JLB 8 (2025) 100299

Contents lists available at ScienceDirect

The Journal of Liquid Biopsy

journal homepage: www.sciencedirect.com/journal/the-journal-of-liquid-biopsy

The impact of liquid biopsy in breast cancer: Redefining the landscape of non-invasive precision oncology

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ARTICLE INFO

Keywords:
BC
Liquid biopsy
Circulating tumor DNA (ctDNA)
Circulating tumor cells (CTCs)
Cell-free nucleic acids
Exosomes

ABSTRACT

Breast cancer (BC) remains a leading cause of morbidity and mortality among women worldwide, necessitating the development of innovative diagnostic and monitoring strategies. Liquid biopsy (LB), a minimally invasive approach that analyzes circulating tumor cells (CTCs), cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), extracellular vesicles (EVs), and other tumor-derived biomarkers in body fluids, has emerged as a transformative tool in BC management. This review comprehensively explores the role of LB in early detection, disease monitoring, treatment stratification, and resistance surveillance in BC. We discuss the latest advancements in LB technologies, including next-generation sequencing (NGS), digital PCR, and single-cell analysis, highlighting their sensitivity and specificity. Additionally, we examine the clinical utility of LB in guiding personalized therapy, particularly in the context of hormone receptor-positive, HER2positive, and triple-negative BC subtypes. Despite its promise, several challenges, including standardization, validation, and integration into clinical practice, remain to be addressed. By summarizing current evidence and future directions, this review underscores the potential of LB to revolutionize BC diagnosis and treatment, paving the way for a more precise and dynamic approach to disease management.

1. Introduction

Breast cancer (BC) continues to pose a substantial global health challenge, ranking among the leading causes of cancer-related mortality in women. In 2022, approximately 2.3 million new cases of invasive BC were diagnosed among females worldwide, resulting in around 670,000 BC-related deaths [1]. Furthermore, the incidence of BC continues to rise, with projections indicating a 61.7 % increase in mortality rates in the Southeast Asia region by 2040, highlighting the growing burden of the disease [2]. Therefore, early detection and accurate disease monitoring are pivotal in enhancing patient outcomes [3]. Historically, tissue biopsy has been the gold standard for cancer diagnosis and management, offering essential insights into the tumor's molecular profile [4]. However, this approach is invasive, often causing patient discomfort and carrying risks of complications. Moreover, tissue biopsies may not fully capture the tumor's heterogeneity, particularly in metastatic contexts, and repeated procedures are frequently impractical, hindering effective monitoring of tumor progression over time [5].

In recent years, liquid biopsy (LB) has emerged as a promising non-invasive diagnostic tool that offers a real-time snapshot of a tumor's genetic and molecular landscape [6]. This technique involves analyzing circulating tumor components found in bodily fluids, primarily blood, including circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes, and other cell-free nucleic acids [6]. Unlike traditional tissue biopsies, LBs are minimally invasive, can be performed repeatedly, and have the potential to capture dynamic changes in the tumor's molecular profile, making them valuable in BC management [7–9].

BC is a highly diverse disease characterized by intricate molecular mechanisms. It is categorized into intrinsic subtypes—such as Luminal A, Luminal B, HER2-enriched, and basal-like (often including triple-negative breast cancer, TNBC)—which are indicative of unique gene expression profiles, clinical patterns, and treatment responses [10]. Common genomic changes in BC include mutations in PIK3CA, TP53, BRCA1/2, ESR1, and the amplification or overexpression of HER2 (ERBB2) [11,12]. These molecular alterations not only inform treatment approaches but also act as vital biomarkers for prognosis, disease tracking, and predicting treatment resistance. Gaining insight into this dynamic molecular landscape is essential for precision oncology and justifies the integration of LB in the management of BC [13].

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https://doi.org/10.1016/j.jlb.2025.100299
Received 15 April 2025; Received in revised form 6 May 2025; Accepted 19 May 2025
Available online 21 May 2025

An increasing number of molecular biomarkers are now utilized in clinical decision-making for BC. Among the clinically validated biomarkers detectable via LB are PIK3CA mutations, which guide the use of PI3K inhibitors like alpelisib in HR+/HER2− advanced BC [14,15]; ESR1 mutations, which indicate resistance to endocrine therapy and suggest therapy adjustments [16,17]; and HER2 amplifications, which continue to facilitate HER2-targeted therapy in both early and advanced cases [18,19]. Alongside the previously mentioned biomarkers, others such as Oncotype DX, MammaPrint, Prosigna ((based on the PAM50 gene signature)), EndoPredict (EP/EPclin score test), and Breast Cancer Index (BCI) are utilized to evaluate the necessity of adjuvant chemotherapy for patients who are ER-positive, HER2-negative, and lymph node-negative [20,21]. These biomarkers analyze recurrence risk and assist in tailoring treatment options. Conversely, several investigational biomarkers are currently being researched for their clinical potential. These include TP53 mutations, which may indicate clonal evolution and aggressive disease; BRCA1/2 reversion mutations, which could forecast resistance to PARP inhibitors; methylation patterns in cell-free DNA; and various non-coding RNAs and exosomal content, showing promise for early detection, response monitoring, and recurrence tracking. Although these emerging biomarkers are not yet standard in clinical practice, they illustrate the future of personalized and minimally invasive BC treatment propelled by LB technologies [22–24].

LB plays a multifaceted role in BC management. It enables early detection of malignancies, potentially identifying cancers before clinical symptoms emerge or tumors become visible through imaging techniques, further offering a critical advantage for high-risk populations where early intervention can significantly influence disease progression [25,26]. Additionally, LB facilitates real-time monitoring of treatment responses, allowing clinicians to adjust therapies based on the tumor's ever-evolving genetic profile [25]. This adaptability is essential for managing resistance to targeted treatments and enhancing the precision of therapeutic decisions. Moreover, the technique can detect minimal residual disease (MRD) and early signs of recurrence, enabling timely interventions that may prevent full-scale metastatic relapse [27]. Furthermore, by providing comprehensive molecular profiling of tumors, LB supports the development of personalized treatment strategies, aligning with the principles of precision medicine [25]. Despite these advantages, challenges such as standardization, sensitivity, and integration into clinical practice persist. Ongoing research and technological advancements are essential to fully realize the potential of liquid biopsy in improving BC outcomes [28].

Building upon the substantial evidence supporting the integration of LB into BC management, this all-inclusive review endeavors to critically evaluate and synthesize current research findings in this domain. The primary objectives are – **(i)** to provide a comprehensive overview of LB modalities and their clinical applications in BC management **(ii)** to conduct a critical assessment of clinical evidence supporting LB across different stages of BC **(iii)** to shed light on limitations of LB, challenges in its clinical integration in routine breast oncological practice, and also draw insights into the possibility of future research on this intriguing novel diagnostic innovation. Through this comprehensive review, we aim to elucidate the transformative potential of LB in revolutionizing BC diagnostics and therapeutics while also delineating the critical areas where further empirical inquiry is warranted to fully harness its clinical benefits.

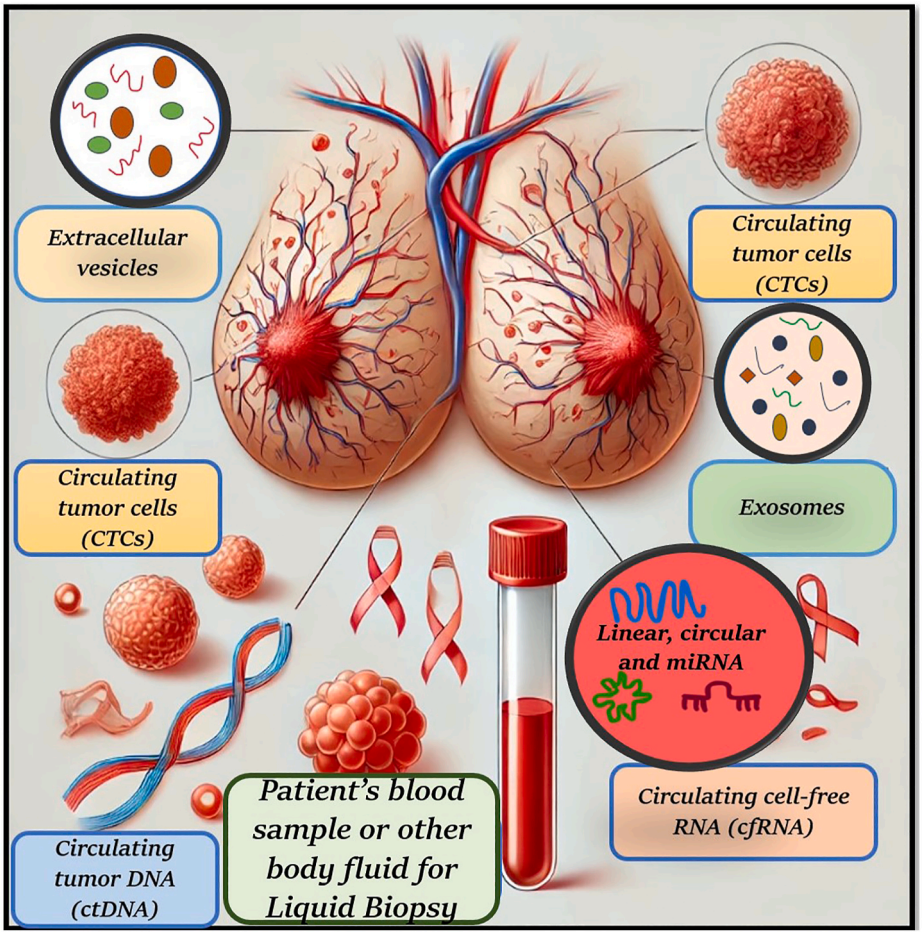

Fig. 1. Illustration depicting the complete spectrum of constituents of liquid biopsy application in breast cancer.

2. Spectrum of liquid biopsy

LB encompasses a variety of techniques that analyze circulating tumor components present in blood and other bodily fluids [25]. These components offer valuable insights into the genetic and molecular characteristics of BC, facilitating non-invasive monitoring of the disease. The primary categories of LB include circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes and extracellular vesicles (EV), and circulating cell-free RNA (cfRNA), as illustrated in Fig. 1 [29,30]. Each of these biomarkers presents distinct advantages as well as unique challenges in their clinical application. Wang H et al., and Pandey S et al. discussed valuable insights on how LB is a minimally invasive, real-time tool for detecting tumor-related biomarkers in body fluids, making it more suitable than traditional biopsies for modern cancer treatments. They also shed light on the important details regarding mutation patterns, tumor heterogeneity, and possible therapeutic resistance which are provided by these vital biomarkers. Furthermore, they even demonstrated that LB has shown promise in clinical practice for detecting actionable mutations, directing individualized treatment plans, and determining MRD [25,31].

2.1. Circulating tumor DNA (ctDNA)

2.1.1. Mechanisms and significance

ctDNA refers to small fragments of DNA that are released into the bloodstream by dying tumor cells, whether through apoptosis or necrosis [32]. These fragments carry crucial genetic information that reflects the genomic alterations of the tumor, including point mutations, copy number variations, and chromosomal rearrangements. Though ctDNA constitutes only a minor fraction of the total cell-free DNA (cfDNA) in the bloodstream, its detection allows for a non-invasive method of assessing tumor burden and monitoring disease dynamics in real time [33,34].

The significance of ctDNA in BC management lies in its capacity to capture tumor heterogeneity and track genetic changes over time. In contrast to tissue biopsies, which provide a limited view of a specific tumor region, ctDNA offers a more comprehensive perspective of the tumor's genetic landscape [35]. This is particularly valuable for detecting MRD following treatment, identifying emerging resistance mutations, and guiding the selection of targeted therapies [36,37].

