Breast Cancer Research

RESEARCH

Open Access

Identification of minimal residual disease using the clonesight test for ultrasensitive ctDNA detection to anticipate late relapse in early breast cancer

Iñaki Comino-Méndez[1,2,3*], Jesús Velasco-Suelto[1,2,3], Javier Pascual[1,2,3], Esperanza López-López[1,2], Maria Elena Quirós-Ortega[1,2,3], Celia Gaona-Romero[1,2], Alejandro Martín-Muñoz[4,5], Patricia Losana[5], Yanira Heredia[5], Emilio Alba[1,2,3,6†] and Angel Guerrero-Zotano[7*†]

Abstract

Background Early-stage breast cancer (BC) diagnosis significantly reduces mortality, yet relapse remains a concern due to undetectable minimal residual disease (MRD). Liquid biopsies offer real-time insights into tumor dynamics, aiding MRD detection and therapy response evaluation. However, MRD detection is challenging due to low tumor DNA levels in circulation.

Methods This prospective study included 20 HR + BC patients who had completed at least 5 years of adjuvant endocrine therapy (ET). Plasma samples were collected every 6 months over a median follow-up period of 2 years. Tumor-specific somatic variants identified through tumor tissue sequencing served as biomarkers for a patient-informed circulating tumor DNA (ctDNA) assay (CloneSight), which utilized a multiplex PCR-based next-generation sequencing (NGS) workflow.

Results ctDNA was detected in patients who experienced clinical relapse, with positivity observed up to 68 months (5.7 years) prior to overt recurrence, highlighting its potential for early relapse identification. In non-relapsed patients, ctDNA remained undetectable in 93% of cases, reflecting a potential high level of specificity. The assay detected ctDNA in 50% of relapsed patients, while no ctDNA signal was identified in the majority of non-relapsed cases.

Conclusion Our exploratory findings indicate that CloneSight could be a promising tool for MRD detection and relapse prediction, providing a cost-effective, patient-informed approach to ctDNA monitoring. The ability of this approach to detect relapse prior to clinical recurrence suggests its potential relevance in improving patient monitoring. These findings suggest that ctDNA-based MRD assays could play a role in future surveillance strategies for HR + BC, though further studies in larger cohorts are needed to confirm their clinical applicability.

Keywords Liquid biopsy, Early breast cancer, Circulating tumor DNA, Minimal residual disease

†Angel Guerrero-Zotano and Emilio Alba contributed equally to this work.

*Correspondence:
Iñaki Comino-Méndez
inaki.comino@ibima.eu
Angel Guerrero-Zotano
angel.guerrero.zotano@gmail.com
Full list of author information is available at the end of the article

Background

Around 95% of breast cancer (BC) patients present with early-stage disease without macroscopic evidence of metastases [1], with a 28% to 65% reduction in mortality rates directly attributable to early diagnosis [2]. However, in luminal BC, the most common subtype, the persistent risk of late relapse remains a significant challenge. Despite extended adjuvant antiestrogen therapy for five or more years, up to 40% of patients experience recurrence, influenced by factors such as tumor grade, size, and axillary node involvement [3]. This highlights the pressing need for improved strategies to manage luminal BC. The risk of recurrence is largely attributed to undetectable minimal residual disease (MRD) that persists following primary treatment [4]. Identifying patients with MRD before it becomes clinically detectable would pave the way for personalized strategies, allowing for early intervention and potentially preventing relapse development.

Liquid biopsy has dramatically revolutionized the field of molecular oncology, emerging as ideal complements and/or substitutes for conventional biopsies. It is increasingly popular due to their accuracy, minimal invasiveness, high reproducibility, and cost-effectiveness for serial analysis [5,6]. Liquid biopsy provides critical real-time insights, with the detection of MRD representing a pivotal element in the framework of early relapse detection. They can be obtained from various bodily fluids such as blood and several tumor materials can be recovered from them, including circulating tumor DNA (ctDNA) among others [6–9].

In early-stage cancers, circulating tumor components are highly diluted in non-tumor material, necessitating the use of resource-intensive methodologies to detect them [[10–14]] Furthermore, early BC, particularly luminal tumors, is characterized by a notably low tumor mutation burden (TMB), which limits the effectiveness of fixed-gene next-generation sequencing (NGS) panels for ctDNA detection [15–17]. While patient-specific panels leveraging somatic single nucleotide variants (SNVs), advanced bioinformatic pipelines, and intensive sequencing processes have demonstrated ultra-high sensitivity for detecting ctDNA in plasma from localized tumors, including BC [4,12,13,18], these approaches often rely on expensive commercial services [4,19].

Here, we present a novel commercial test designed to address these limitations, enabling the detection of late relapses at earlier time points through personalized ctDNA tracking, potentially extending the window for clinical intervention. This test applies advanced yet practical methodologies, aiming to facilitate the integration of ctDNA detection techniques into routine clinical settings. By demonstrating the feasibility of implementing liquid biopsy technologies, this study contributes to ongoing efforts to enhance early cancer detection and disease monitoring.

Methods

Patients and Samples

Patients diagnosed with stage III HR + BC treated and followed at Instituto Valenciano of Oncology (Valencia, Spain) were recruited. All patients in this cohort have completed at least 5 years of adjuvant endocrine therapy (ET), are free of relapse and have given their consent to the biobank for plasma collection and NGS analysis of their primary tumor. Patients were followed for a median of 7.21 years (range 4.33–14.50 years) from the start of the study period (first blood sample), with plasma samples collected every 4 to 6 months during the initial 2 years of follow-up. Some samples were collected during ongoing ET, while others were obtained after its completion, in accordance with routine clinical practice at our institution (Fig. 1). This practice includes regular physical examinations, periodic blood sampling, and annual breast imaging. Following the conclusion of adjuvant ET, patient follow-up transitions to annual visits, with the same procedures maintained to ensure consistent monitoring. Samples were analyzed retrospectively, and both patients and their treating physicians remained blinded to the results.

