, et al. Assessment of molecular relapse detection in early-stage breast cancer. JAMA Oncol 2019;5(10):1473–8. https://doi.org/10.1001/jamaoncol.2019.1838.

[27] Radovich Milan, Jiang Guanglong, Hancock Bradley A, Chitambar Christopher, Nanda Rita, Falkson Carla, Lynce Filipa C, et al. Association of circulating tumor DNA and circulating tumor cells after neoadjuvant chemotherapy with disease recurrence in patients with triple-negative breast cancer: preplanned secondary analysis of the BRE12-158 randomized clinical trial. JAMA Oncol 2020;6(9): 1410–5. https://doi.org/10.1001/jamaoncol.2020.2295.

[28] Turner NC, Swift C, Jenkins B, Kilburn L, Coakley M, Beaney M, Fox L, et al. Results of the C-TRAK TN trial: a clinical trial utilising CtDNA mutation tracking to detect molecular residual disease and trigger intervention in patients with moderate- and

high-risk early-stage triple-negative breast cancer. Ann Oncol 2023;34(2):200–11. https://doi.org/10.1016/j.annonc.2022.11.005.
[29] Pessoa Luciana Santos, Heringer Manoela, Ferrer Valéria Pereira. CtDNA as a cancer biomarker: a broad overview. Crit Rev Oncol Hematol 2020;155(November): 103109. https://doi.org/10.1016/j.critrevonc.2020.103109.
[30] Vasseur Antoine, Kiavue Nicolas, Bidard François-Clément, Pierga Jean-Yves, Cabel Luc. Clinical utility of circulating tumor cells: an update. Mol Oncol 2021; 15(6):1647–66. https://doi.org/10.1002/1878-0261.12869.
[31] Chemi Francesca, Mohan Sumitra, Guevara Tatiana, Clipson Alexandra, Rothwell Dominic G, Dive Caroline. Early dissemination of circulating tumor cells: biological and clinical insights. Front Oncol 2021;11. https://www.frontiersin.org/articles/10.3389/fonc.2021.672195.
[32] Cristofanilli Massimo, Pierga Jean-Yves, James Reuben, Rademaker Alfred, Davis Andrew A, Peeters Dieter J, Fehm Tanja, et al. The clinical use of circulating tumor cells (CTCs) enumeration for staging of metastatic breast cancer (MBC): international expert consensus paper. Crit Rev Oncol Hematol 2019;134(February): 39–45. https://doi.org/10.1016/j.critrevonc.2018.12.004.
[33] Cristofanilli Massimo, Budd G Thomas, Ellis Matthew J, Stopeck Alison, Matera Jeri, Craig Miller M, Reuben James M, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351(8): 781–91. https://doi.org/10.1056/NEJMoa040766.
[34] Krishnamurthy Savitri, Cristofanilli Massimo, Singh Balraj, James Reuben, Gao Hui, Cohen Evan N, Andreopoulou Eleni, et al. Detection of minimal residual disease in blood and bone marrow in early stage breast cancer. Cancer 2010;116(14):3330–7. https://doi.org/10.1002/cncr.25145.
[35] Janni Wolfgang J, Brigitte Rack Leon, Terstappen WMM, Pierga Jean-Yves, Taran Florin-Andrei, Fehm Tanja, Hall Carolyn, et al. Pooled analysis of the prognostic relevance of circulating tumor cells in primary breast cancer. Clin Cancer Res 2016;22(10):2583–93. https://doi.org/10.1158/1078-0432.CCR-15-1603.
[36] Pierga Jean-Yves, Bidard François-Clément, Mathiot Claire, Brain Etienne, Delaloge Suzette, Giachetti Sylvie, de Cremoux Patricia, Salmon Rémy, Vincent-Salomon Anne, Marty Michel. Circulating tumor cell detection predicts early metastatic relapse after neoadjuvant chemotherapy in large operable and locally advanced breast cancer in a phase II randomized trial. Clin Cancer Res 2008;14(21): 7004–10. https://doi.org/10.1158/1078-0432.CCR-08-0030.
[37] Bidard François-Clément, Belin Lisa, Delaloge Suzette, Lerebours Florence, Ngo Charlotte, Reyal Fabien, Alran Séverine, et al. Time-dependent prognostic impact of circulating tumor cells detection in non-metastatic breast cancer: 70-month analysis of the REMAGUS02 study. Int J Breast Cancer 2013;2013:e130470. https://doi.org/10.1155/2013/130470. May.