In a study by Magbanua et al., the authors investigated the clinical significance and biological differences in ctDNA shedding in early-stage BC patients undergoing neoadjuvant chemotherapy (NAC) in the I-SPY2 trial. By analyzing ctDNA in hormone receptor (HR)-positive/HER2-negative and triple-negative BC (TNBC) subtypes, the researchers observed higher ctDNA positivity rates in TNBC across all treatment stages. Notably, early ctDNA clearance—measured three weeks after treatment initiation—predicts a favorable NAC response exclusively in TNBC patients. Additionally, ctDNA positivity correlates with reduced distant recurrence-free survival in both subtypes, whereas ctDNA negativity post-NAC is associated with improved prognosis, even in cases with significant residual disease [38]. Wang R et al. investigated the utility of serial ctDNA as a biomarker for monitoring treatment response and predicting residual disease in BC patients undergoing primary systemic therapy (PST). In a cohort of 72 stage II–III BC patients, 208 plasma samples were analyzed at three time points using next-generation sequencing. ctDNA alterations were detected in 51.4 % of patients at baseline, and a greater reduction in ctDNA levels during PST correlated with better treatment response. Complete responders showed a median variant allele fraction (VAF) reduction of −97.4 %, compared to −46.7 % in partial responders and +21.1 % in non-responders ($p = 0.0012$). Early VAF changes predicted tumor response (AUC = 0.7448, $p = 0.02$), and a significant early decrease in ctDNA was associated with longer recurrence-free survival (HR = 12.54, $p = 0.0063$). These findings highlight the potential of ctDNA as a tool for guiding perioperative management in BC [39]. Dickinson et al. conducted a systematic review and meta-analysis to examine the association between ctDNA detection and survival outcomes in metastatic BC (MBC). Analyzing data from 37 studies encompassing 4264 female patients, the study finds a significant correlation between ctDNA presence and worse survival outcomes (hazard ratio: 1.40; 95 % CI, 1.22–1.58). Subgroup analysis highlighted that TP53 and ESR1 alterations were linked to poorer survival, while PIK3CA alterations showed no significant association. Additionally, ctDNA detection via next-generation sequencing and digital PCR correlates with worse survival. The findings suggest that ctDNA may serve as a prognostic biomarker in MBC, providing real-time insights into tumor biology beyond static tissue biopsies [40].

2.1.2. Detection methods

The detection and quantification of ctDNA necessitate highly sensitive and specific techniques due to its low abundance in the bloodstream [41]. The two primary approaches for ctDNA analysis include.

a) Polymerase chain reaction (PCR) Based Methods:

- **Digital Droplet PCR (ddPCR):** This technique partitions a DNA sample into thousands of droplets, allowing for the highly sensitive detection of specific mutations by amplifying target sequences within each droplet [42]. ddPCR is particularly effective for quantifying known mutations, such as those found in the PIK3CA or ESR1 genes, which are significant in BC [43,44]. Kodahl et al. demonstrated that droplet ddPCRbased ctDNA analysis is a sensitive and noninvasive method for detecting PIK3CA mutations in advanced BC, showing 83 % concordance with tumor tissue. Serial ctDNA monitoring correlated mutation levels with treatment response, supporting its potential for guiding PI3K inhibitor therapy and complementing imaging [45]. Li et al. demonstrated that longitudinal ctDNA monitoring effectively detects ESR1 mutations as a biomarker of endocrine resistance in ER + MBC, showing high concordance ($r = 0.96$, $P < 0.0001$) between plasma and tissue samples. ESR1 mutations emerged in 17.8 % of patients post-aromatase inhibitor therapy, with allele frequency changes correlating with acquired resistance. Patients receiving everolimus with endocrine therapy had longer progression-free survival, highlighting ctDNA-based ESR1 monitoring as a valuable tool for guiding treatment strategies in ER + MBC [46]. Sánchez-Martín et al. compared QX200 droplet digital PCR (QX200 ddPCR) and absolute Q plate-based digital PCR (pdPCR) for ctDNA analysis in early-stage BC, finding over 90 % concordance in ctDNA positivity. While both systems showed comparable sensitivity, ddPCR had higher variability and a longer workflow. ctDNA levels were significantly elevated in patients with high Ki67 scores and aggressive BC subtypes, supporting the clinical utility of both dPCR platforms [47].
- **Beads, Emulsion, Amplification, and Magnetics (BEAMing):** BEAMing integrates PCR and flow cytometry to achieve high sensitivity in detecting and quantifying mutations [48,49]. It is commonly utilized to monitor specific mutations in ctDNA, especially in clinical trials evaluating responses to targeted therapies. For the detection and measurement of target DNA copies, it combines flow cytometry and emulsion PCR with magnetic beads [50,51]. Each droplet has a bead covered with thousands of copies of the single DNA molecule following the amplification stage. After that, the beads are magnetically collected and examined using optical scanning or flow cytometry equipment in a matter of minutes. This makes it possible to characterize the DNA variety found in the template population precisely and use it to calculate the percentage of mutant DNA [48,52]. O'Leary et al. compared BEAMing and ddPCR for detecting ESR1 and PIK3CA mutations in ctDNA from advanced BC patients in the PALOMA-3 trial. Both methods showed high concordance ($\kappa = 0.91$ for ESR1, $\kappa = 0.87$ for PIK3CA), with minor discordance (3.9 % for ESR1, 5.0 % for PIK3CA), mainly at allele frequencies <1 %. The study confirms that both techniques are reliable for ctDNA mutation

detection, with sampling variability contributing to discrepancies [53]. Balakrishnan et al. developed a microfluidic platform using superparamagnetic (SPM) beads for efficient extraction and separation of ctDNA from stage I and II cancer patients. Their simulation-based approach achieved a ctDNA yield of 5.7 ng per 10 μL of plasma, with a sensitivity of 65.57 % and specificity of 95.38 %. These findings highlight the potential of microfluidic-based liquid biopsy for early cancer detection and precision medicine [54].

b) Next-Generation Sequencing (NGS)-Based Methods

- **Targeted NGS Panels:** These panels concentrate on a pre-defined set of genes that are relevant to BC, enabling the simultaneous detection of multiple mutations. Targeted NGS is particularly useful for identifying actionable mutations and tracking clonal evolution in metastatic BC [55,56]. Shim H et al. analyzed the genomic profile of ctDNA in BC patients and its clinical implications. Targeted sequencing using the Oncomine Breast cfDNA panel was performed on 38 patients, with whole-exome sequencing on matched tumor DNA (n = 20). Survival analysis and chemotherapy response were evaluated, with validation and serial monitoring of genomic variants in five patients using ddPCR. ctDNA alterations were detected in 82 % of patients, with TP53 (50 %), PIK3CA (15 %), and ESR1 (14 %) as the most common variants, though the concordance rate with matched tumor DNA was only 9.7 % among positives. Patients with TP53 mutations had significantly worse overall survival (HR = 3.90, 95 % CI: 1.10–13.84, P = 0.035), with statistical significance maintained in multivariate analysis. Serial monitoring of somatic variants (PIK3CA, TP53) in ctDNA revealed that changes in allele frequency correlated with chemotherapy response. These findings suggest that ctDNA profiling provides additional genomic insights beyond tumor DNA analysis, and its longitudinal monitoring can aid in prognosis and treatment response evaluation in BC management [57]. Sun et al. explored ctDNA as a biomarker for monitoring and predicting outcomes in MBC using targeted NGS. Plasma samples from 54 patients were analyzed before and after chemotherapy, with paired lymphocytes to exclude clonal hematopoiesis. They identified 1182 nonsynonymous mutations in 419 genes, with higher detection in tumors >3 cm (p = 0.035) and HER2(−) patients (p = 0.029). HER2 status was significantly linked to mutation burden (p = 0.025). Baseline ctDNA showed higher sensitivity and specificity than post-chemotherapy samples, and elevated ctDNA levels correlated with poor survival (p < 0.001), highlighting its potential as a prognostic biomarker [58]. Yoshinami T et al. used molecular barcode NGS (MB-NGS) to detect ctDNA with high sensitivity in early-stage BC. Sequencing 13 frequently mutated genes in stage I/II tumors identified 95 mutations in 62 % of cases. Plasma DNA analysis detected ctDNA in 16.1 % of patients, which correlated with aggressive tumor features and worse distant disease-free survival (P < 0.001). These findings suggest that personalized MB-NGS can serve as a valuable prognostic marker in early BC [59]. Smith et al. developed and validated MammaSeq, a BC-specific NGS panel targeting 79 genes and 1369 mutations for both primary and metastatic cases. Performance evaluation involved sequencing 46 solid tumor and 14 plasma ctDNA samples, achieving high mean depths of 2311 × and 1820 × , respectively. The analysis identified 592 mutations in solid tumors and 43 in ctDNA, with median mutations per sample of 3 and 2.5, respectively. Copy number alterations included 46 amplifications and 35 deletions in solid tumors, while 40 % of solid tumors harbored 26 clinically actionable variants (OncoKB levels 1–3). Furthermore, ESR1 and FOXA1 mutation allele frequencies correlated with CA.27.29 levels in matched blood samples [60]. Wang et al. ctDNA as an early biomarker of therapeutic efficacy and prognosis in 72 patients with stage II–III BC undergoing primary systemic therapy (PST). Using NGS of a 128-gene panel, ctDNA was analyzed at three time points—before treatment, after two cycles, and prior to surgery. The study found that baseline ctDNA positivity was associated with more aggressive tumor features, and a significant early decline in ctDNA levels after two cycles of therapy strongly correlated with achieving pathological complete response (pCR). Additionally, persistent ctDNA or insufficient early decline was linked to a higher risk of disease recurrence [39].
- **Whole-Genome Sequencing (WGS):** WGS provides a comprehensive analysis of the entire tumor genome, facilitating the discovery of novel mutations and structural variants [61,62]. Garcia-Murillas et al. evaluated a WGS-based ctDNA platform (NeXT Personal MRD) for detecting molecular residual disease and predicting relapse in early BC. Analyzing 617 plasma samples from 78 patients, the assay detected ctDNA in 98 % at diagnosis and identified molecular residual disease in all relapse cases, with a median lead time of 15 months. ctDNA positivity correlated with higher relapse risk and lower survival (P < 0.0001). This approach showed superior sensitivity over exome-based molecular residual disease assays, emphasizing its potential for early relapse detection and treatment guidance [63]. Saal LH et al. evaluated a personalized tumor-informed digital PCR (dPCR) assay targeting structural variants (SVs) in ctDNA to monitor MRD and predict relapse in early BC. In this interim analysis of the prospective SCAN-B study, 46 patients underwent WGS-based assay design, with ctDNA detected pre-surgery in 93 % and persisting post-NAT in 24 %, significantly increasing relapse risk (P = 0.002). Postoperative ctDNA detection preceded clinical recurrence by a median of 11.8 months and was linked to worse survival (P < 0.0001). These findings highlight the assay's high sensitivity and potential for early relapse detection and personalized treatment guidance [64]. Table 1 thoroughly summarizes the methods employed for the detection of ctDNA in BC. While WGS is highly informative, it is less commonly employed in routine clinical practice due to its cost and complexity [65].

2.2. Circulating tumor cells (CTCs)

2.2.1. Detection and enumeration

CTCs are integral complete tumor cells that have separated from the primary tumor or metastatic locations and have entered the bloodstream [66,67]. In contrast to ctDNA, which is made up of fragmented DNA, CTCs are intact cells that can be examined for genetic and phenotypic traits [67,68]. The identification and counting of CTCs offer valuable prognostic insights and can help track treatment responses. CTC detection is challenging due to the rarity of these cells in the blood, often occurring at frequencies as low as one CTC per billion blood cells [69–71]. Several technologies have been developed to isolate and enumerate CTCs.

- **CellSearch System:** The CellSearch system is the only FDA-approved method for CTC detection in BC. It uses immunomagnetic separation to isolate CTCs based on the expression of the epithelial cell adhesion molecule (EpCAM). The number of CTCs detected is associated with prognosis; higher CTC counts are linked to poorer outcomes [71]. Riethdorf S et al. validated the CellSearch system for detecting CTCs in metastatic BC across three laboratories. The assay demonstrated high precision, with >95 % of controls within expected ranges and an 80–82 % recovery rate. CTCs were detected in 70 % of patients, with stable sample integrity for 72 h under various conditions. Strong inter-instrument agreement confirmed its reliability, supporting CellSearch as a robust tool for routine clinical assessment of metastatic BC [72]. Dirix et al. compared the CellSearch® immunomagnetic method with a new filtration-based platform for detecting CTCs in metastatic BC. In 60 patients, CTC positivity was 56.7 % with CellSearch® and 66.7 % with the filtration method, showing strong correlation. Both methods demonstrated a significant association between CTC presence and reduced overall survival (p < 0.001). The filtration-based system proved to be a viable alternative, reinforcing CTC enumeration as a valuable prognostic tool for guiding treatment and monitoring

Table 1
Detection methods of circulating tumor DNA (ctDNA) in breast cancer.