Pre-treatment primary tumor tissue (FFPE) was obtained for the patients. Immunohistochemical (IHC) analysis was performed to quantify expression of human epidermal growth factor receptor 2 (HER2), hormone receptors (HR), and Ki67. Estrogenic receptor (ER) and progesterone receptor (PR) were considered positive in tumors presenting more than 1% nuclear-stained cells. HER2 staining was scored according to the guidelines [20]. HER2 status was considered positive when graded as 3+, while 0 to 1+ were negative and 2+ was an inconclusive result and silver in situ hybridization was performed. The tumor tissues were stained with hematoxylin–eosin and marked for tumor content by a qualified pathologist to achieve >20% in tumor cells in the macro-dissected area. Tumor tissue DNA was extracted using the RecoverAll™ Total Nucleic Acid Isolation Kit (ThermoFisher Scientific).

Blood samples from the study participants were collected in EDTA blood tubes and processed within 2 h following venipuncture. The plasma supernatant was isolated by centrifugation for 10 min at 3,000 rpm at room temperature and subsequently stored at −80 °C until the extraction of cell-free DNA (cfDNA).

cfDNA was obtained from plasma samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen) following the manufacturer's instructions. The cfDNA fragment

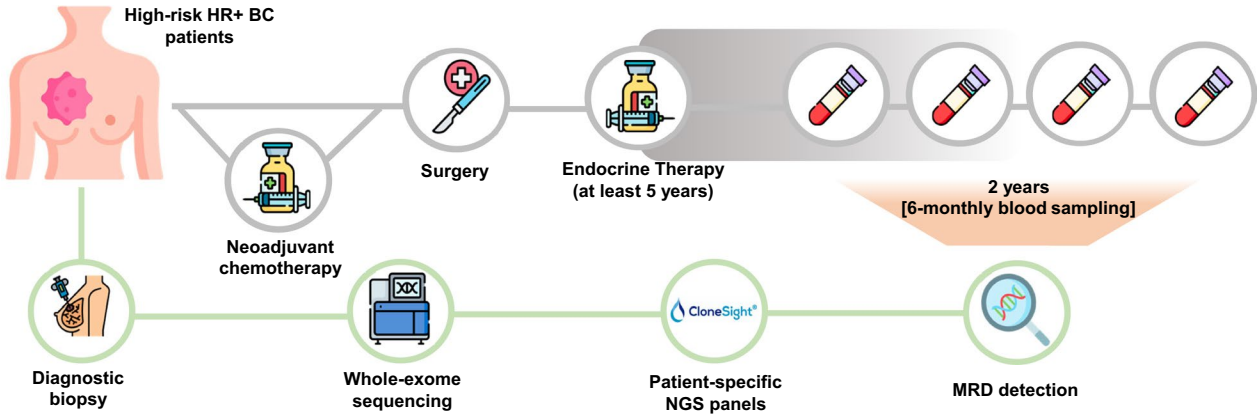

Fig. 1 CloneSight assay workflow. Schematic representation of the clinical pathway for HR + breast cancer (BC) patients, including treatment and blood collection. Patientsunderwent neoadjuvant chemotherapy (if applicable) and received at least 5 years of endocrine therapy. Plasma samples were collected during and/or after endocrine therapy at 6-month intervals during 2 years. Tumor tissue obtained at the time of initial BC diagnosis was evaluated for suitability. DNA was extracted from these samples, and whole exome sequencing was performed to identify somatic mutations unique to each patient. These mutations were subsequently used to develop personalized CloneSight assays for minimal residual disease (MRD) detection

size and percentage were determined through electrophoresis using the Cell-free DNA ScreenTape Analysis on the TapeStation system (Agilent Technologies, Inc.), with cfDNA fragments defined as those ranging between 50 and 700 bp.

Germline DNA from each patient was extracted from the isolated peripheral blood mononuclear cells (PBMCs) using the QIAamp DNA Blood Mini Kit (Qiagen). This DNA was employed to generate WES libraries and permit the selection of exclusive somatic mutations to be employed as ctDNA biomarkers. DNA quantification was performed using the RNAse P assay (ThermoFisher Scientific).

For each patient plasma sample, the entire cfDNA amount was employed to determine MRD positivity.

Baseline mutational screening from matched tumor-normal WES

Both tumor and germline DNA from the 20 patients was subjected to whole exome sequencing (WES) using the Agilent V6 exome kit (Agilent) to construct sequencing libraries. Samples were tested for integrity and purity and fragmented using the Covaris system. Then, 150 to 250 bp fragments were selected from the fragmented genome using magnetic beads. The fragments were subjected to end-repair, 3' adenylation, and adapter ligation. The selected fragments were amplified and hybridized with probes capturing the whole exome. The captured fragments were amplified and circularized to be sequenced in the DNBSEQ-G400 platform (BGI genomics). DNA from tumor was sequenced at 100X and germline DNA at 50X.

FASTQ files from tumor and normal samples were processed using the Python-based samtools library [21] for alignment, sorting, and duplicate marking. Somatic variants in tumor samples were identified using three different pipelines: i) the combination of Manta for structural variants [22] and Strelka for small variants [23], ii) VarNet [24], and iii) Mutect2 [25]. To minimize the inclusion of artifacts, only variants detected by at least two of the three pipelines were selected. The workflow was implemented using Snakemake to ensure reproducibility and traceability. Selected variants were annotated with the IonReporter platform (Thermo Fisher Scientific Inc.). Intronic, synonymous or low-support variants (less than 6 independent reads) were excluded from further analysis. Final candidates were manually reviewed in IGV to confirm both quality and authenticity. Variants were prioritized using a proprietary algorithm that considers coverage, variant allele frequency (VAF), pathogenicity, functional impact, and the likelihood of deamination artifacts. Variants meeting or exceeding the minimum acceptance score were selected as suitable MRD biomarkers.

