



REVIEW ARTICLE

Metastasis

The breast is yet to come: current and future utility of circulating tumour DNA in breast cancer

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Advances in genomic strategies and the development of targeted therapies have enabled precision medicine to revolutionise the field of oncology. Precision medicine uses patient-specific genetic and molecular information, traditionally obtained from tumour biopsy samples, to classify tumours and treat them accordingly. However, biopsy samples often fail to provide complete tumour profiling, and the technique is expensive and, of course, relatively invasive. Advances in genomic techniques have led to improvements in the isolation and detection of circulating tumour DNA (ctDNA), a component of a peripheral blood draw/liquid biopsy. Liquid biopsy offers a minimally invasive method to gather genetic information that is representative of a global snapshot of both primary and metastatic sites and can thereby provide invaluable information for potential targeted therapies and methods for tumour surveillance. However, a lack of prospective clinical trials showing direct patient benefit has limited the implementation of liquid biopsies in standard clinical applications. Here, we review the potential of ctDNA obtained by liquid biopsy to revolutionise personalised medicine and discuss current applications of ctDNA both at the benchtop and bedside.

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BACKGROUND

Advances in methods to identify and detect tumour-specific molecular biomarkers over the past two decades have led to an increased potential for personalised treatment. The focus on precision medicine in the field of oncology has vastly improved the development of targeted therapies and overall patient outcome^{1,2}. Moreover, despite the initial success of targeted therapies, cancers often develop distinct mechanisms of resistance and patients eventually relapse, making the need to detect and monitor molecular biomarkers in response to treatment important. Although tissue/tumour biopsy samples remain the gold standard from which molecular information is gathered, the presence of tumour heterogeneity or multiple tumour sites presents a significant challenge. Furthermore, tissue biopsies can be expensive, invasive, and difficult to perform³. The development of liquid biopsies, and advances in the detection of circulating tumour DNA (ctDNA)—fragments of DNA derived from tumour cells—have provided clinicians with an effective method to address the shortcomings associated with traditional biopsy samples.

Liquid biopsy samples refer to harvested bodily fluids, often blood, and the associated components (RNA, DNA, circulating tumour cells, extracellular vesicles, etc.), which provide information on potential biomarkers⁴. In this review, we will focus specifically on the potential clinical use of ctDNA detected in liquid biopsy samples. The presence of ctDNA in liquid biopsy samples enables clinicians to serially collect and quantify genetic information from multiple tumour sites. Consequently, this benefit, among others, has led to an explosion in research to determine the many potential applications of liquid biopsy in

screening, prognostics, treatment monitoring, and clinical decision making, and a number of FDA-approved techniques that assess ctDNA are currently in use (Box 1).

However, despite the numerous potential benefits of ctDNA-based liquid biopsy, several ongoing concerns exist. For example, the mutational profiles obtained from tumour biopsy samples are not always concordant with those present in ctDNA, which potentially raises concerns about sensitivity and specificity^{5,6}. These discrepancies often result from the low levels of tumour-derived mutations in blood, along with the presence of naturally occurring variants that are not associated with the tumour, including clonal haematopoiesis, in which blood cells derived from a single clone are overrepresented. The low abundance of ctDNA in the blood might also fail to provide an accurate representation of tumour heterogeneity. Sample processing issues can further exacerbate issues of low ctDNA concentration and contamination by non-cancer associated variants^{7–9}. Unfortunately, a consensus on appropriate pre-analytical steps to alleviate these difficulties has yet to be determined. To address these overarching concerns, scientists are working to improve techniques used throughout ctDNA pipelines, including isolation, detection and analysis. However, until these issues are sufficiently addressed and benefit is demonstrated in clinical trials, the use of ctDNA-based tests is best limited to specific situations. Currently, qualitative mutation detection for personalised treatment choices, particularly for late-stage disease, is the most prominent clinical use. However, translational research laboratories and companies have demonstrated the increasing potential of ctDNA analysis in applications such as disease surveillance and identification, which could significantly impact the mortality rates of individual cancers.