S. No.	Method	Principle/Technology	Key Features	Clinical Applications & Insights	Representative Studies
1.	Digital Droplet PCR (ddPCR)	Partitioning DNA into thousands of droplets for mutation-specific amplification	High sensitivity and quantification of known mutations (e.g., PIK3CA, ESR1)	- Detection of PIK3CA mutations with 83 % concordance to tumor tissue - Longitudinal tracking of ESR1 mutations as markers of endocrine resistance - Correlation of ctDNA with aggressive phenotypes and treatment response	Kodahl et al. [45] Li et al. [46] Sánchez-Martín et al. [47]
2.	BEAMing (Beads, Emulsion, Amplification, Magnetics)	Combines emulsion PCR with flow cytometry and magnetic beads for mutation quantification	Ultra-sensitive, can detect low-frequency mutations, suitable for clinical trials	- High concordance with ddPCR for PIK3CA and ESR1 detection - Used in PALOMA-3 trial for response monitoring - Microfluidic-based BEAMing improves ctDNA extraction in early cancers	O'Leary et al. [53] Balakrishnan et al. [54]
3.	Targeted Next-Generation Sequencing (NGS)	Panels targeting breast cancer-specific genes (e.g., TP53, PIK3CA, ESR1)	Enables multiplex mutation detection and clonal evolution analysis	- Detection of multiple somatic variants in 82 % of patients - Poor prognosis linked to TP53 mutations - Higher ctDNA burden in HER2(−) tumors and those >3 cm - ESR1/FOXA1 allele frequencies correlated with tumor burden markers	Shim et al. [57] Sun et al. [58] Yoshinami T et al. [59] Smith et al. [60] Wang et al. [39]
4.	Whole-Genome Sequencing (WGS)	Sequencing the entire genome for comprehensive variant detection	Broad genomic scope, detects novel SNVs, CNAs, SVs, high cost and complexity	- MRD detection with long lead times before clinical relapse - ctDNA positivity pre/post-surgery linked to relapse risk and poor survival - ML-based ctDNA signatures capture phenotypic traits (e.g., proliferation, ER signalling)	Garcia-Murillas et al. [63] Saal LH et al. (2023) [64]

disease progression [73]. Huebner H et al. validated the CellSearch system for detecting CTCs in metastatic BC across three laboratories. The assay showed high precision, with >95 % of controls within expected ranges and an 80–82 % recovery rate. CTCs were detected in ~70 % of patients, with stable counts for 72 h under various conditions. Strong inter-instrument agreement confirmed its reliability, supporting CellSearch as a robust tool for routine clinical assessment of metastatic BC [74].

- **Microfluidic Devices:** These devices capture CTCs by exploiting their physical properties, such as size and deformability, or by using antibody-coated surfaces to bind specific markers on CTCs. Microfluidic technologies offer high sensitivity and can be used to capture viable CTCs for downstream analysis [75–79]. Zhang et al. evaluated a size-based microfluidic chip for detecting CTCs in BC, independent of EpCAM expression. Tested in 129 patients and 50 controls, it showed 73.6 % sensitivity and 82.0 % specificity. CTC counts correlated with TNM stage and metastasis ($P < 0.005$) but not with age or tumor size. The optimal cut-off was 3.5 cells/mL (AUC-ROC = 0.845). Combining CTC detection with tumor markers improved screening. This method offers a sensitive, antibody-independent approach for diagnosis, prognosis, and treatment monitoring [80]. Hassanzadeh-Barforoushi et al. developed a microfluidic sequential trapping array for rapid, label-free isolation of CTCs in BC. The system captured CTCs based on size and deformability, maintaining cell viability. Tested on patient and control samples, it showed high sensitivity and specificity, detecting both single CTCs and clusters. The study concluded that this method enables efficient CTC detection, offering a promising tool for real-time cancer monitoring [81]. Macaraniag et al. developed a microfluidic system for isolating CTCs from small blood volumes in a mouse model of BC. This approach aimed to provide a minimally invasive and efficient method for detecting CTCs, which serve as biomarkers for cancer progression and metastasis. The system utilized size-based filtration to selectively capture CTCs while allowing other blood components to pass through. Performance testing with blood samples from tumor-bearing mice demonstrated high sensitivity and efficiency in isolating CTCs across different tumor progression stages. The method required minimal sample volumes, making it particularly suitable for longitudinal monitoring in preclinical cancer research [82].
- **Immunofluorescence and Flow Cytometry:** These methods use fluorescent antibodies to identify CTCs based on surface markers. They are often employed in research settings to study CTC heterogeneity and to explore their role in metastasis [83–85]. Muchlińska et al. explored the simultaneous detection of CTCs and circulating cancer-associated fibroblasts (cCAFs) in BC patients using imaging flow cytometry (imFC) and multimarker immunofluorescent staining. Analyzing blood samples from 210 patients, the study identified various CTC phenotypes, including epithelial and epithelial-mesenchymal transition (EMT)-related subtypes, with CTCs detected in 27.6 % of cases, particularly in metastatic patients. cCAFs were co-detected with CTCs in 3.3 % of patients and linked to visceral metastases. The findings highlight the potential of imFC in liquid biopsy and emphasize the importance of a multimarker approach for improved cancer monitoring and risk assessment [86]. Wang et al. developed a flow cytometry-based method for detecting CTCs in BC patients by quantifying CK19 expression in peripheral blood. Analyzing 73 samples, including 48 from breast carcinoma or benign tumor patients and 25 from healthy controls, the method demonstrated high sensitivity, detecting a single cancer cell among 10^4 white blood cells. CK19 expression was found in 27 % of BC cases and correlated with disease progression, peaking in stage IV. In chemotherapy-monitored patients, CK19 levels declined post-treatment, suggesting its potential for disease monitoring and therapy assessment [87]. Bansal et al. assessed CTC detection in BC

patients compared to benign breast disease and healthy controls, examining associations with clinicopathological parameters, hormonal profiles, and microRNA polymorphisms. Among 114 BC cases, 108 benign cases, and 182 controls, CTCs were detected in 9.64 % of cancer patients but were absent in non-cancer groups. CTC positivity correlated with tumor size, grade, histologic type, metastasis, and skin infiltration but not with immunohistochemical profiles or microRNA polymorphisms. However, further research with larger sample sizes is needed to validate these findings [88]. Liu et al. evaluated CTC detection in BC patients using multiparameter flow cytometry (FCM) and assessed its clinical relevance. CTCs were identified in 53.2 % of patients but not in healthy controls. Chemotherapy reduced CTC positivity from 72.7 % to 30.3 % after two cycles ($P < 0.05$). CTC rates correlated with TNM stage, Ki-67, and HER-2 status but not with ER/PR expression. HER-2-amplified and triple-negative subtypes had the highest CTC positivity. The study concluded that ultra-high-speed FCM is a sensitive method for CTC detection, aiding micrometastatic risk assessment in BC subtypes [89]. Hu et al. evaluated multiparameter flow cytometry for CTC detection in BC and its prognostic significance for overall survival (OS). This method showed higher specificity than RT-PCR, with a sensitivity limit of 10^{-5}. Among 45 patients, those with CTCs $\geq$5 had significantly shorter OS (65.5 vs. 95 weeks, $P < 0.05$). Kaplan-Meier and Cox regression analyses confirmed CTC count, metastasis, and age as key OS predictors [90]. Table 2 provides an all-encompassing summary, shedding light on the key detection methods of CTCs in BC.

2.2.2. Clinical relevance in BC

In cases of BC, CTCs are clinically significant [91,92], disease stage, response to treatment, and overall prognosis are all correlated with their blood levels. Treatment modifications for metastatic BC may be guided by changes in the CTC count, which can act as an early signal of therapeutic efficacy or resistance. Furthermore, the molecular characterization of CTCs can reveal information about the biology of tumors and assist in locating targets for individualized therapy [93,94]. For instance, HER2-targeted treatments may be useful in treating patients who were previously categorized as HER2-negative since the expression of HER2 on CTCs may differ from that of the original tumor [95]. In a comprehensive review, Nicolò E et al. discussed that the HER2 is a critical biomarker in BC, influencing therapeutic decisions. Currently, HER2 status is evaluated through immunohistochemistry and in situ hybridization on tissue biopsies. However, given the challenges associated with tissue sampling, there is a growing need for a non-invasive, real-time method to assess HER2 status. CTCs have emerged as promising biomarkers for this purpose, allowing HER2 evaluation at genomic, transcriptomic, and protein levels on both bulk and single-cell analyses. A major limitation in current research is the lack of a standardized definition of HER2-positive CTCs, complicating both clinical and investigative applications. Studies have reported discrepancies between the HER2 status of primary tumors and corresponding CTCs, with some HER2-negative BC patients exhibiting HER2-positive CTCs and vice versa. These findings have led to investigations into the prognostic and predictive value of HER2 expression in CTCs in both early and metastatic BC, potentially expanding the use of anti-HER2 therapies to additional patient groups and providing insights into treatment resistance mechanisms [96].

2.3. Extracellular vesicles (EVs) and exosomes

2.3.1. Role in cancer biology

Extracellular vesicles (EVs), including exosomes and microvesicles, have gained significant attention as minimally invasive biomarkers in LB for BC [97,98]. BC-derived EVs contain tumor-specific markers such as EpCAM, HER2, and MUC1, along with oncogenic microRNAs (e.g., miR-1246, miR-21, and miR-373) that contribute to tumor progression and drug resistance [99,100]. The application of advanced detection techniques, including flow cytometry, nanoparticle tracking analysis, and microfluidic platforms, has improved EV isolation and characterization, enhancing diagnostic accuracy [101–103]. Exosomes are small, membrane-bound extracellular vesicles (30–150 nm in diameter) secreted by various cell types, including cancer cells. They carry a cargo of proteins, lipids, and nucleic acids, including DNA, RNA, and microRNAs, which can modulate the tumor microenvironment and facilitate metastasis. Exosomes are actively involved in cell-to-cell communication, transferring oncogenic signals that promote tumor growth, angiogenesis, and immune evasion [97,98]. In BC, exosomes have been implicated in processes such as drug resistance by transferring drug efflux pumps or resistance-related microRNAs between cells. They also play a role in the establishment of pre-metastatic niches, where they prepare distant sites for tumor cell colonization [101–103].

Table 2
Detection methods of circulating tumor cells (CTCs) in breast cancer.

S. No.	Method	Principle	Advantages	Limitations	Key Study Insights
1.	**CellSearch System** (FDA-approved)	Immunomagnetic capture using anti-EpCAM antibodies and cytokeratin staining	Clinically validated, reproducible, FDA-approved, prognostic relevance	Limited to EpCAM + cells, may miss mesenchymal CTCs	Riethdorf et al.: >95 % precision, 70 % detection rate, stable for 72h. [72], Dirix et al. - Significant prognostic correlation, filtration method slightly outperformed CellSearch [73]. Huebner et al.: Consistent recovery rates (~80 %) across labs [74].
2.	**Microfluidic Devices**	CTC isolation via size, deformability, or surface marker binding	Label-free options, preserves cell viability, suitable for downstream analysis	Device-specific variability, limited scalability	Zhang et al.: 73.6 % sensitivity, AUC-ROC = 0.845, correlated with TNM stage [80], Hassanzadeh-Barforoushi et al.: High sensitivity/specificity with viable capture [81]. Macaraniag et al.: Effective in mouse models, enabling longitudinal tracking [82].
3.	**Immunofluorescence**	Antibody-based staining of CTCs using epithelial and/or mesenchymal markers	Allows phenotypic profiling, identifies EMT-related CTCs	Labor-intensive, lacks standardization	Muchlińska et al.: Detected EMT-related CTCs and cancer-associated fibroblasts, CTCs in 27.6 % of patients, mostly metastatic [86].
4.	**Flow Cytometry**	Fluorescent-labelled antibodies (e.g., CK19, EpCAM) identify and quantify CTCs in blood	High throughput, quantifiable, useful for serial monitoring	May miss low-abundance CTCs, requires cell-specific markers	Wang et al.: CK19+ CTCs detected in 27 %, correlated with disease stage [87], Liu et al.: 53.2 % detection, dropped post-chemo, associated with TNM and HER2 status [89], Hu et al.: CTC ≥5 predicted poor OS, validated by Cox regression [81], Bansal et al. - 9.64 % detection in BC only, no signal in controls [90].