ctDNA detection by personalized NGS test

The tumor-informed ctDNA assay called CloneSight (Altum Sequencing Co.) was performed using a multiplex PCR (mPCR)-based NGS workflow for ctDNA detection and quantification. Mutations were selected as biomarkers based on sequencing quality and VAF. An average of 12 somatic, patient-specific, high-ranking SNVs or INDELS derived from tumor tissue WES were selected for mPCR testing (range 1–31 mutations per

Table 1 Clinicopathological and treatment characteristics of the patients enrolled in the study

Clinicopathological characteristics	MRD-positive (%)	MRD-negative (%)
Age at diagnosis		
50–60	1 (33.33)	4 (23.52)
> 60	2 (66.66)	13 (76.47)
Tumor type		
IDC	2 (66.66)	13 (76.47)
ILC	1 (33.33)	3 (17.64)
Other	0 (0)	1 (5.88)
Tumor size		
T1	1 (33.33)	1 (5.88)
T2	1 (33.33)	13 (76.47)
T3	1 (33.33)	2 (11.76)
T4	0 (0)	1 (5.88)
Lymph nodes		
N0	0 (0)	1 (5.88)
N1	0 (0)	2 (11.76)
N2	1 (33.33)	9 (52.94)
N3	2 (66.66)	5 (29.41)
Surgery		
Breast conserving	2 (66.66)	11 (64.70)
Mastectomy	1 (33.33)	5 (29.41)
Bilateral mastectomy	0 (0)	1 (5.88)
Adjuvant Radiation		
Y	3 (100)	17 (100)
N	0 (0)	0 (0)
Chemotherapy		
Y	3 (100)	15 (88.23)
N	0 (0)	2 (11.76)
Adjuvant Endocrine Therapy		
Y	3 (100)	17 (100)
N	0 (0)	0 (0)
Type of Endocrine Therapy		
TAM	0 (0)	0 (0)
AI	3 (100)	12 (70.58)
TAM + AI	0 (0)	5 (29.41)
Time in endocrine therapy		
> 5 years and ongoing	0 (0)	2 (11.76)
< 5 years and ongoing	0 (0)	0 (0)
> 5 years and completed	3 (100)	15 (88.23)
< 5 years and completed	0 (0)	0 (0)
Treated with CDK4/6 inhibitors		
Y	0 (0)	0 (0)
N	3 (100)	17 (100)

patient). Primers for mPCR were designed and applied to cfDNA to track all selected biomarkers following the previously described protocol [26,27]. An average of 45 ng of cfDNA per patient was used for library preparation (range 2.78—666 ng). Libraries were sequenced on the Ion S5 System platform (Life Technologies, Thermo Fisher Scientific Inc.) with a targeted coverage of 500,000 ×per amplicon. Same workflow was applied to three healthy control donor DNA samples to obtain the limit of detection (LOD) and limit of quantification (LOQ) for each biomarker.

Statistical analyses

Plasma samples with at least two variants detected were defined as ctDNA-positive. MRD status (CloneSight value) was defined by the mutation with the highest VAF.

Results

Patient baseline characteristics

A total of 20 BC patients were included in this study, all of whom had sufficient tumor tissue for sequencing and clinical follow-up data. The median age at diagnosis was 66 years (range 53–85 years), with over 75% of patients aged more than 60 years. Most tumors were of the invasive ductal carcinoma (IDC) subtype (75%), while a smaller proportion were invasive lobular carcinoma (ILC) (20%) or other histology (5%). Tumor size distribution showed that 70% of patients presented with T2 tumors, with smaller proportions classified as T1 (10%), T3 (15%), or T4 (5%). Regarding nodal status, 50% of patients had N2 involvement, 35% had N3, and fewer presented with N1 (10%) or N0 (5%). All patients underwent surgical treatment, with 70% undergoing breast-conserving surgery and 30% receiving mastectomy (including unilateral or bilateral procedures). Adjuvant radiation therapy was administered to all patients, and chemotherapy was given to 90%, with all MRD-positive cases receiving chemotherapy. ET was part of the treatment protocol for all patients. Among them, 75% received aromatase inhibitors (AI), while 25% received a combination of tamoxifen and AI. At the time of the study, 90% had completed more than 5 years of ET, with two patients still receiving treatment. No patients in the cohort were treated with CDK4/6 inhibitors (Fig. 1**,** Table 1).

The median clinical follow-up (from diagnosis) was 161 months (range 78–256 months) (Fig. 2). Three patients (15%) were identified as MRD-positive during the study, while the remaining 17 patients (85%) were MRD-negative. Detailed associations between MRD status and clinicopathological characteristics are provided in Table 1**.**