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Box 1 FDA-approved ctDNA-based assays for cancer profiling

The number of FDA-approved biopsies that profile ctDNA is steadily growing. At the time of this review four different assays, with different goals, are approved for ctDNA detection. The first test to be approved was the cobas® EGFR Mutation Test v2. This test was initially approved for detecting *EGFR* mutations in tissue to help identify patients eligible for erlotinib. However, over time the test has been developed and adapted. In 2016 this test it was also approved for use on plasma specimens to identify patients eligible for osimertinib, and in 2020 was further expanded to all five currently approved *EGFR* inhibitors. A test similar to the cobas® test is the therascreen® PIK3CA RGQ PCR Kit, a companion diagnostic for the PI3K inhibitor, alpelisib, that detects *PIK3CA* mutations through quantitative PCR in both tissue and blood. Distinct from these tests is the Epi proColon® test. Instead of indicating a drug, this test is used to aid in the early identification of colorectal cancer by detecting the methylation of *SEPT9* by real-time PCR in those refusing or unable to undergo a colonoscopy. These tests query only one gene for specific modifications. However, the next generation of ctDNA-based liquid biopsy tests profile many genes at once through sequencing. These tests, such as Guardant360® CDx and FoundationOne® Liquid CDx, are approved as companion diagnostics for the use of specific drugs, as well as for general tumour profiling. The ability to develop a mutational profile from a single blood sample can revolutionise patient care and may identify potential off-label uses of targeted therapies upon failure of traditional therapies. The continuous improvement and utilisation of liquid biopsy tests has the potential to improve patient outcomes, both through recommending specific treatments as most of these tests imply, as well as alternative uses such as early cancer detection as Epi proColon® implies. Information was collected from FDA approvals and company websites.

Presently, the clinical utility of ctDNA-based liquid biopsy approaches is being heavily explored in clinical breast cancer research. Breast cancer genetics, genomics and mutational profiles have been well-studied and defined over the years, and researchers are focusing on using ctDNA in conjunction with additional diagnostic tests in an effort to improve detection and treatment. Furthermore, the multiple subtypes of breast cancer each have their own molecular targets with well-known resistance mutations, for which ctDNA analysis could inform treatment decisions. In this review we seek to highlight commonly used ctDNA detection methods, survey the literature on the use of ctDNA in breast cancer and delineate areas of continuing development in ctDNA-based liquid biopsy towards providing personalised care for patients with breast cancer.

SAMPLE PROCESSING

The extraction and isolation of cell free DNA from plasma is an important step in the ability to utilise liquid biopsies for genetic information. Improper sample processing can lead to potential contamination from the rapid lysis of white blood cells. Although EDTA collection tubes may be used, they require sample processing within 6 h of sample collection or the sample is compromised. Streck Cell-Free DNA BCT collection tubes allow for stable storage blood for up to 14 days at room temperature and are ideal for circulating free DNA (cfDNA) isolation¹⁰. Blood is centrifuged to separate the plasma, which contains ctDNA, and the buffy coat, which contains germline DNA¹¹. Plasma is centrifuged a second time to remove cellular contaminants and processed using a circulating nucleic acid isolation kit (i.e. QIAamp® Circulating Nucleic Acid Kit). The sample is then ready to be analysed. An overview of sample processing for ctDNA is shown in Fig. 1.

MEASURING CTDNA OBTAINED BY LIQUID BIOPSY

Techniques for measuring cfDNA were first introduced in the early 1990s with a technique that utilised allele-specific PCR¹². As technologies for detecting cfDNA expanded, they developed into two distinct but broad categories: PCR-based techniques and next generation sequencing (NGS)-based techniques. These techniques each have their benefits and disadvantages. Below is a broad overview of a few cfDNA technologies to

date (Fig. 2). It is important to note that the list of ctDNA technologies is expansive and new technologies are constantly being developed.

PCR-based techniques

PCR-based techniques—including real-time quantitative PCR (qPCR), Beads, Emulsion, Amplifying and Magnetics (BEAMing) and droplet-digital PCR (ddPCR)—offer the simplest approach for the detection and quantification of ctDNA. PCR-based methods generally use target-specific probes that are designed to identify single-nucleotide variants. Thus, only known variants can be queried, and only a few variants can be probed at one time. Consequently, these techniques are typically applied to detect mutations that are highly prevalent or to track previously verified mutations.

qPCR. qPCR is a rapid and cheap method that most often uses a quenched fluorescent probe, which is released during amplification and emits a detectable fluorescent signal. Although the specificity of qPCR is quite high, significant variation can occur between repeated runs¹³, which has led to the development of new qPCR-based techniques to improve assay performance. However, these detection techniques have a relatively low threshold of mutation detection (0.1%) and cannot accurately detect extremely rare variants^{14,15}.