2.3.2. Potential as biomarkers

Exosomes hold promise as biomarkers for BC due to their stability in bodily fluids and their ability to reflect the molecular composition of the tumor [104]. Their contents can be analyzed to detect specific mutations, gene expression profiles, or protein signatures associated with BC. Exosomes can be isolated from blood, urine, or other body fluids using ultracentrifugation, immunoaffinity capture, or size-exclusion chromatography [105–107].

Several studies have explored the use of exosomal microRNAs and proteins as diagnostic and prognostic biomarkers in BC [108,109]. For instance, the detection of specific microRNAs (e.g., miR-21, miR-1246) in exosomes has been associated with poor prognosis and resistance to chemotherapy [110–112]. Li et al. validated exosomal miR-1246 as a potential serum biomarker for BC and its role in tumor progression. miR-1246 was highly expressed in metastatic BC cells and transferred via exosomes, promoting tumor survival, migration, and chemotherapy resistance by suppressing the tumor suppressor CCNG2. Exosomes from metastatic cells enhanced the invasiveness of non-malignant cells. The study highlights miR-1246 as a promising biomarker for early detection and a potential target for miRNA-based therapies [113]. Ongoing research is focused on developing exosome-based lLBs that can provide non-invasive, real-time insights into tumor biology and guide personalized treatment strategies. Jia et al. developed a machine learning-based exosomal RNA profiling platform for multi-cancer detection and localization. In a multi-center study, RNA from plasma-derived exosomes was analyzed across 818 participants, identifying 12 exosomal tumor RNA signatures (ETR.sig). A Random Forest model demonstrated high accuracy (AUC = 0.915) in distinguishing cancer cases from controls, with robust performance in classifying eight cancer types (AUC >0.85). Integration with tissue RNA sequencing and clinical data reinforced biomarker relevance [114]. Wang et al. developed a novel microfluidic platform for rapid and efficient exosome capture and enrichment, overcoming limitations of existing exosome-based diagnostics. Using antibody-conjugated microbeads, this method enhances sensitivity, enables multi-biomarker detection, and requires only 50 μL of plasma with a 35-min processing time. In BC patients, EpCAM- and MUC1-positive exosomes achieved AUCs of 0.98 and 0.99, respectively, with multi-biomarker integration reaching an AUC of 1.0. This platform offers a highly accurate and efficient approach for exosome-based LB [115]. Xu et al. in their multicenter cohort study analyzed tumor-derived EVs using a novel detection method based on dual DNA tetrahedral nanostructures. A total of 512 BC patients and 198 nonneoplastic individuals were recruited to assess the diagnostic and prognostic value of EV levels. The study found that tumor-derived EV levels were significantly elevated in newly diagnosed BC patients compared to nonneoplastic individuals, with a diagnostic cutoff value of 3.58 U/μL. Additionally, for metastasis monitoring, BC patients with metastases exhibited significantly higher EV levels than those without, with a threshold of 3.91 U/μL. This biomarker demonstrated superior efficacy in both diagnosis and metastasis surveillance compared to traditional tumor markers [116]. Bandini E et al. evaluated the diagnostic and prognostic potential of extracellular vesicle (EV)-based biomarkers in BC, emphasizing their utility in early detection and disease monitoring. The study found significantly elevated tumor-derived EV levels in BC patients compared to non-neoplastic individuals, with a diagnostic cutoff of 3.58 U/μL. EV levels were also higher in metastatic patients, with a metastasis monitoring threshold of 3.91 U/μL. EV-based biomarkers demonstrated superior diagnostic accuracy and metastasis prediction compared to traditional tumor markers. Using advanced EV isolation and molecular profiling techniques, the study underscores the clinical relevance of EVs as a non-invasive, highly specific tool for BC detection. These findings advocate for the integration of EV-based LB strategies into clinical practice for improved and personalized cancer management [117].

2.4. Circulating cell-free RNA (cfRNA)

2.4.1. Emerging applications

cfRNA includes messenger RNA (mRNA), microRNA (miRNA), and long non-coding RNA (lncRNA) released into the bloodstream by tumor cells [118]. Unlike ctDNA, which provides genomic information, cfRNA reflects the active gene expression profile of the tumor, offering a dynamic view of the tumor's functional state [118,119]. Emerging applications of cfRNA in BC include its use as a diagnostic tool, particularly for detecting early-stage disease. cfRNA can also be used to monitor treatment response by tracking changes in the expression of specific genes associated with therapeutic targets or resistance mechanisms [118–120]. For example, detecting cfRNA transcripts encoding HER2 or ER can provide insights into the tumor's receptor status, guiding decisions on targeted therapies [121]. Larson et al. performed the first transcriptome-wide characterization of cfRNA in stage III breast (n = 46) and lung (n = 30) cancer patients, as well as non-cancer participants (n = 89), using data from the Circulating Cell-free Genome Atlas (NCT02889978). Analysis revealed that 68 % (39,564 out of 57,820) of annotated genes were undetectable in cfRNA from non-cancer individuals. Within these low-noise regions, the study identified tissue- and cancer-specific genes, termed "dark channel biomarker" (DCB) genes, which were consistently detected in cancer patients. DCB levels in plasma correlated with tumor shedding rates and RNA expression in matched tumor tissue, suggesting that highly expressed DCBs in tumors could improve cancer detection, particularly in patients with low levels of circulating tumor DNA. Overall, the findings demonstrated that cfRNA could serve as a valuable tool for cancer detection, tumor tissue-of-origin prediction, and cancer subtype classification [122]. Schwarzenbach et al. explored the potential of nucleic acid quantification and genetic alterations in cell-free DNA as minimally invasive tools for BC screening. The study analyzed preoperative serum samples from 102 BC patients, 32 individuals with benign breast disease, and 53 healthy controls, with a mean follow-up of 6.2 years for cancer patients. Serum DNA and RNA levels were quantified, and loss of heterozygosity (LOH) at four polymorphic markers (D13S159, D13S280, D13S282 at 13q31-33, and D10S1765 at PTEN region 10q23.31) was assessed. DNA ($p = 0.016$) and RNA ($p = 0.001$) levels distinguished cancer patients from healthy individuals but did not differentiate malignant from benign lesions. Elevated serum DNA levels correlated with poorer overall ($p = 0.021$) and disease-free survival ($p = 0.025$), while LOH at all analyzed markers was associated with lymph node metastasis ($p = 0.026$). Additionally, LOH at D13S280 ($p = 0.047$) and D13S159 ($p = 0.046$) was linked to overall and disease-free survival, respectively. These findings support the diagnostic and prognostic value of cell-free tumor DNA in BC, with LOH at 13q31-33 potentially indicating lymphatic tumor cell dissemination [123]. Lasham et al. evaluated the prognostic value of circulating RNAs and a protein biomarker in BC patients as complementary tools to existing clinical tests. Microarray profiling of plasma samples from 30 BC patients and 10 controls identified small noncoding RNAs, including microRNA-923 (miR-923). In an expanded cohort of 253 BC patients, miR-923 levels were quantified using ddPCR, alongside cancer antigen (CA) 15-3 protein measurements. Cox regression survival analysis demonstrated that both miR-923 and CA 15-3 levels at surgery were independently associated with patient prognosis ($P = 3.9 \times 10^{-3}$ and $P = 1.9 \times 10^{-9}$, respectively). Integrating these biomarkers with standard clinicopathological features significantly improved recurrence prediction (AUC at 3 years: 0.858 vs. 0.770; $P = 0.017$) [124]. Nguyen et al. highlighted the potential of plasma cfRNA as a biomarker for early BC detection, addressing the limitations of ctDNA, which has low sensitivity due to its low fraction and molecular heterogeneity. Unlike ctDNA, cfRNA—including cell-free mRNA (cfmRNA)—captures transcriptomic alterations from both tumor cells and the tumor microenvironment. The study conducted transcriptomic profiling of cfmRNAs in 24 BC patients and 33 healthy individuals using next-generation sequencing. Differential expression analysis identified

10,955 differentially expressed cfmRNAs (DEMs), including established BC markers such as LAMP3, HSD11B1, PRTG, and LPL. Pathway enrichment analysis revealed 49 significantly enriched pathways, with immune-related DEMs (CD3D, CD8B, CD274, CTLA4, FOXP3, IL2RA) linked to tumor-infiltrating lymphocytes, emphasizing their role in immune interactions. The combination of tumor-specific and immune-related DEMs effectively distinguished BC patients from healthy individuals. These findings underscore the clinical utility of cfmRNAs as a minimally invasive biomarker for detecting BC cases that shed low amounts of ctDNA [125].

2.4.2. Technical challenges

The analysis of cfRNA presents several technical challenges that must be addressed to harness its full potential as a biomarker [126,127]. One major challenge is its stability, as cfRNA is inherently less stable than DNA in circulation and is highly susceptible to degradation by ribonucleases (RNases). This necessitates meticulous sample handling and processing to preserve RNA integrity and ensure reliable [126,127]. Sensitivity is another critical issue, given the low abundance of cfRNA in the bloodstream [128]. Detecting such minute quantities requires highly sensitive techniques, including reverse transcription quantitative PCR (RT-qPCR) and digital PCR, both of which must be carefully optimized to minimize the risk of false negatives. Additionally, a significant obstacle to cfRNA research is the lack of standardized protocols for its extraction, quantification, and analysis. Variability in methodologies across studies can lead to inconsistencies, reducing the reproducibility and comparability of findings [119,129]. Despite these technical hurdles, cfRNA holds great promise as a biomarker for BC, particularly in the realm of personalized medicine. As detection technologies continue to advance and efforts toward standardization progress, cfRNA is poised to become an essential component of LB-based strategies for BC detection, prognosis, and treatment monitoring [129,130].

Table 3 provides a holistic overview of the various techniques of liquid biopsy and their clinical implementation in BC.

3. Applications of liquid biopsy in BC

LB is a powerful tool that offers a range of applications in BC management [131,132]. Its non-invasive nature and ability to provide real-time insights into tumor biology make it an attractive alternative to traditional tissue biopsies. Here, we explore the key applications of LB in BC, including early detection and screening, prognostic assessment, monitoring treatment response, detecting minimal residual disease, and identifying resistance mechanisms [133–135].

3.1. Early detection and screening

3.1.1. Potential role in high-risk populations

Early detection of BC is crucial for improving survival rates, particularly in high-risk populations such as those with a family history of the disease, genetic predispositions (e.g., BRCA1/2 mutations), or prior personal history of BC [136]. LB, particularly through the analysis of ctDNA and CTCs, has shown potential for detecting BC at an early stage, even before clinical symptoms or abnormalities are visible through imaging [137,138]. Research suggests that ctDNA levels can be elevated in the early stages of BC, making it a potential biomarker for early detection. In high-risk populations, regular LB testing could complement existing screening methods, offering an additional layer of monitoring and potentially catching tumors at a more treatable stage [139,140]. Cailleux et al. investigated the potential of ctDNA as a relapse indicator in early-stage BC patients following neoadjuvant chemotherapy. Using a tumor-informed next-generation sequencing assay, they analyzed plasma samples from 44 patients at multiple time points. Baseline ctDNA was detected in 58 % of cases and was associated with high Ki67 levels and MYC copy-number gain. However, ctDNA detection rates dropped to 5 % at presurgery and last follow-up. Notably, ctDNA presence at these later stages was strongly linked to shorter event-free survival (EFS), with high hazard ratios, whereas baseline ctDNA detection did not significantly predict EFS. These findings highlight the potential of ctDNA monitoring for early relapse detection, warranting further research in interventional trials [141].

3.1.2. Comparison with traditional screening methods (mammography)

Mammography remains the gold standard for BC screening, particularly in asymptomatic women [142]. However, it has notable limitations, including false positives, false negatives, and challenges in detecting certain aggressive or molecular subtypes of BC that may not be readily visible on imaging [142,143]. In contrast, LB presents several advantages that could complement or enhance current screening methods [31,144]. One key benefit is its non-invasive nature, as it requires only a blood draw, eliminating the need for radiation exposure associated with mammography. Additionally, LB has the potential for early detection by identifying cancer at a molecular level before it becomes radiologically apparent. Another significant advantage is its ability to provide real-time insights into tumor biology, allowing for the monitoring of tumor dynamics and potential changes in its molecular profile before they become detectable through imaging [31,144]. These advantages highlight the promise of LB as a complementary tool in BC screening and management. Studies are ongoing to evaluate the

Table 3
An integrative table encompassing the various techniques of liquid biopsy and their clinical implementation in breast cancer.