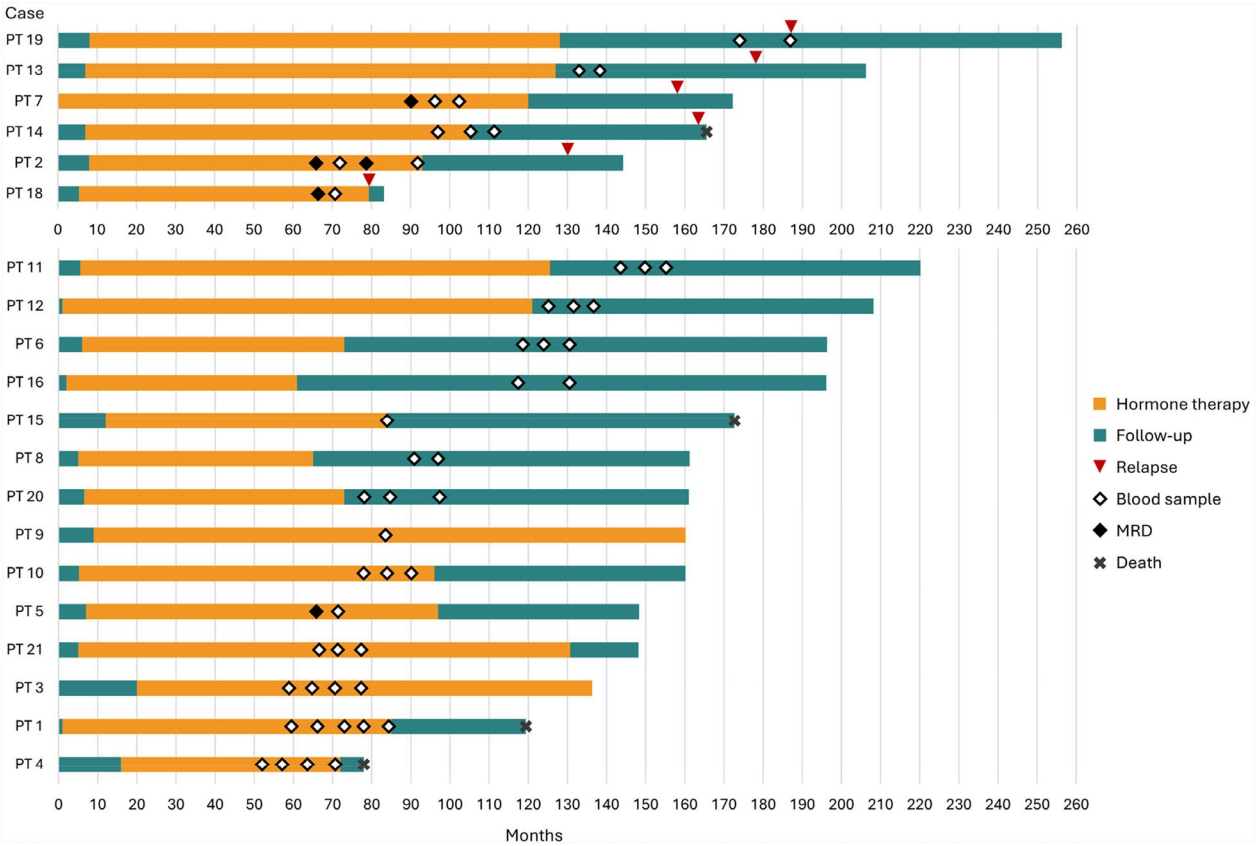

Fig. 2 Swimmer plot for the breast cancer (BC) patients included in the study Visualization of clinical follow-up timelines for all study participants. The upper section represents patients who experienced clinical relapse (n = 6), while the lower section shows those without relapse (n = 14). Orange bars indicate the duration of hormone therapy (HT). Blood sampling events (n = 55) are represented by diamonds, with filled diamonds indicating minimal residual disease (MRD) positivity based on ctDNA detection

Ultrasensitive ctDNA detection with the Clonesight test predicts late relapses ahead of clinical progression

A total of 55 plasma samples were sequenced using patient-specific panels (median 3 samples, range 1–5). The median time from diagnosis to the first blood collection was 86.54 months (range 52.00–173.98), while the median time from the first blood sampling to the last clinical follow-up was 77.41 months (range 16.89–82.85). Among the cohort, 6 patients (30%) experienced relapse with either metastatic or localized disease (median time from diagnosis to relapse was 161 months, range 83–187) (Table 2). Notably, ctDNA was detected as biomarker for MRD in plasma samples from 3 out of these 6 relapsed patients (50%): PT2, PT7, and PT18 (Fig. 2 and 3). Detection of ctDNA was observed with a median VAF

Table 2 Clinical and molecular characteristics of relapsed patients, including ctDNA detection dynamics

Patient	Type of recurrence (metastatic/local)	Metastatic site	Time from diagnosis to relapse (months)	Lead time to clinical relapse (months)	ctDNA detected in first blood draw (Yes/No)	ctDNA detected in all blood draws	ctDNA detected in any blood draw
2	Local + Metastatic	Bone (multiple)	130	64	Yes	No	Yes
7	Local	Breast	158	68	Yes	No	No
13	Metastatic	Bone	178	–	–	–	–
14	Metastatic	Liver (multiple)	164	–	–	–	–
18	Metastatic	Brain	83	17	Yes	No	No
19	Local	Breast (Axillary lymph node)	187	–	–	–	–