BEAMing. BEAMing is a digital PCR method that combines the techniques of PCR and flow cytometry¹⁶. Magnetic beads are tagged with bait DNA and mixed with oil, PCR reagents and target DNA to form an emulsion. Each oil droplet is capable of undergoing PCR-mediated amplification of the DNA that is bound to the bait DNA. The emulsions are then magnetically purified and opened to isolate the amplified target DNA, which is identified with hybridised fluorescent probes and enumerated by flow cytometry. BEAMing has been reported to detect 1 mutant DNA molecule among 10,000 normal molecules, which is 10x more sensitive than improved qPCR assays¹⁷. However, despite the improved sensitivity, BEAMing is laborious and expensive, and only a few laboratories and commercial entities are capable of efficiently employing the technology.

ddPCR. The widely used technique of ddPCR incorporates the methodology of both qPCR and BEAMing¹⁸. ddPCR involves an emulsion of quenched fluorescent DNA probes, PCR components and sample DNA. Theoretically, the emulsion is diluted to contain ≤ 1 DNA molecule per oil droplet. PCR amplification removes the probe's quencher, and fluorescent droplets are quantified using flow cytometry. Compared to BEAMing and qPCR, ddPCR is a relatively inexpensive method and offers improved performance metrics^{13,19,20}.

Next generation sequencing (NGS)-based techniques

Next-generation sequencing (NGS) is technically complicated but offers the ability to multiplex samples in a single run, thereby significantly improving scalability and allowing for the detection of rare or unknown mutations—that is, a priori knowledge of the mutation is not required. The inner workings of NGS are beyond the scope of this review and have been discussed elsewhere^{21,22}. Generally, however, NGS in the context of ctDNA evaluation involves sequencing the exons and the intron/exon junctions of a subset of cancer-related genes to identify both known and unknown mutations. Although this approach can detect mutant alleles at frequencies as low as 0.1%, many sequencing platforms have a random error rate of the same frequency²³. Therefore, standard versions of this technique might not be sufficient for analysing low amounts of ctDNA, such as those in early cancer patients or post-surgery. However, advanced barcoding systems, improved hybrid capture techniques, and/or greater sequencing

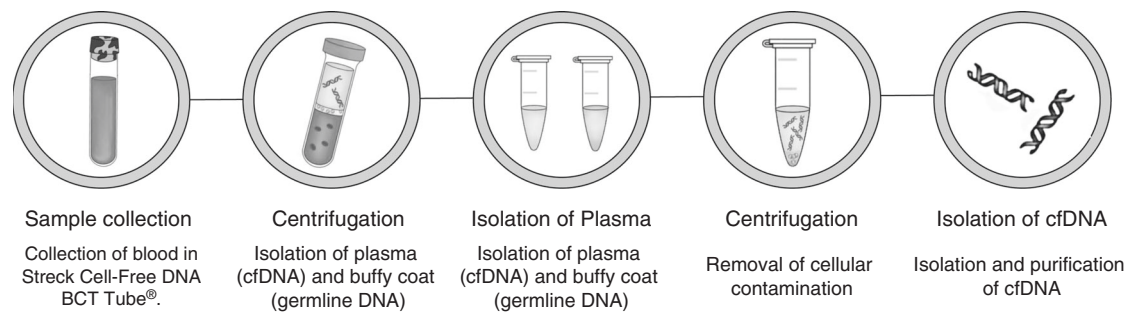


Fig. 1 Schematic of sample processing for isolation of cfDNA. Blood samples are collected in tubes made specifically to stabilize all cfDNA. The collected sample is then centrifuged and cfDNA-containing plasma is isolated. Remaining cellular contamination is eliminated with a final centrifugation, and cfDNA is isolated and purified through a variety of commercial kits.

read depth have been implemented to minimise errors and maximise detection.