Liquid Biopsy Component	Technique	Clinical Applications in Breast Cancer	Clinical Status
Circulating Tumor DNA (ctDNA)	- Droplet Digital PCR (ddPCR) - Next-Generation Sequencing (NGS)	- Detection of actionable mutations (*PIK3CA*, *ESR1*, *BRCA1/2*) - Monitoring treatment response - Detection of minimal residual disease (MRD) - Early relapse prediction	- FDA-approved for *PIK3CA* mutation detection (e.g., FoundationOne® Liquid CDx) - MRD detection under clinical investigation (e.g., Signatera™)
Circulating Tumor Cells (CTCs)	- CellSearch® system - Microfluidic capture platforms	- Prognostic marker in metastatic breast cancer - Research into receptor profiling (ER/PR/HER2) and resistance mechanisms - Potential predictive biomarker in trials	- FDA-approved for CTC enumeration (prognostic only) - Molecular characterization remains investigational
Extracellular Vesicles (EVs) (including Microvesicles)	- Ultracentrifugation - Immunoaffinity capture	- Potential early detection biomarker - Insight into tumor progression and metastasis - Carriage of DNA, RNA, and proteins reflecting tumor status	- Research-stage; not yet clinically approved
Exosomes (specific subtype of EVs)	- Isolation via ultracentrifugation, size-exclusion chromatography, or immunoprecipitation	- Potential biomarkers for diagnosis, prognosis, and therapy resistance - Study of exosomal miRNA, lncRNA, and proteins for therapeutic monitoring	- Under preclinical and early clinical research; not yet standard of care
Circulating Cell-Free RNA (cfRNA)	- RT-qPCR - RNA sequencing	- Analysis of tumor-derived mRNAs and non-coding RNAs (e.g., miRNA, lncRNA) - Potential markers of early relapse, resistance	- Research phase; limited clinical validation

effectiveness of LB in routine screening and its potential to complement or even replace mammography in certain high-risk populations. For now, it is used as an adjunct tool rather than a replacement for traditional screening methods [28,145,146]. Freitas et al. investigated a spectrochemical approach combined with multivariate classification techniques as a bio-analytical tool for BC screening via liquid biopsy. Using attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy, they analyzed plasma samples from 476 patients over two years, capturing biochemical signatures of nucleic acids, carbohydrates, lipids, and proteins. The method demonstrated high diagnostic accuracy, with 94 % sensitivity and 91 % specificity, comparable to traditional mammography. Additionally, it offered advantages such as improved prognosis, enhanced treatment effectiveness, reduced false positives and negatives, lower costs, and higher analytical throughput. These findings highlight the potential of spectrochemical LB as a promising screening tool for early BC detection, warranting further clinical validation [147].

3.2. Prognostic biomarkers

3.2.1. Use of liquid biopsy for predicting disease progression

Prognostic biomarkers are crucial for predicting the likely course of the disease and informing treatment decisions. LB provides valuable information on the tumor's genetic and molecular profile, which can be used to assess disease prognosis [7,25]. ctDNA levels and the presence of CTCs can offer insights into tumor burden, metastatic potential, and overall disease progression. Elevated ctDNA levels have been associated with advanced disease stages and poorer outcomes. Similarly, the presence and quantity of CTCs in the blood correlate with disease progression and metastasis. By monitoring these biomarkers, clinicians can gain a better understanding of the disease trajectory and adjust treatment strategies accordingly [31,37,148]. Visvanathan et al. assessed the clinical utility of a novel LB-based BC methylation (LBx-BCM) assay for the early detection of disease progression in metastatic BC (MBC). This prototype assay, utilizing a 9-marker methylation panel on the GeneXpert cartridge system, was evaluated in the TBCRC 005 prospective biomarker study. Plasma samples from 144 MBC patients were collected at baseline, week 4, and week 8. At week 4, patients with high cumulative methylation (CM) levels had significantly shorter median progression-free survival (PFS) (2.88 months vs. 6.60 months, $P = 0.001$) and overall survival (OS) (14.52 months vs. 22.44 months, $P = 0.005$) compared to those with low CM. In multivariable analysis, high CM remained associated with shorter PFS (HR, 1.90; 95 % CI, 1.20–3.01; $P = 0.006$). Additionally, an increase in CM from baseline to week 4 (OR, 4.60; 95 % CI, 1.77–11.93; $P = 0.002$) and high CM at week 4 (OR, 2.78; 95 % CI, 1.29–5.99; $P = 0.009$) were predictive of progressive disease at the first restaging. A risk model developed using week 4 CM levels successfully predicted disease progression within three months of treatment initiation. These findings indicated that the automated LBx-BCM assay could serve as a valuable tool for monitoring early disease progression in MBC patients undergoing routine treatment. Further validation is necessary to establish its clinical applicability across different therapeutic settings [149].

3.2.2. ctDNA and CTCs as prognostic markers

- **ctDNA:** ctDNA can reflect the tumor's genetic mutations and copy number variations. Higher levels of ctDNA have been linked to more aggressive disease and worse prognosis. Tracking ctDNA over time can provide insights into disease progression, response to therapy, and risk of relapse [150,151]. Guo et al. conducted a systematic review assessing the prognostic value of ctDNA in BC throughout the treatment cycle. Analyzing 30 studies published between 2016 and 2022, they found that baseline ctDNA positivity was associated with a lower objective response rate (ORR) and that ctDNA detected during neoadjuvant therapy correlated with reduced pathological complete response (pCR) rates. Additionally, ctDNA presence after surgery was significantly linked to shorter relapse-free survival (RFS) and a higher relapse risk. Both pre-operative and post-operative ctDNA levels were predictive of overall survival (OS), with post-operative ctDNA showing a particularly strong association. These findings highlight ctDNA as a promising prognostic biomarker for monitoring treatment response and disease progression in BC. However, further standardization and validation are needed to ensure its clinical applicability [152]. Papakonstantinou et al. conducted a systematic review and meta-analysis to evaluate the prognostic value of ctDNA in early BC patients undergoing neoadjuvant therapy (NAT). Analyzing 11 eligible studies from a pool of 2,908, they found that ctDNA detection at both baseline and post-NAT was significantly associated with worse relapse-free survival (RFS) and overall survival (OS). Specifically, post-NAT ctDNA presence had a strong correlation with poor outcomes, with hazard ratios of 5.67 for RFS and 4.00 for OS. However, ctDNA detection did not predict the likelihood of achieving a pathological complete response (pCR). These findings suggest that ctDNA assessment during NAT could serve as a risk stratification tool, highlighting the need for further prospective studies to optimize treatment individualization for EBC patients [153].
- **CTCs:** The enumeration and characterization of CTCs offer prognostic information. A higher number of CTCs correlates with a greater risk of disease progression and poor prognosis. Changes in CTC levels during treatment can indicate response or resistance, guiding therapeutic decisions [154,155]. Moussavi et al. reviewed the evidence on CTCs as markers of disease progression in metastatic BC. Using immunohistochemistry-based isolation techniques, particularly the FDA-approved CellSearch® system, they analyzed clinical studies assessing the prognostic and predictive value of CTC enumeration. The review found that CTC-positive patients had significantly shorter overall survival (OS) and, in many cases, lower progression-free survival (PFS) compared to CTC-negative patients. While the findings supported the prognostic utility of CTCs, further research was needed to establish their role in guiding treatment decisions. Ongoing clinical trials were exploring the potential of CTC enumeration for therapeutic stratification, highlighting the need for continued investigation to define its clinical relevance [156]. Pierga et al. evaluated the prognostic significance of CTCs compared to serum tumor markers in metastatic BC patients undergoing first-line chemotherapy with or without targeted therapy. Using the CellSearch® system, CTCs were enumerated at baseline, before cycle 2 (C2), and at cycle 3 or 4 (C3/4) in 267 patients. Baseline CTC detection rates were 65 % at $\geq$1 CTC/7.5 ml and 44 % at $\geq$5 CTC/7.5 ml, independent of BC subtypes. CTC presence correlated with tumor burden, bone/liver involvement, and performance status. A threshold of $\geq$1 CTC/7.5 ml was strongly prognostic for progression-free survival (PFS), while $\geq$5 CTCs/7.5 ml was significantly associated with both PFS and overall survival (OS). Among patients with $\geq$5 CTCs/7.5 ml at baseline, 50 % showed a reduction to <5 CTCs/7.5 ml at C2, correlating with improved survival outcomes. Notably, all patients receiving anti-HER2 therapy had <5 CTCs/7.5 ml after three treatment cycles. This study, the largest prospective validation of CTCs as independent prognostic markers, demonstrated that early CTC enumeration could predict poor PFS and OS, supporting its potential use in monitoring treatment response [157].

3.3. Predictive biomarkers

The identification of targetable biomarkers in breast cancer has markedly influenced the development of precision therapies. Established biomarkers such as ER, PR, and HER2 are routinely evaluated to stratify patients for endocrine therapy, HER2-targeted agents (e.g., trastuzumab, pertuzumab), or chemotherapy [158]. In addition, somatic

mutations in PIK3CA and alterations in BRCA1/2 genes have emerged as important therapeutic targets, with agents like alpelisib (for PIK3CA-mutated tumors) and PARP inhibitors (for germline BRCA-mutated cancers) showing clinical benefit [159–161]. Other biomarkers, including PD-L1 expression in TNBC and androgen receptor (AR) expression, are under active investigation for their potential to guide immunotherapy and anti-androgen strategies, respectively [162, 163]. The ability to therapeutically target these molecular alterations has not only improved progression-free survival in specific patient subsets but has also reiterated the need for dynamic biomarker evaluation throughout disease evolution [22].

Liquid biopsy approaches, including the analysis of ctDNA and CTCs, provide non-invasive means to assess these biomarkers in real-time [164]. Techniques such as (ddPCR) and NGS enable sensitive detection of actionable mutations like PIK3CA, ESR1 (associated with endocrine resistance), and copy number variations such as HER2 amplification in ctDNA. Similarly, molecular characterization of CTCs can reveal receptor status (ER, PR, HER2) and immune checkpoint marker expression (e.g., PD-L1), offering insights into tumor heterogeneity and therapeutic resistance [164,165]. Clinically, the use of LB facilitates early detection of minimal residual disease, monitoring of treatment response, and identification of emerging resistance mechanisms, particularly in metastatic breast cancer where repeat tissue biopsies are often impractical. As evidence supporting the clinical utility of LB grows, its integration into routine practice is expected to enhance precision oncology and improve patient outcomes [166,167].

3.4. Monitoring treatment response

3.4.1. Real-time assessment of treatment efficacy

One of the significant advantages of LB is its ability to provide real-time assessments of treatment efficacy. By analyzing ctDNA and CTC levels, clinicians can monitor how well a treatment is working and make timely adjustments if needed [168]. For example, a decrease in ctDNA levels often correlates with a positive response to treatment, while stable or increasing levels may suggest treatment resistance or disease progression. Similarly, changes in the number of CTCs can provide early indicators of how well a therapy is working, potentially allowing for quicker modifications to the treatment regimen [27,169,170].

3.5. Detection of minimal residual disease (MRD)

3.5.1. Role of liquid biopsy in detecting MRD post-treatment

Minimal residual disease (MRD) refers to the small number of cancer cells that remain after treatment, which can lead to relapse [171]. Liquid biopsy has shown promise in detecting MRD by identifying residual ctDNA or CTCs that may not be detectable through imaging alone. This capability allows for earlier intervention and closer monitoring in the post-treatment phase [172].

For example, studies have demonstrated that the presence of ctDNA after initial treatment is associated with a higher risk of relapse, indicating the potential of liquid biopsy to guide subsequent treatment decisions and follow-up strategies [172,173]. Stergiopoulou et al. conducted a long-term follow-up study on operable BC patients using comprehensive LB analysis to assess MRD, metastasis biology, and therapy resistance. Peripheral blood samples from 13 early-stage BC patients were analyzed over ten years using multiple LB techniques, including CTC enumeration, phenotypic characterization, gene expression analysis, mutation profiling, and DNA methylation assessment. Among the patients, 77 % remained LB-negative throughout follow-up and did not relapse, whereas 23 % who tested positive for at least one LB marker experienced relapse. Molecular characteristics of CTCs varied over time and increased before clinical relapse, with LB markers detecting MRD up to four years before metastasis became clinically evident. These findings underscore the potential of LB for early relapse detection and personalized treatment, reinforcing its clinical value in long-term BC monitoring [174].