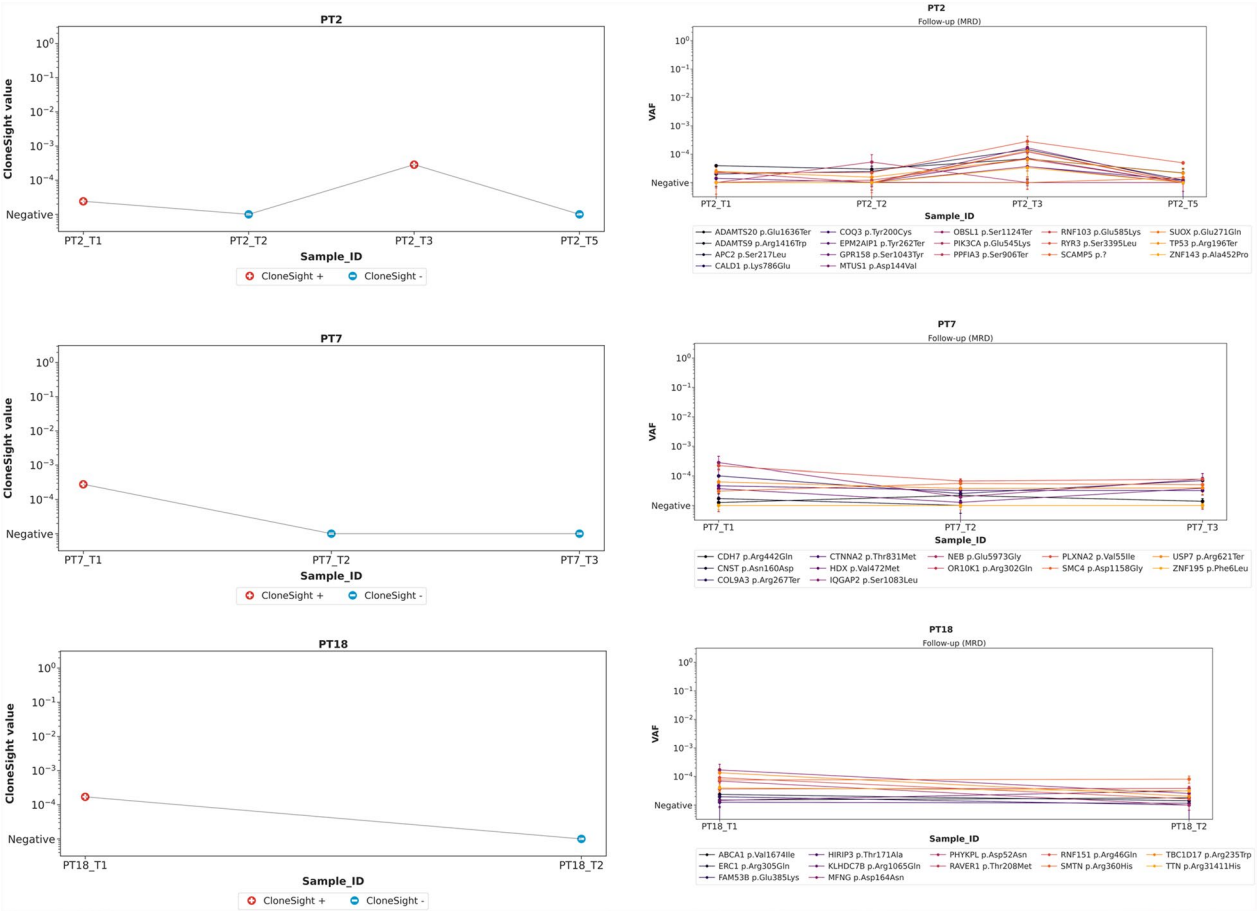

Fig. 3 Graphical representation of the MRD-positive patients. The left-hand graphs illustrate MRD status (positive or negative) at each time point, accompanied by the corresponding CloneSight values. The right-hand graphs depict the variant allele frequency (VAF) of individual biomarkers included in the patient-specific panels at each time point. Each data point reflects the dynamics of ctDNA biomarkers over time, providing a detailed view of MRD detection and progression

of 0.028% (range 0.0024–0.036). Here in, MRD positivity antedated clinical relapse in up to 68 months (range 17–68) with detection in the first blood sample collected from the positive patients. Specifically, PT2 had two MRD-positive plasma samples during follow-up, indicating an ongoing ctDNA presence prior to clinical progression. This patient showed MRD positivity in 2 out of the 4 plasma samples tested (50%) and presented with local recurrence and multiple bone metastases. PT7 experienced relapse with local disease and showed MRD positivity in the first blood sample collected during ET. Interestingly, PT18 had a recurrence with a single brain metastasis that was detected more than a year before clinical relapse (Fig. 2 and 3**,** Table 2).

Conversely, three patients (PT13, PT14, and PT19; 50%) experienced relapse without detectable MRD in their tested plasma samples (Fig. 2**,** Table 2**)**. PT13, who relapsed with an isolated bone lesion. Only two plasma samples were collected from PT13 during follow-up, one of which had a lower cfDNA quantity (< 20 ng), which may have impacted MRD detection sensitivity. This patient presented with an isolated bone metastasis and is currently without evidence of progressive disease. PT14 relapsed and subsequently died from liver metastasis more than four years after the last MRD test. In this case, the MRD assay had been limited to only 4 SNVs as biomarkers, which may have contributed to the absence of detectable ctDNA prior to relapse. PT19 experienced a recurrence in an isolated lymph node originating from the breast and currently shows no signs of PD. For this patient, two plasma samples were tested, with one presenting a very low cfDNA yield (2 ng), which could have impacted the detection capability.

In contrast, MRD positivity was observed in patient PT5, who exhibited a single positive plasma sample during monitoring (Supplementary Fig. 1). Despite this detection, PT5 remained disease-free after completing ET and continues to be regularly clinical monitored.

Notably, the clinical follow-up duration for this patient (< 150 months) is shorter than the time from initial BC diagnosis to relapse observed in other patients in the cohort (median = 161 months, range 83–187 months) (Fig. 2, Table 2).

Among non-relapsed patients, ctDNA remained undetectable in 93% of cases, supporting its potential role in MRD assessment. Notably, MRD positivity was consistently associated with disease recurrence, whereas ctDNA negativity was observed in most patients who remained relapse-free. Of note, two non-relapsed patients with negative MRD detection (PT8 and PT12) were included in the analysis, but their panels contained fewer than two biomarkers, which should be considered when interpreting their results.

Discussion

This study investigates the use of plasma ctDNA detection to MRD in patients with high-risk HR + BC who were 5 or more years post-diagnosis. Employing a tumor-informed assay that tracks multiple somatic mutations, we explored the relationship between MRD detection and the occurrence of both distant and local recurrences. The selection of this high-risk cohort was guided by the rationale that these patients are most likely to harbor MRD, thus providing an optimal context for assessing ctDNA prognostic value and clinical relevance.

The findings from this study align with previous research underscoring the prognostic value of ctDNA in early-stage BC [4,19,28,29]. The results highlight the potential of patient-specific ctDNA assays, such as the CloneSight test, to predict relapse significantly ahead of clinical detection, offering a window for early therapeutic intervention. Notably, CloneSight's ability to detect ctDNA up to 68 months before clinical progression demonstrates an unprecedented lead time compared to similar studies [4, 19], that could redefine monitoring protocols.