[38] Hall Carolyn, Karhade Mandar, Laubacher Barbara, Anderson Amber, Henry Kuerer, DeSynder Sarah, Anthony Lucci. Circulating tumor cells after neoadjuvant chemotherapy in stage I–III triple-negative breast cancer. Ann Surg Oncol 2015; 22(3):552–8. https://doi.org/10.1245/s10434-015-4600-6.
[39] Ward Mark P, Kane Laura E, Norris Lucy A, Mohamed Bashir M, Kelly Tanya, Bates Mark, Clarke Andres, et al. Platelets, immune cells and the coagulation cascade; friend or foe of the circulating tumour cell? Mol Cancer 2021;20(1):59. https://doi.org/10.1186/s12943-021-01347-1.
[40] Barradas AMC, Terstappen LWMM. Towards the biological understanding of CTC: capture technologies, definitions and potential to create metastasis. Cancers 2013; 5(4):1619–42. https://doi.org/10.3390/cancers5041619.
[41] Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, et al. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 2004;10(20):6897–904. https://doi.org/10.1158/1078-0432.CCR-04-0378.
[42] Rack Brigitte, Schindlbeck Christian, Julia Jückstock, Ulrich Andergassen, Hepp Philip, Zwingers Thomas, Thomas W, Friedl P, et al. Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst: J Natl Cancer Inst 2014;106(5):dju066. https://doi.org/10.1093/jnci/dju066.
[43] Janni Wolfgang, Rack Brigitte Kathrin, Fasching Peter A, Haeberle Lothar, Tesch Hans, Lorenz Ralf, Schochter Fabienne, et al. Persistence of circulating tumor cells in high risk early breast cancer patients five years after adjuvant chemotherapy and late recurrence: results from the adjuvant SUCCESS A trial. J Clin Oncol 2018; 36(15_suppl):515. https://doi.org/10.1200/JCO.2018.36.15_suppl.515. 515.
[44] Sparano Joseph, O'Neill Anne, Alpaugh Katherine, Wolff Antonio C, Northfelt Donald W, Dang Chau T, Sledge George W, Miller Kathy D. Association of circulating tumor cells with late recurrence of estrogen receptor–positive breast cancer: a secondary analysis of a randomized clinical trial. JAMA Oncol 2018;4(12): 1700–6. https://doi.org/10.1001/jamaoncol.2018.2574.
[45] van Dalum G, van der Stam GJ, Tibbe AG, Franken B, Mastboom WJ, Vermes I, et al. Circulating tumor cells before and during follow-up after breast cancer surgery. Int J Oncol 2015 Jan;46(1):407–13. https://doi.org/10.3892/ijo.2014.2694.
[46] Goodman Chelain R, Seagle Brandon-Luke L, Friedl Thomas WP, Rack Brigitte, Lato Krisztian, Fink Visnja, Cristofanilli Massimo, et al. Association of circulating tumor cell status with benefit of radiotherapy and survival in early-stage breast cancer. JAMA Oncol 2018;4(8):e180163. https://doi.org/10.1001/jamaoncol.2018.0163.
[47] Deng Zicheng, Wu Shengming, Wang Yilong, Shi Donglu. Circulating tumor cell isolation for cancer diagnosis and prognosis. EBioMedicine 2022;83(September): 104237. https://doi.org/10.1016/j.ebiom.2022.104237.
[48] Chang Chun-Li, Huang Wanfeng, Jalal Shadia I, Chan Bin-Da, Mahmood Aamer, Shahda Safi, O'Neil Bert H, Matei Daniela E, Savran Cagri A. Circulating tumor cell detection using a parallel flow micro-aperture chip system. Lab Chip 2015;15(7): 1677–88. https://doi.org/10.1039/C5LC00100E.
[49] Lin Danfeng, Shen Lesang, Luo Meng, Zhang Kun, Li Jinfan, Qi Yang, Zhu Fangfang, et al. Circulating tumor cells: biology and clinical significance. Signal Transduct Targeted Ther 2021;6(1):1–24. https://doi.org/10.1038/s41392-021-00817-8.
[50] Reduzzi Carolina, Di Cosimo Serena, Gerratana Lorenzo, Motta Rosita, Martinetti Antonia, Vingiani Andrea, D'Amico Paolo, et al. Circulating tumor cell clusters are frequently detected in women with early-stage breast cancer. Cancers 2021;13(10):2356. https://doi.org/10.3390/cancers13102356.