3.5.2. Impact on clinical decision-making

The ability to detect MRD using LB can significantly influence clinical decision-making by enabling early intervention, tailored monitoring, and personalized treatment plans [172]. Detecting MRD before clinical relapse allows for timely therapeutic interventions, potentially improving patient outcomes by preventing disease progression [175]. Additionally, patients identified as high-risk for relapse can be monitored more closely, ensuring immediate action if signs of recurrence emerge. Furthermore, MRD detection aids in refining treatment strategies, allowing clinicians to adjust therapies based on an individual's relapse risk, ultimately leading to a more personalized and effective approach to BC management [176,177].

3.6. Identification of resistance mechanisms

3.6.1. Liquid biopsy for identifying emerging resistance mutations

One of the most valuable applications of LB is its ability to identify resistance mutations that emerge during treatment [25,178]. Tumors can evolve and develop new mutations that confer resistance to current therapies. LB enables the detection of these mutations in ctDNA, providing insights into why a treatment may no longer be effective [179]. For instance, mutations in the ESR1 gene can develop in hormone receptor-positive BC, leading to resistance to endocrine therapies. Detection of such mutations through LB allows for timely adjustments in treatment strategies, such as switching to alternative therapies or combining treatments to overcome resistance [180,181]. Sandbothe et al. conducted a study to evaluate the utility of circulating cfDNA analysis from plasma samples of metastatic BC patients to guide therapy. Using a NGS assay integrated into routine molecular diagnostics, they targeted four key genes (ESR1, PIK3CA, ERBB2, and TP53) associated with therapy resistance and prognosis. Among 162 liquid biopsy samples and 25 paired metastatic tissue samples, ESR1 mutations were found in 25.9 % of cases, ERBB2 mutations in 3.7 %, and TP53 mutations in 17 %, informing potential shifts to treatments like fulvestrant, elacestrant, or neratinib. The study confirmed that liquid biopsy is a sensitive and non-invasive tool for detecting resistance mutations, thereby aiding in personalized treatment strategies and potentially improving outcomes in metastatic BC patients [182]. The ability to detect MRD using liquid biopsy can significantly influence clinical decision-making by enabling early intervention, tailored monitoring, and personalized treatment plans. Detecting MRD before clinical relapse allows for timely therapeutic interventions, potentially improving patient outcomes by preventing disease progression. Additionally, patients identified as high-risk for relapse can be monitored more closely, ensuring immediate action if signs of recurrence emerge. Furthermore, MRD detection aids in refining treatment strategies, allowing clinicians to adjust therapies based on an individual's relapse risk, ultimately leading to a more personalized and effective approach to BC management [183,184].

3.6.2. Implications for targeted therapy adjustments

Identifying resistance mechanisms plays a crucial role in optimizing targeted therapy by enabling early detection of resistance mutations, facilitating personalized treatment adjustments, and ultimately improving patient outcomes [185,186]. Detecting resistance mutations at an early stage allows clinicians to modify treatment strategies before disease progression occurs, potentially enhancing therapeutic efficacy. Additionally, understanding specific resistance mechanisms enables a more tailored approach, allowing for the use of alternative or combination therapies suited to the patient's evolving tumor profile. By making timely adjustments based on resistance detection, clinicians can achieve better disease management, leading to improved survival and overall patient outcomes [187,188].

4. Technological advances and challenges

The field of LB is rapidly evolving, driven by advancements in detection technologies and a growing understanding of its clinical applications. However, several challenges remain that must be addressed to fully integrate LB into routine clinical practice [28,148]. This section explores recent technological advancements, challenges in standardization, and economic considerations related to the widespread adoption of LB.

4.1. Advancements in detection technologies

4.1.1. Improved sensitivity and specificity

Recent technological advancements have significantly enhanced the sensitivity and specificity of LB assays. Key developments include.

- **Digital Droplet PCR (ddPCR):** ddPCR has emerged as a highly sensitive technique for detecting low-abundance ctDNA mutations. By partitioning a sample into thousands of droplets, ddPCR allows for precise quantification of rare mutations with high sensitivity and specificity. This advancement is particularly valuable for monitoring MRD and detecting early signs of relapse [189,190].
- **Enhanced Next-Generation Sequencing (NGS):** NGS technologies have greatly improved the ability to detect a wide range of genetic alterations in ctDNA, including single nucleotide variations, insertions and deletions, and copy number variations. Recent advancements in NGS have led to increased sensitivity for detecting low-frequency mutations and greater depth of coverage, allowing for more comprehensive tumor profiling [191,192].
- **Microfluidic Technologies:** Microfluidic devices have advanced the isolation and analysis of CTCs and exosomes from blood samples. These devices use micro-scale channels and automated processes to capture and analyze rare cells and extracellular vesicles with high efficiency and sensitivity. Innovations in microfluidics have led to improvements in the accuracy and speed of CTC detection and characterization [193,194].
- **Integrated Assays:** New integrated assays combine multiple LB components, such as ctDNA, CTCs, and exosomes, to provide a more comprehensive assessment of tumor biology. These multi-analyte approaches offer a more holistic view of the disease and enhance the ability to monitor treatment response and detect resistance mechanisms [195,196].

4.1.2. Integration with next-generation sequencing (NGS)

NGS has transformed liquid biopsy by enabling the simultaneous analysis of multiple genetic targets from ctDNA [197]. Its integration with LB technologies allows for comprehensive mutation profiling, facilitating the detection of a wide range of genetic alterations, including both established driver mutations and novel variants. This broad profiling supports personalized treatment strategies and the identification of potential therapeutic targets [198]. Additionally, NGS enables the tracking of tumor evolution by analyzing serial ctDNA samples over time, offering valuable insights into tumor progression, treatment response, and the emergence of resistance mutations. Furthermore, advancements in NGS have enhanced the ability to generate high-quality genomic data from minimal ctDNA quantities, making LB a feasible and minimally invasive approach for real-time cancer monitoring [41,199,200].

4.2. Current status of liquid biopsy and its implication in clinical management of BC

Several liquid biopsy strategies have achieved clinical approval or recommendation for use in BC management, particularly in the metastatic setting [28,168]. ctDNA analysis is now employed for the detection of PIK3CA mutations to guide the use of alpelisib in hormone receptor-positive, HER2-negative metastatic breast cancer, based on FDA approval [161]. Platforms such as the FoundationOne® Liquid CDx and Guardant360® CDx are validated assays that detect actionable genomic alterations from plasma [201,202]. Additionally, CellSearch® remains the only FDA-approved platform for enumeration of CTCs as a prognostic marker in metastatic BC [203]. Although CTC enumeration is prognostic rather than predictive of therapeutic response, its clinical relevance has been well documented. These advancements highlight the role of LB not only in providing molecular profiles for targeted therapy selection but also in offering real-time monitoring of disease burden and progression [204,205].

Beyond these approved applications, several LB techniques are under active investigation in clinical trials to expand their utility in breast cancer. ctDNA-based assays are being evaluated for the detection of MRD post-curative surgery and adjuvant therapy, with the aim of early relapse prediction before clinical or radiologic evidence of disease. Personalized, tumor-informed assays such as Signatera™ are being studied for this purpose [206]. Furthermore, research is ongoing into profiling CTCs beyond enumeration, including molecular characterization of receptor status (ER, PR, HER2) and expression of immune checkpoint markers such as PD-L1, which could inform dynamic treatment strategies [203,207]. Exosomal RNA, tumor-educated platelets, and methylation-based ctDNA assays are also emerging as promising biomarkers, with the potential to provide broader insights into tumor biology and therapeutic resistance [6,28,208]. If validated, these strategies could redefine surveillance, treatment adaptation, and early intervention paradigms in BC management [166].

4.3. Challenges in standardization

4.3.1. Variability in methodologies

Despite technological advancements, variability in liquid biopsy methodologies remains a significant challenge, impacting the reliability and comparability of results [26,184]. Different detection technologies, such as ddPCR, NGS, and microfluidics, exhibit variations in sensitivity, specificity, and data interpretation, leading to inconsistencies across studies and clinical settings [42,209,210]. Additionally, differences in sample handling, processing, and storage can affect the quality and quantity of analytes, influencing test outcomes. Standardizing these procedures is crucial to ensuring accuracy and reproducibility. Furthermore, data interpretation remains a critical issue, as the clinical significance of ctDNA levels or CTC counts can vary depending on the assay used and the clinical context. Establishing standardized criteria for result interpretation is essential for improving clinical decision-making and the broader adoption of LB in oncology [67,211].

4.3.2. Need for consensus guidelines

To address these challenges, there is a need for consensus guidelines and best practices for liquid biopsy. Establishing standardized protocols for blood collection, cfDNA extraction, and storage is essential for minimizing variability and ensuring reproducibility [212,213]. Consensus on assay validation methods, including sensitivity, specificity, and analytical performance, is needed to ensure the reliability of LB tests. Guidelines for the clinical use of LB, including indications, interpretation of results, and integration with existing diagnostic and monitoring practices, will help standardize its application in patient care [149,214].

5. Clinical trials and evidence

LB is increasingly being evaluated in clinical trials to determine its effectiveness and utility in BC management. This section provides a summary of key clinical trials involving LB, examines its impact on patient outcomes, and discusses the limitations and gaps in current research.

5.1. Summary of key clinical trials involving liquid biopsy in BC

Several key clinical trials have explored the role of LB in BC, focusing on its potential for early detection, prognosis, and treatment monitoring. Table 4 elucidates a comprehensive summary of key clinical trials on LB in BC.

5.2. Impact of liquid biopsy on patient outcomes

The integration of LB into clinical practice has the potential to significantly impact patient outcomes in several ways [220]. LB has demonstrated promise in detecting BC at earlier stages compared to traditional imaging methods. Early detection allows for timely intervention and may improve survival rates by addressing the disease before it becomes more advanced [28,220]. LB enables the identification of specific genetic mutations and alterations that can guide personalized treatment strategies. By tailoring therapies based on the tumor's molecular profile, clinicians can improve treatment efficacy and reduce adverse effects [25]. LB provides real-time monitoring of tumor dynamics, allowing for the adjustment of treatment plans based on the patient's response [221]. This capability helps in identifying resistance early and adapting therapies to improve outcomes. The non-invasive nature of LB reduces the need for frequent tissue biopsies, which can be uncomfortable and risky [25,221]. This aspect is particularly beneficial for patients requiring regular monitoring or those with metastatic disease.

5.3. Limitations and gaps in current research

Despite the advancements and potential benefits of LB, several limitations and gaps in current research remain. Variability in results due to differences in assay methods, sample processing, and interpretation can affect the reliability of findings, highlighting the need for standardized methodologies and interpretation criteria [222,223]. While technological advancements have improved sensitivity and specificity, challenges persist in detecting low-abundance biomarkers and distinguishing tumor-derived signals from background noise, necessitating further assay refinement [224]. Additionally, many studies are limited by small sample sizes or a focus on specific patient subgroups, underscoring the need for large-scale, multicenter trials to validate LB's clinical utility across diverse populations. Cost and accessibility also present barriers, particularly in resource-limited settings where specialized equipment may not be readily available, emphasizing the importance of economic evaluations and cost-reduction strategies [144,225]. Furthermore, the absence of established guidelines and regulatory approvals for many LB tests hampers their integration into routine clinical practice, requiring the development of clear regulatory pathways and standardized protocols [148,226]. Finally, biological variability in ctDNA, CTCs, and other biomarkers across different patients and tumor types complicates result interpretation, necessitating further research into tumor biology to optimize LB applications [29,227].

Despite the expanding role of LB in BC management, clinical implementation remains largely limited to ctDNA and CTC-based assays, with significant barriers hindering broader adoption of other analytes [28,148]. While ctDNA analysis has achieved regulatory approval for detecting actionable mutations like PIK3CA, and CTC enumeration has been validated as a prognostic tool, most liquid biopsy technologies have not yet reached the necessary levels of analytical and clinical validation [28,228,229]. Technical challenges include the heterogeneity of circulating analytes, the low abundance of tumor-derived material in early-stage disease, and the lack of standardized pre-analytical and analytical protocols across laboratories [26,165]. Furthermore, issues such as assay sensitivity, specificity, and reproducibility across diverse clinical settings limit the translation of promising investigational biomarkers into routine practice [144,169]. These limitations are especially critical when considering applications such as early detection, minimal residual disease monitoring, and real-time therapeutic guidance, where even small inaccuracies can lead to significant clinical consequences [26].