In our series, we observed MRD positivity even in patients with local relapse and brain metastases, clinical situations where ctDNA detection is more challenging [18,19,30,31]. However, the variability in MRD detection among HR + BC patients reflect reported challenges, were MRD-positive patients did not present clinical evidence of relapse [4,19,32]. In this regard, patient PT5 is the only individual with a positive MRD test who has not yet developed a clinical relapse. While this patient exhibited a single positive plasma sample during monitoring, she remained disease-free following the completion of ET. However, the follow-up duration for PT5 is notably shorter (< 150 months) than the median time to relapse observed in other relapsed patients (161 months, range 83–187 months). This raises the possibility that PT5 may still be at risk of recurrence, underscoring the importance of long-term monitoring in MRD-positive patients. In this regard, prior studies suggest that transient ctDNA positivity without confirmed relapse, as seen in some HR + BC patients, might represent shedding from indolent micrometastases or tumor activity modulated by treatment, rather than false positives [4,19]. For instance, intermittent ctDNA release during ET may occur, suggesting ongoing tumor suppression rather than growth. Further longitudinal studies are warranted to better understand the prognostic implications of isolated MRD positivity and its potential role as an early indicator of late recurrence.

On the other hand, cases where patients developed recurrent disease without ctDNA detection may be influenced by the lower levels of ctDNA typically associated with indolent disease. The underlying tumor biology of HR + BC, including a lower proliferation rate, frequent relapse sites such as bone or brain, and ongoing treatment with ET, may contribute to these discrepancies. The clinical implications of these findings suggest that repeated and consistent ctDNA monitoring is vital for accurate MRD assessment. Serial sampling could capture intermittent ctDNA release, potentially improving the sensitivity of MRD detection, especially for HR + BC patients.

The findings of this study provide valuable insights into the performance of the MRD assay for detecting clinical recurrences in a cohort of HR + early BC patients. Notably, ctDNA was detected in 50% of relapsed patients, while remaining undetectable in the majority of non-relapsed cases. Previous studies have reported varying MRD detection rates across different BC subtypes, with lower detection frequencies typically observed in HR +/HER2- disease [4,19]. This may be attributed to the biological characteristics of HR + tumors, including lower ctDNA shedding and typically lower TMB, which may pose challenges for ctDNA-based MRD detection. Despite these limitations, the ability of the assay to detect late recurrences months to years before clinical progression highlights its potential for identifying patients at risk of relapse and informing long-term disease monitoring strategies.

The detection of MRD using ctDNA has been extensively studied across different BC subtypes, revealing substantial differences in both MRD detection rates and lead time to recurrence. In this study, CloneSight, a WES-based MRD assay, demonstrated a limit of detection (LOD) of 0.0024% VAF and the ability to detect ctDNA up to 68 months before clinical progression. This lead time is considerably longer than those reported in previous studies evaluating RaDaR, Signatera, NeXT Personal, and PCM, particularly in HR +/HER2- early BC,

where MRD detection remains challenging due to the biological characteristics of these tumors.

The study by Lipsyc-Sharf et al. evaluated RaDaR (NeoGenomics/Inivata) in a high-risk HR +/HER2- early BC cohort, reporting a lead time of up to 37.6 months and an LOD ranging from 0.0023% to 0.8019% VAF [19]. Similarly, Coombes et al. assessed Signatera (Natera) in HR +/HER2- early BC, demonstrating a lead time of up to 24 months and an LOD of 0.01% VAF [12]. Both studies provide a direct clinical comparison for CloneSight, as they focus on late recurrence detection in HR +/HER2- patients, where dormant residual disease may persist for extended periods before relapse.

In this regard, Garcia-Murillas et al. analyzed NeXT Personal (Personalis), a whole-genome sequencing (WGS)-based assay, which tracks a median of 1,451 mutations and achieved an LOD of $\leq$0.001% VAF with a lead time of up to 61.5 months. However, this study included a mixed cohort of BC subtypes (HR +/HER2-, HER2 +, and TNBC) and was not specifically designed to assess MRD detection prior to late relapse in HR +/HER2- patients, making direct comparisons more complex. Notably, the longest lead times in this study were observed in TNBC patients, likely reflecting the higher ctDNA shedding typically associated with more aggressive tumor biology [33].

Recently, Garcia-Murillas et al., also evaluated the PCM assay (Invitae) in a cohort of early BC patients representing different subtypes. The study reported a lead time up to 58.9 months. Again, the study demonstrated that lead time to relapse varies significantly across BC subtypes, with the upper end of the range observed in TNBC patients [34].

The ability of CloneSight to detect ctDNA up to 68 months before clinical progression suggests a potential advantage in identifying late recurrences in HR +/HER2- early BC, where ctDNA levels are typically lower, and MRD detection is less frequent. The longer lead time observed in this study, compared to RaDaR, Signatera, and PCM, may be indicative of the potential sensitivity of CloneSight in detecting MRD in this challenging clinical context.

This study highlights potential therapeutic considerations for high-risk luminal BC patients, particularly those with detectable MRD. Early ctDNA detection creates a critical window for timely intervention with therapies such as CDK4/6 inhibitors, targeted agents, or extended ET, potentially altering the trajectory of disease relapse. Additionally, considering the enduring risk of late recurrence in luminal BC, long-term and consistent MRD monitoring is crucial. High-risk patients, especially those with indolent disease, could benefit from personalized surveillance strategies, enhancing early detection and optimizing treatment outcomes.

The limitations of our study include several factors that may impact the robustness of our findings. First, the small sample size limits the statistical power and generalizability of the results. Second, the relatively short duration of plasma follow-up reduces the ability to capture late recurrences, which could affect the long-term prognostic value of the MRD test in this cohort. Third, the lack of concurrent imaging data is a limitation, as patients were not routinely imaged unless symptomatic. As a result, we cannot confirm whether MRD-positive patients had radiographically detectable metastases at the time of ctDNA detection. Additionally, the blood volume collected for analysis represents another limitation, as larger volumes could increase the likelihood of detecting highly diluted ctDNA in plasma samples. Future studies should incorporate regular imaging and optimize blood collection protocols to enhance the sensitivity and correlation of MRD findings with clinical outcomes.