[51] Krol Ilona, Schwab Fabienne D, Carbone Roberta, Ritter Mathilde, Picocci Sabrina, Marzia L, De Marni, Stepien Grazyna, et al. Detection of clustered circulating tumour cells in early breast cancer. Br J Cancer 2021;125(1):23–7. https://doi.org/10.1038/s41416-021-01327-8.
[52] Fernández Santiago, Cristóbal Rafael López-López, Piñeiro Roberto. Chapter three - models to study CTCs and CTC culture methods. In: Reduzzi Carolina, Gerratana Lorenzo, editors. International review of cell and molecular biology. Massimo cristofanilli, 381:57–98. Circulating tumor cells, from biotech innovation to clinical utility. Academic Press; 2023. https://doi.org/10.1016/bs.ircmb.2023.07.007.
[53] Sharma Sandhya, Zhuang Rachel, Long Marisa, Pavlovic Mirjana, Kang Yunqing, Azhar Ilyas, Asghar Waseem. Circulating tumor cell isolation, culture, and downstream molecular analysis. Biotechnol Adv 2018;36(4):1063–78. https://doi.org/10.1016/j.biotechadv.2018.03.007.
[54] Zhu Ziqing, Hu Erya, Shen Hong, Tan Jun, Zeng Shan. The functional and clinical roles of liquid biopsy in patient-derived models. J Hematol Oncol 2023;16(April): 36. https://doi.org/10.1186/s13045-023-01433-5.
[55] Silvestri Marco, Dugo Matteo, Vismara Marta, De Cecco Loris, Lanzoni Davide, Vingiani Andrea, Folli Secondo, et al. Copy number alterations analysis of primary tumor tissue and circulating tumor cells from patients with early-stage triple negative breast cancer. Sci Rep 2022;12(1):1470. https://doi.org/10.1038/s41598-022-05502-6.
[56] Georgoulias V, Bozionelou V, Agelaki S, Perraki M, Apostolaki S, Kallergi G, Kalbakis K, Xyrafas A, Mavroudis D. Trastuzumab decreases the incidence of clinical relapses in patients with early breast cancer presenting chemotherapy-resistant CK-19mRNA-Positive circulating tumor cells: results of a randomized phase II study. Ann Oncol 2012;23(7):1744–50. https://doi.org/10.1093/annonc/mds020.
[57] Zhou Ying, Zhou Jinmei, Hao Xiaopeng, Shi Haoyuan, Li Xuejie, Wang Anqi, Hu Zhiyuan, Yang Yanlian, Jiang Zefei, Wang Tao. Efficacy relevance of PD-L1 expression on circulating tumor cells in metastatic breast cancer patients treated with anti-PD-1 immunotherapy. Breast Cancer Res Treat 2023;200(2):281–91. https://doi.org/10.1007/s10549-023-06972-6.
[58] Riethdorf Sabine, Müller Volkmar, Zhang Liling, Rau Thomas, Loibl Sibylle, Komor Martina, Roller Marc, et al. Detection and HER2 expression of circulating tumor cells: prospective monitoring in breast cancer patients treated in the neoadjuvant GeparQuattro trial. Clin Cancer Res 2010;16(9):2634–45. https://doi.org/10.1158/1078-0432.CCR-09-2042.
[59] Jaeger BaS, Neugebauer J, Andergassen U, Melcher C, Schochter F, Mouarrawy D, Ziemendorff G, et al. The HER2 phenotype of circulating tumor cells in HER2-positive early breast cancer: a translational research project of a prospective randomized phase III trial. PLoS One 2017;12(6):e0173593. https://doi.org/10.1371/journal.pone.0173593.
[60] Aceto Nicola, Bardia Aditya, Miyamoto David T, Donaldson Maria C, Wittner Ben S, Spencer Joel A, Yu Min, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell 2014;158(5):1110–22. https://doi.org/10.1016/j.cell.2014.07.013.
[61] WHO Classification of Tumours Editorial Board. Breast Tumours. 2019. https://publications.iarc.fr/Book-And-Report-Series/Who-Classification-Of-Tumours/Breast-Tumours-2019.
[62] Edge Stephen B, Compton Carolyn C. The American joint committee on cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010;17(6):1471–4. https://doi.org/10.1245/s10434-010-0985-4.