The use of circulating cfRNA in LB exemplifies these challenges even more acutely [119,230]. Although cfRNA offers unique biological information, capturing real-time gene expression changes and microenvironmental dynamics, its inherent instability poses a major technical hurdle. cfRNA molecules are rapidly degraded by circulating RNases, requiring meticulous sample handling, immediate processing, and highly sensitive detection methods such as RT-qPCR or RNA sequencing acutely [120,230,231]. Moreover, cfRNA abundance is often extremely low, particularly in early-stage breast cancer, leading to difficulties in achieving consistent, reproducible results [119]. Another limitation is the biological complexity of cfRNA, which includes heterogeneous populations of mRNA, miRNA, and long non-coding RNAs, complicating data interpretation [230,231]. Currently, no cfRNA-based assays are approved for clinical use in breast cancer, and the field lacks large, prospective validation studies demonstrating clinical utility. Until technical standardization is achieved and robust clinical evidence is generated, cfRNA applications in breast cancer will likely remain confined to experimental and investigational settings [231].

6. Future directions and emerging trends

As the field of liquid biopsy continues to evolve, several promising directions and emerging trends are shaping its future applications in BC management. These developments hold the potential to revolutionize how we approach diagnosis, treatment, and monitoring of the disease. This section explores the role of LB in personalized medicine, its integration with artificial intelligence (AI), its combination with other biomarkers, and the regulatory and ethical considerations that accompany its use.

6.1. Personalized medicine

6.1.1. Role of liquid biopsy in the era of personalized treatment

LB is poised to play a critical role in the era of personalized medicine by offering detailed insights into the genetic and molecular characteristics of tumors [169]. Personalized medicine aims to tailor treatments to the individual's unique tumor profile, optimizing therapeutic efficacy and minimizing side effects [25,169]. LB enables this approach by providing real-time information about tumor-specific mutations, gene expression patterns, and molecular alterations through non-invasive blood samples [25,169].

One of the key benefits of LB in personalized treatment is its ability to monitor changes in tumor genetics over time. As tumors evolve, they may acquire new mutations or develop resistance to therapies [232]. LB allows for continuous tracking of these changes, enabling clinicians to adjust treatment strategies promptly based on the latest tumor profile. This dynamic approach to treatment can enhance the effectiveness of personalized therapies and improve patient outcomes [31]. Moreover, LB facilitates the identification of novel therapeutic targets by detecting previously undiagnosed genetic alterations. This capability supports the development of targeted therapies tailored to specific tumor characteristics, advancing the field of precision oncology [233].

6.2. Integration with artificial intelligence (AI)

6.2.1. Use of AI in analyzing liquid biopsy data

AI and machine learning (ML) technologies are increasingly being integrated into the analysis of LB data, offering significant potential to enhance diagnostic accuracy and treatment planning. AI algorithms can process vast amounts of data from LB assays, identifying patterns and correlations that may be challenging for human analysts to discern [234].

AI can improve the interpretation of complex genetic and molecular

Table 4
Key clinical trials on liquid biopsy in breast cancer.

S. No.	Trial Name/No/ Reference	Study Objective	Biomarker Analyzed	Patient Population	Key Findings	Clinical Implications
1	**STIC CTC METABREAST Trial (NCT01710605)**	To evaluate whether CTC count–based treatment decision-making can improve outcomes in patients with hormone receptor-positive, HER2- MBC.	CTCs	Hormone receptor-positive, HER2-negative metastatic breast cancer patients; Patients with available CTC counts at baseline.	Patients with high CTC counts (>5 CTCs/7.5 mL of blood) who were switched to chemotherapy had improved PFS compared to those who continued endocrine therapy. CTC-based treatment decisions resulted in clinical benefits for a subset of patients.	CTCs may serve as a predictive biomarker for guiding first-line treatment decisions in HR+/HER2- metastatic breast cancer. Implementing CTC-based decision-making could optimize therapy selection and improve patient outcomes.
2.	**SWOG 0500 Trial (NCT00382018)**	To determine whether early switching from initial chemotherapy to an alternative regimen based on persistently high CTC counts improves overall survival in metastatic breast cancer.	CTCs	Patients with metastatic breast cancer receiving first-line chemotherapy; Patients with persistently elevated CTC counts after one cycle of chemotherapy	Persistently high CTC count after the first cycle of chemotherapy was associated with poor prognosis. However, switching chemotherapy early based on high CTC levels did not improve overall survival compared to continuing initial therapy.	While CTCs are a strong prognostic biomarker, they may not be effective as a predictive tool for guiding early chemotherapy changes. Standard clinical and radiographic assessment remains essential in treatment decision-making.
3.	**CirCe01 Trial (NCT01349842)**	To assess whether monitoring CTCs can guide chemotherapy decisions in patients with MBC who have already received multiple lines of treatment.	CTCs	MBC patients; Patients who had previously received at least two lines of chemotherapy; Patients with CTC count >5 per 7.5 mL of blood	Persistent high CTC levels indicated poor prognosis. Early switching of chemotherapy based on high CTC levels did not improve overall survival.	CTCs are useful as a prognostic marker but may not be effective in guiding chemotherapy changes in late-line metastatic settings. Standard clinical assessment remains critical for treatment decisions.
4.	**Treat CTC Trial (NCT01548677)**	To evaluate whether administering secondary adjuvant chemotherapy in early-stage breast cancer patients with detectable CTCs after primary treatment can improve outcomes.	CTCs	Patients with early-stage breast cancer; Patients who had completed standard primary treatment (surgery ± chemotherapy/ radiotherapy); Patients with persistent CTCs detected post-t/t	Detectable CTCs post-treatment was associated with a higher risk of recurrence. The trial aimed to assess the benefit of additional chemotherapy, but results are still awaited.	If positive, the findings could support CTCs as a tool for identifying patients who might benefit from additional treatment. May lead to personalized adjuvant therapy strategies based on CTC status.
5.	**DETECT III Trial (NCT01619111)**	To assess whether targeted HER2-directed therapy benefits patients with HER2-negative metastatic breast cancer who have HER2-positive circulating tumor cells.	CTCs with HER2 expression	Patients with HER2-metastatic breast cancer; Patients with HER2-positive CTCs detected in blood samples	HER2+ CTCs were detected in some patients with HER2- primary tumors. The impact of adding HER2- targeted therapy (lapatinib ± chemotherapy) is being evaluated, with potential clinical benefits in this subgroup.	If successful, this study could change treatment paradigms by incorporating CTC analysis to guide HER2-targeted therapy in MBC patients. May help personalize treatment for a subset of patients with HER2- disease but HER2+ CTCs.
6.	**ALCINA** (NCT02866149)	To evaluate whether changes in ctDNA levels can serve as an early indicator of treatment response in patients with hormone receptor-positive, HER2-negative metastatic breast cancer receiving palbociclib and fulvestrant.	ctDNA, particularly mutations in ESR1, PIK3CA, and other relevant genes	Patients with HR+/ HER2- metastatic breast cancer Patients receiving palbociclib (CDK4/6 inhibitor) and fulvestrant (endocrine therapy) Patients with detectable ctDNA at baseline	A rapid decline in ctDNA levels after treatment initiation correlated with better PFS. Persistent or rising ctDNA levels were associated with early disease progression and resistance to therapy. Specific mutations, such as ESR1 mutations, were linked to resistance to endocrine therapy.	ctDNA serves as a real-time, non-invasive biomarker for monitoring t/t response and detecting resistance early. Its use enables personalized t/t adjustments by identifying patients unlikely to benefit from therapies like palbociclib and fulvestrant. Additionally, ctDNA analysis may facilitate earlier intervention strategies, allowing therapy modifications before radiographic progression is detected.
7.	NCT02448771	To evaluate the clinical efficacy and safety of combining bazedoxifene, a third-generation selective ER receptor modulator and degrader, with palbociclib, a CDK4/6 inhibitor, in patients with advanced hormone receptor–positive (HR+),	ctDNA; Mutations in genes such as PIK3CA and ESR1	36 patients with advanced HR+/HER2− breast cancer All had experienced disease progression on prior endocrine therapy. A heavily pretreated cohort, with many having received	The combination therapy demonstrated a clinical benefit rate (CBR) of 33.3 %, with stable disease observed in 56 % of the intent-to-treat population. The median progression-free survival (PFS) was 3.6 months. Patients with activating	The combination of bazedoxifene and palbociclib shows potential efficacy in heavily pretreated HR+/ HER2− advanced breast cancer patients, warranting further investigation. Monitoring PIK3CA mutations through ctDNA

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Table 4 (*continued*)

S. No.	Trial Name/No/ Reference	Study Objective	Biomarker Analyzed	Patient Population	Key Findings	Clinical Implications
		HER2- breast cancer who have progressed on prior endocrine therapy. Additionally, the study aimed to utilize whole-exome sequencing (WES) of ctDNA from liquid biopsies to monitor tumor heterogeneity and genetic evolution during t/t.		multiple lines of therapy	PIK3CA mutations at baseline had a shorter PFS, indicating potential resistance to the combination therapy. Longitudinal WES of ctDNA revealed tumor heterogeneity, sub-clonal genetic evolution, and identified actionable mutations acquired during t/t.	could serve as a predictive biomarker for resistance, aiding in personalized treatment strategies. Utilizing WES of liquid biopsies offers a non-invasive method to track tumor evolution and adapt therapeutic approaches accordingly.
8.	Visvanathan et al. [149]	To assess the clinical utility of the LBx-BCM prototype assay, an automated liquid biopsy test detecting circulating methylated DNA, for early prediction of disease progression and survival in patients with metastatic breast cancer (MBC).	Circulating cell-free methylated DNA (ccfDNA) assessed through a 9-marker panel using the LBx-BCM assay.	144 women with metastatic breast cancer. Plasma samples collected at baseline, week 4, and week 8.	At week 4, patients with high cumulative methylation (CM) had significantly shorter median PFS (2.88 months) compared to those with low CM (6.60 months) (P = 0.001). Overall survival (OS) was also shorter in the high CM group (14.52 months) versus the low CM group (22.44 months) (P = 0.005). High CM levels at week 4 were associated with a higher risk of disease progression at first restaging (OR, 2.78; 95 % CI, 1.29–5.99; P = 0.009). A robust risk model based on week 4 circulating CM levels was developed to predict disease progression as early as 3 months after initiating a new treatment	The LBx-BCM assay shows promise as a clinical tool for early detection of disease progression in MBC patients. Early identification of patients at higher risk of progression could allow for timely modifications to treatment strategies. Further validation is needed to confirm the assay's utility across different treatment regimens.
9.	Shah et al. [215]	To evaluate the efficacy of palbociclib (a CDK4/6 inhibitor) in combination with trastuzumab in HER2-positive metastatic breast cancer (MBC) with brain metastases (BM). To analyze circulating tumor DNA (ctDNA) in patients with active BM to assess its potential as a biomarker for disease progression.	Circulating tumor DNA (ctDNA)	Twelve patients with HER2-positive MBC and active BM. Among them, four had hormone receptor-positive HR + disease, and eight had HR− disease.	Six patients achieved stable disease, while the remaining six experienced disease progression. - The median progression-free survival (PFS) was 2.2 months. Analysis of ctDNA revealed that patients with progressive BM but stable or responding systemic disease had low variant allele frequency (VAF) and a lower number of detectable genetic alterations in ctDNA from blood samples.	Palbociclib, in combination with trastuzumab, did not demonstrate significant activity in treating HER2-positive MBC with BM. The low VAF and fewer detectable alterations in ctDNA among patients with progressive BM suggest that ctDNA analysis from blood may have limited utility in monitoring intracranial disease progression in this context. Alternative therapeutic strategies and more effective biomarkers are needed for managing and monitoring HER2+ MBC patients with brain metastases
10.	**Cohen et al.** [216]	To evaluate the efficacy of the Parsortix® PC1 System, an FDA-cleared microfluidic device, in capturing and harvesting circulating tumor cells (CTCs) from the peripheral blood of metastatic breast cancer (MBC) patients. The study also aimed to characterize these CTCs using immunofluorescence (IF) and Wright-Giemsa (WG) staining methods.	Circulating Tumor Cells (CTCs)	76 metastatic breast cancer (MBC) patients 76 self-declared female healthy volunteers (HVs)	CTCs were identified in: 64.5 % of MBC patients using immunofluorescence (IF) staining 61.8 % of MBC patients using Wright-Giemsa (WG) staining CTCs were detected in: 5.3 % of healthy volunteers (HVs) using IF staining 2.6 % of HVs using WG staining The Parsortix® PC1 System demonstrated linear and reproducible performance in harvesting tumor cells from blood samples, with a detection range of 1 to approximately 100 cells.	The Parsortix® PC1 System effectively captures and harvests CTCs from MBC patients, enabling further characterization and potential use in personalized medicine. The system's epitope-independent mechanism allows for the capture of CTCs with diverse phenotypes, based on cell size and deformability, which may provide a more comprehensive understanding of tumor heterogeneity. The low incidence of CTC detection in healthy volunteers suggests high specificity of the Parsortix® PC1 System for identifying CTCs in MBC patients.