Conclusion

In conclusion, ctDNA monitoring represents a highly promising approach for the early detection of relapse in BC. This study highlights the potential of CloneSight, a novel MRD-detection assay, to provide enhanced monitoring capabilities. CloneSight enables the detection of relapse at earlier time points than previously reported in similar studies, highlighting its potential for identifying patients at risk of late recurrence and improving long-term disease monitoring. By focusing exclusively on previously identified tumor-specific markers, CloneSight achieves highly targeted, deep sequencing coverage while significantly reducing sequencing costs. Unlike generic panels or broad-spectrum sequencing methods, this tailored approach enables patient-specific MRD detection, optimizing ctDNA tracking for long-term disease monitoring, underscoring its potential as a pivotal tool for improving patient outcomes through earlier intervention and the refinement of personalized treatment strategies. While these findings are promising, further research is necessary to validate its potential advantages and determine its optimal clinical application.

Abbreviations

BC	Breast cancer
cfDNA	Cell-free DNA
ctDNA	Circulating tumor DNA
ER	Estrogenic receptor
ET	Endocrine therapy
FFPE	Formalin-fixed paraffin-embedded
HER2	Human epidermal growth factor receptor 2
HR	Hormone receptor
IHC	Immunohistochemistry
INDEL	Insertion and deletion
LOD	Limit of detection

mPCR	Multiplex polymerase chain reaction
MRD	Minimal residual disease
NGS	Next-generation sequencing
NPV	Negative predictive value
PBMCs	Peripheral blood mononuclear cells
PPV	Positive predictive value
PR	Progesterone receptor
SD	Standard deviation
SNVs	Single nucleotide variants
TMB	Tumor mutation burden
VAF	Variant allele frequency
WES	Whole exome sequencing
WGS	Whole genome sequencing

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13058-025-02016-7.

Additional file1

Acknowledgements
The authors extend their gratitude to all the women who participated in this study. We also sincerely thank the funding organizations, "Ayuda RYC2023-044581-I financiada por MICIU/AEI/https://doi.org/10.13039/501100011033 y por el FSE+" and CIBERONC, for their support in funding the contracts of I.C-M. and J.V.-S.

Author contributions
Conceived and designed study: A.G.-Z., I.C.-M., E.A., J.P.; Conducting experiments: I.C.-M., J.V.-S., M.E.Q.-O.; Data analysis: A.G.-Z., I.C.-M., J.V.-S., C.G.-R., E.L.-L., A.M., P.L., Y.H.; Writing, reviewing and editing: I.C.-M., A.G.-Z., E.A., J.P. J.V.-S, A.M., Y.H. All authors read and approved the final manuscript.

Funding
This work was supported by a Pfizer Grant (Grant ID: 53234235). The funder had no role in study design data collection and analysis, decision to publish or preparation of the manuscript.

Availability of data and materials
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All participants provided written informed consent prior to enrolment in the study. The study protocol was reviewed and approved by the Ethics Committee of the "Instituto Valenciano de Oncología", complying with all relevant ethical regulations including the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Medical Oncology Department, Hospital Universitario Virgen de la Victoria, 29010 Malaga, Spain. [2]The Biomedical Research Institute of Málaga, IBIMA-CIMES-UMA), 29010 Malaga, Spain. [3]Centro de Investigación Biomédica en Red de Cancer (CIBERONC - CB16, 12/00481), 28029 Madrid, Spain. [4]Hematological Malignancies Clinical Research Unit, Centro Nacional de Investigaciones Oncologicas (CNIO), Madrid, Spain. [5]Altum Sequencing Co, Madrid, Spain. [6]Faculty of Medicine, University of Málaga, 29010 Malaga, Spain. [7]Medical Oncology Department, Fundación Instituto Valenciano de Oncología, Valencia, Spain.