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Table 4 (*continued*)

S. No.	Trial Name/No/ Reference	Study Objective	Biomarker Analyzed	Patient Population	Key Findings	Clinical Implications
11.	**NCT04488614**	To identify new biomarkers that enable detection of systemic recurrences at the molecular level in early-stage breast cancer patients. The study also aims to integrate patient-reported outcome measures (PROMs) to assess quality of life and fatigue, facilitating a comprehensive understanding of patient well-being alongside biological monitoring.	Circulating tumor cells (CTCs), Circulating tumor DNA (ctDNA), Exosomal micro-RNA (miRNA), miRNA in tumor-educated platelets, Metabolomic profiles	1455 patients with early-stage breast cancer enrolled between 2011 and 2030 at two university hospitals in Western Norway. A control group comprising 200 women without cancer, aged 25–70 years, providing the same data for comparison.	As this is an ongoing longitudinal observational study, specific findings are yet to be reported. The study protocol outlines methodologies for collecting and analyzing liquid biopsies and PROMs to monitor disease progression and patient well-being over time.	The integration of liquid biopsy analyses with patient-reported outcomes may provide a comprehensive monitoring approach for early-stage breast cancer patients. Identifying molecular biomarkers associated with systemic recurrence could lead to earlier interventions, while PROMs can inform supportive care strategies to enhance quality of life. The study's findings have the potential to improve personalized treatment plans and survivorship care.
12.	**NCT01917279**	To explore the role of the molecular tumor burden index (mTBI) in ctDNA as a therapeutic response and prognostic biomarker in MBC patients.	Circulating tumor DNA (ctDNA)	125 patients with MBC	Pretreatment mTBI values correlated with tumor burden ($P = 0.025$). Patients with high-level pretreatment mTBI had shorter overall survival compared to those with low-level pretreatment mTBI (median overall survival: 40.9 months vs. 68.4 months, $P = 0.011$).	The mTBI in ctDNA can potentially be used as a response evaluation criterion in breast cancer, aiding in prognosis and therapeutic response assessment.
13.	**NCT02549430**	To evaluate the prognostic role of CTC counts and RB1 gene expression in patients with estrogen receptor-positive (ER+), human epidermal growth factor receptor 2-negative (HER2−) advanced breast cancer (ABC) undergoing treatment with palbociclib, either alone or in combination with endocrine therapy.	CTCs and RB1 gene expression on CTCs	46 patients with ER-positive, HER2-negative advanced breast cancer (ABC)	CTCs were detected in 50 % of patients before starting treatment (T0). Patients with ≥5 CTCs/7.5 mL of blood at T0 had a shorter PFS compared to those with <5 CTCs (median PFS: 3.5 months vs. 9.2 months; $P = 0.002$). An increase of ≥3 CTCs after the first treatment cycle (T1) was associated with worse PFS (median PFS: 3.7 months vs. 9.2 months; $P = 0.006$). RB1 gene expression analysis on CTCs was feasible and provided additional prognostic information.	CTC count is a promising modality for monitoring response to palbociclib treatment in patients with ER+, HER2- advanced breast cancer. CTC count at the time of progression could predict clinical outcomes post-palbociclib treatment. RB1 expression analysis on CTCs may provide additional prognostic information, although results should be interpreted with caution due to the small sample size.
14.	**CirCe T-DM1 trial (NCT01975142)**	To assess the efficacy of trastuzumab-emtansine (T-DM1) in patients with HER2-negative metastatic breast cancer (MBC) who have HER2-amplified circulating tumor cells (CTCs).	HER2 amplification in CTCs.	154 women with HER2-negative metastatic breast cancer, previously treated with at least two lines of chemotherapy and having measurable disease.	CTCs were detected in 78.7 % (118/154) of patients. HER2 amplification in CTCs was found in 9.1 % (14/154) of patients. Among 11 patients treated with T-DM1, only one achieved a confirmed partial response.	HER2 amplification in CTCs is rare among HER2-negative MBC patients. Treatment with T-DM1 showed limited efficacy in this subset, suggesting that HER2-amplified CTCs may not be a reliable biomarker for T-DM1 responsiveness in HER2-negative MBC.
15.	**Horimoto et al.** [217]	To investigate circulating tumor cells (CTCs), including their epithelial-mesenchymal transition (EMT) status, in patients with metastatic breast cancer undergoing eribulin-based treatment, aiming to assess the potential of CTCs as predictive markers for treatment efficacy.	CTCs, Epithelial and mesenchymal markers on CTCs.	22 patients with MBC receiving eribulin-based treatment	CTCs were detected in 68.2 % (15/22) of patients before treatment initiation. Patients with <5 CTCs/7.5 mL of blood had a median progression-free survival (PFS) of 4.8 months, while those with ≥5 CTCs had a median PFS of 2.1 months. Patients with ≥5 mesenchymal CTCs/7.5 mL of blood had a significantly shorter PFS compared to those with <5 mesenchymal CTCs (median PFS: 1.2 months vs. 4.8 months; $P = 0.008$).	Determining both mesenchymal and epithelial CTCs at baseline may serve as a predictive tool for eribulin responsiveness in metastatic breast cancer patients. Evaluation of mesenchymal CTCs could be considered in larger studies, as current clinical trials often focus solely on detecting epithelial markers.
16.	**Paoletti et al.** [218]	To explore early changes in circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) as potential noninvasive tools to assess	CTCs, including ER and Ki67 expression. ctDNA for ESR1 ligand-binding domain	43 patients with ER-positive, HER2-negative metastatic breast cancer.	Before starting AZD9496, 25 % (11/43) of patients had ≥5 CTCs per 7.5 mL of whole blood, none of whom experienced a reduction to <5	Elevated baseline CTC counts are a strong prognostic factor in this cohort. Early changes in CTC-ER+ and ESR1LBDm +

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Table 4 (*continued*)

S. No.	Trial Name/No/ Reference	Study Objective	Biomarker Analyzed	Patient Population	Key Findings	Clinical Implications
		pharmacodynamics and early efficacy of the oral selective estrogen receptor degrader (SERD) AZD9496 in patients with estrogen receptor-positive (ER+), metastatic breast cancer (MBC)	mutations (ESR1LBDm)		CTCs during treatment. Patients with ≥5 CTCs at baseline had worse progression-free survival (PFS) compared to those with <5 CTCs (P = 0.0003). 31 % (14/45) of patients had ESR1LBDm + ctDNA at baseline, with five patients exhibiting ≥2 unique mutations. Early on-treatment changes were observed in CTC-ER+ and ESR1LBDm + ctDNA, but not in overall CTC number.	ctDNA during treatment may serve as potential pharmacodynamic markers. Integrating multiple circulating biomarkers in prospective trials may improve outcome prediction and identification of endocrine therapy resistance mechanisms over relying on a single biomarker.
17.	**Ligthart ST et al.** [219]	To quantitatively assess HER2 protein expression on circulating tumor cells (CTCs) in patients with metastatic (M1) and non-metastatic (M0) breast cancer using the CellSearch® system.	HER2 protein expression on CTCs	103 patients with metastatic breast cancer (M1), 88 patients with non-metastatic breast cancer (M0)	CTCs were detected in 52 % of M1 patients and 22 % of M0 patients. HER2-positive CTCs were found in 30 % of M1 patients and 12 % of M0 patients. There was a significant correlation between HER2 expression on CTCs and primary tumor HER2 status.	Quantitative assessment of HER2 expression on CTCs can provide additional information beyond primary tumor HER2 status, potentially guiding targeted therapies in both metastatic and non-metastatic breast cancer patients.

data by identifying biomarkers associated with disease progression, treatment response, and resistance mechanisms. ML models can also predict patient outcomes based on historical data and real-time LB results, providing personalized prognostic information [235].

Furthermore, AI-driven analysis can streamline the workflow of LB testing, from sample processing to result interpretation. Automated algorithms can reduce the time required for data analysis and minimize human error, leading to faster and more reliable results. The integration of AI into liquid biopsy workflows has the potential to enhance the efficiency and accuracy of cancer diagnostics and monitoring [235,236].

6.3. Potential for combination with other biomarkers

6.3.1. Synergistic use of liquid biopsy with traditional tissue biopsy

Combining LB with traditional tissue biopsy offers a synergistic approach to cancer diagnosis and management [25]. While tissue biopsy remains the gold standard for obtaining comprehensive tumor samples, LB provides a complementary, non-invasive method for monitoring tumor dynamics and treatment response [25,169].

The combination of these two approaches can offer a more complete picture of the tumor's genetic landscape. For instance, tissue biopsy can provide detailed information on tumor histology and specific mutations, while LB can track changes in ctDNA, CTCs, and exosomes over time [237]. Integrating data from both sources can enhance the accuracy of diagnosis, prognostication, and treatment planning.

Additionally, using LB to monitor MRD and detect emerging resistance mutations can inform decisions on whether additional tissue biopsies are needed or if treatment adjustments should be made [27,238]. This combined approach can lead to more informed and timely clinical decisions, improving overall patient management.

6.4. Regulatory and ethical considerations

6.4.1. Challenges in regulatory approval

The integration of LB into routine clinical practice faces several regulatory challenges. As a relatively new technology, LB assays must undergo rigorous validation to ensure their accuracy, reliability, and clinical utility. Regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) require extensive clinical evidence before granting approvals for new diagnostic tests [148].

Developing standardized protocols and performance metrics for LB tests is essential for gaining regulatory approval [239]. The establishment of clear guidelines for assay validation, quality control, and clinical use will support the adoption of LB in various healthcare settings [239].

6.4.2. Ethical implications of liquid biopsy in clinical practice

The use of LB raises several ethical considerations, including issues related to patient consent, privacy, and data security [25,237]. As LB generates detailed molecular profiles, ensuring that patients fully understand the implications of the results and provide informed consent is crucial [237].

Furthermore, the handling and storage of sensitive genetic information must adhere to stringent privacy and data protection standards. Ethical considerations also extend to the potential for incidental findings, where unexpected genetic information may be discovered that could have implications for the patient or their family members [238].

7. Conclusion

7.1. Summary of findings

LB has emerged as a transformative tool in BC management, offering a non-invasive method for early detection, monitoring treatment response, and personalizing therapy. Key findings from clinical trials highlight its potential to improve patient outcomes by providing real-time insights into tumor dynamics and facilitating tailored treatment strategies.

7.2. Clinical implications of liquid biopsy in BC management

The clinical implications of liquid biopsy are profound, with the potential to enhance early detection, guide personalized treatment, and monitor disease progression more effectively than traditional methods. By integrating LB with other diagnostic tools and leveraging advancements in AI, clinicians can achieve more accurate and timely management of BC.

7.3. Future research directions

Future research should focus on addressing the current limitations of LB, including variability in results, sensitivity issues, and the need for large-scale validation studies. Continued development of standardized methodologies and cost-effective solutions will be crucial for broader adoption. Additionally, exploring the potential for integrating LB with emerging technologies and biomarkers will further enhance its role in personalized medicine. Addressing regulatory and ethical challenges will also be essential to ensuring the responsible and effective use of LB in clinical practice.

This paper has been prepared by the abovementioned authors and reviewed and agreed upon for submission. The requirements for authorship as stated above in this document have been met, and that each author believes that the manuscript represents honest work.

Declarations.

Data availability statement

Data sharing does not apply to this article as no new data were created or analyzed in this study.

Funding

NIL.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: No conflict of Intrests If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

NIL.

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