Received: 21 January 2025 Accepted: 4 April 2025
Published online: 01 May 2025

References

1. Howlader N, Altekruse SF, Li CI, Chen VW, Clarke CA, Ries LAG, Cronin KA. US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. JNCI: J Natl Cancer Inst. 2014. https://doi.org/10.1093/jnci/dju055.
2. Berry DA, Cronin KA, Plevritis SK, Fryback DG, Clarke L, Zelen M, et al. Effect of screening and adjuvant therapy on mortality from breast cancer. N Engl J Med. 2005;353(17):1784–92.
3. Pan H, Gray R, Braybrooke J, Davies C, Taylor C, McGale P, et al. 20-Year Risks of Breast-Cancer Recurrence after Stopping Endocrine Therapy at 5 Years. N Engl J Med. 2017;377(19):1836–46.
4. Shaw JA, Page K, Wren E, de Bruin EC, Kalashnikova E, Hastings R, et al. Serial Postoperative Circulating Tumor DNA Assessment Has Strong Prognostic Value During Long-Term Follow-Up in Patients With Breast Cancer. JCO Precis Oncol. 2024;8: e2300456.
5. MJ IJ, Berghuis AMS, de Bono JS, Terstappen L. (2018) Health economic impact of liquid biopsies in cancer management. Expert Rev Pharmacoecon Outcomes Res:1–7 593
6. Alba-Bernal A, Lavado-Valenzuela R, Dominguez-Recio ME, Jimenez-Rodriguez B, Queipo-Ortuno MI, Alba E, et al. Challenges and achievements of liquid biopsy technologies employed in early breast cancer. EBioMedicine. 2020;62: 103100.
7. Heidrich I, Ackar L, Mossahebi Mohammadi P, Pantel K. Liquid biopsies: Potential and challenges. Int J Cancer. 2020.
8. Vaidyanathan R, Soon RH, Zhang P, Jiang K, Lim CT. Cancer diagnosis: from tumor to liquid biopsy and beyond. Lab Chip. 2018;19(1):11–34.
9. De Rubis G, Rajeev Krishnan S, Bebawy M. Liquid Biopsies in Cancer Diagnosis, Monitoring, and Prognosis. Trends Pharmacol Sci. 2019;40(3):172–86.
10. Chin RI, Chen K, Usmani A, Chua C, Harris PK, Binkley MS, et al. Detection of Solid Tumor Molecular Residual Disease (MRD) Using Circulating Tumor DNA (ctDNA). Mol Diagn Ther. 2019.
11. Pantel K, Alix-Panabieres C. Liquid biopsy and minimal residual disease - latest advances and implications for cure. Nat Rev Clin Oncol. 2019;16(7):409–24.
12. Coombes RC, Page K, Salari R, Hastings RK, Armstrong A, Ahmed S, et al. Personalized Detection of Circulating Tumor DNA Antedates Breast Cancer Metastatic Recurrence. Clin Cancer Res. 2019;25(14):4255–63.
13. McDonald BR, Contente-Cuomo T, Sammut SJ, Odenheimer-Bergman A, Ernst B, Perdigones N, Chin SF, Farooq M, Mejia R, Cronin PA, Anderson KS, Kosiorek HE, Northfelt DW, McCullough AE, Patel BK, Weitzel JN, Slavin TP, Caldas C, Pockaj BA, Murtaza M. Personalized circulating tumor DNA analysis to detect residual disease after neoadjuvant therapy in breast cancer. Sci Trans Medicine. 2019. https://doi.org/10.1126/scitranslmed.aax7392.
14. Alba-Bernal A, Godoy-Ortiz A, Dominguez-Recio ME, Lopez-Lopez E, Quiros-Ortega ME, Sanchez-Martin V, et al. Increased blood draws for ultrasensitive ctDNA and CTCs detection in early breast cancer patients. NPJ Breast Cancer. 2024;10(1):36.
15. Martincorena I, Campbell PJ. Somatic mutation in cancer and normal cells. Science. 2015;349(6255):1483–9.
16. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018.
17. Jimenez-Rodriguez B, Alba-Bernal A, Lopez-Lopez E, Quiros-Ortega ME, Carbajosa G, Garrido-Aranda A, et al. Development of a Novel NGS Methodology for Ultrasensitive Circulating Tumor DNA Detection as a Tool for Early-Stage Breast Cancer Diagnosis. Int J Mol Sci. 2022;24(1).
18. Parsons HA, Rhoades J, Reed SC, Gydush G, Ram P, Exman P, et al. Sensitive Detection of Minimal Residual Disease in Patients Treated for Early-Stage Breast Cancer. Clin Cancer Res. 2020;26(11):2556–64.
19. Lipsyc-Sharf M, de Bruin EC, Santos K, McEwen R, Stetson D, Patel A, et al. Circulating Tumor DNA and Late Recurrence in High-Risk Hormone Receptor-Positive, Human Epidermal Growth Factor Receptor 2-Negative Breast Cancer. J Clin Oncol. 2022;40(22):2408–19.

20. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol. 2007;25(1):118–45.
21. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25(16):2078–9.
22. Chen X, Schulz-Trieglaff O, Shaw R, Barnes B, Schlesinger F, Kallberg M, et al. Manta: rapid detection of structural variants and indels for germline and cancer sequencing applications. Bioinformatics. 2016;32(8):1220–2.
23. Saunders CT, Wong WS, Swamy S, Becq J, Murray LJ, Cheetham RK. Strelka: accurate somatic small-variant calling from sequenced tumor-normal sample pairs. Bioinformatics. 2012;28(14):1811–7.
24. Krishnamachari K, Lu D, Swift-Scott A, Yeraliyev A, Lee K, Huang W, et al. Accurate somatic variant detection using weakly supervised deep learning. Nat Commun. 2022;13(1):4248.
25. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol. 2013;31(3):213–9.
26. Jimenez-Ubieto A, Poza M, Martin-Munoz A, Ruiz-Heredia Y, Dorado S, Figaredo G, et al. Real-life disease monitoring in follicular lymphoma patients using liquid biopsy ultra-deep sequencing and PET/CT. Leukemia. 2023;37(3):659–69.
27. Alvarez N, Martin A, Dorado S, Colmenares R, Rufian L, Rodriguez M, et al. Detection of minimal residual disease in acute myeloid leukemia: evaluating utility and challenges. Front Immunol. 2024;15:1252258.
28. Garcia-Murillas I, Cutts RJ, Walsh-Crestani G, Phillips E, Hrebien S, Dunne K, et al. Longitudinal monitoring of circulating tumor DNA to detect relapse early and predict outcome in early breast cancer. Breast Cancer Res Treat. 2024.
29. Cutts R, Ulrich L, Beaney M, Robert M, Coakley M, Bunce C, et al. Association of post-operative ctDNA detection with outcomes of patients with early breast cancers. ESMO Open. 2024;9(9): 103687.
30. Garcia-Murillas I, Chopra N, Comino-Mendez I, Beaney M, Tovey H, Cutts RJ, et al. Assessment of Molecular Relapse Detection in Early-Stage Breast Cancer. JAMA Oncol. 2019;5(10):1473–8.
31. Magbanua MJM, Swigart LB, Wu HT, Hirst GL, Yau C, Wolf DM, et al. Circulating tumor DNA in neoadjuvant-treated breast cancer reflects response and survival. Ann Oncol. 2021;32(2):229–39.
32. Olsson E, Winter C, George A, Chen Y, Howlin J, Tang MH, et al. Serial monitoring of circulating tumor DNA in patients with primary breast cancer for detection of occult metastatic disease. EMBO Mol Med. 2015;7(8):1034–47.
33. Garcia-Murillas I, Abbott CW, Cutts RJ, Boyle SM, Pugh J, Keough KC, et al. Whole genome sequencing powered ctDNA sequencing for breast cancer detection. Ann Oncol. 2025.
34. Garcia-Murillas I, Cutts RJ, Walsh-Crestani G, Phillips E, Hrebien S, Dunne K, et al. Longitudinal monitoring of circulating tumor DNA to detect relapse early and predict outcome in early breast cancer. Breast Cancer Res Treat. 2025;209(3):493–502.

Publisher's Note