The first such technology to be developed was dubbed ‘Safe-Seqs’ for Safe-Sequencing System²⁴. Additional iterations, including duplex sequencing²⁵ and Targeted Error Correction Sequencing (TEC-seq)²⁶, among others, ensued. All of these techniques use barcoded cfDNA sequencing libraries to generate redundant sequences of the same barcoded molecule. For clarity, cfDNA refers to all the DNA that is derived from a liquid biopsy—that is, DNA that originates from both normal and cancer cells—whereas ctDNA refers to DNA originating from cancer cells only (thus, ctDNA is a subset of cfDNA). These cfDNA molecules can then be consolidated to determine if any mutations found are bona fide mutations or whether they are due to sequencing errors, as true mutations should be present in multiple NGS reads containing the same barcodes. Integrated Digital Error Suppression for Cancer Personalised Profiling by deep sequencing (iDES-CAPP-seq) involves both single-stranded and double-stranded molecular barcoding with in silico error suppression, which enables the results to be optimised for molecule recovery and to detect sequencing mistakes, respectively²⁷.

The development of these technologies significantly improves the sensitivity of NGS-based techniques while enabling the interrogation of thousands of genomic positions at once. Although promising, the clinical validity of NGS-based techniques for the detection of ctDNA still requires further evaluation, and their clinical utility—that is, whether these tests can change outcomes to help guide care for breast cancer patients—has yet to be demonstrated.

CTDNA IN EARLY DISEASE DETECTION

The early detection of breast cancer is critical to patient outcome: detection at the localised stage confers a 99% 5-year relative survival rate compared with a 27% 5-year relative survival rate for distant metastases²⁸. Currently, mammography is the primary screening mode for breast cancer and is recommended by all leading medical organisations for women of average risk between the ages of 50 and 75 years. However, overdiagnosis and false positives can be associated with mammography²⁹. Similar issues have been reported for screening modalities for other cancer types, such as prostate-specific antigen (PSA) screens for prostate cancer. On the basis of the potential survival benefits derived from early detection, research groups have therefore sought to design more accurate methods of detection for early-stage disease. Currently, many approaches to detect ctDNA are being studied to determine their role(s) in early disease detection (Table 1).

Total cfDNA levels

One of the simplest approaches used to assess early stages of disease is the analysis of total cfDNA levels. Cancer patients have higher levels of total cfDNA (comprising DNA from normal

and cancer cells) compared with control populations, typically determined using qPCR or a fluorescent DNA quantification assay. Agassi et al. used a fluorescent assay to confirm that patients with early-stage breast cancer had levels of cfDNA that were higher, pre-surgery, than those measured in patients post-surgery, in healthy patients or in patients with pre-cancerous lesions. This method outperformed the blood-based breast cancer protein biomarker CA15-3, as the sensitivity and specificity of increased levels of cfDNA to detect breast cancer were 72% and 75% respectively while CA15-3 only identified 11% of patients. Notably, sensitivity of the cfDNA assay correlated with later stage disease, identifying 100% of patients with lymph node involvement³⁰. Another study using cfDNA as a biomarker demonstrated that a qPCR-based assay could correctly identify 89 out of 100 patients with early-stage breast cancer, with a false positive rate of only 6%³¹. To address the discrepancies in total cfDNA-based classifications, Yu et al. performed a meta-analysis, and determined that the pooled sensitivity and specificity of multiple techniques was 87%³². It is important to note, however, that elevated cfDNA levels are not exclusive to tumours and might be affected by other comorbidities, resulting in false positive results. The measurement of cfDNA levels has been explored as a possible adjunct test with mammography to decrease false positives, by distinguishing benign from malignant lesions, and thereby prevent overtreatment. However, Peled et al. reported that the assessment of total cfDNA in suspected breast cancer patients was unable to discriminate between benign and malignant lesions and that additional biomarkers might be required³³.

ctDNA detection

Techniques to detect ctDNA specific mutations from cfDNA, including targeted PCR-based approaches and NGS, have vastly improved the use of cfDNA in cancer detection. Targeted techniques used for screening, such as BEAMing and ddPCR, are best applied to highly prevalent mutations in breast cancer. For example, Beaver et al. used ddPCR to detect hotspot *PIK3CA* mutations from within the blood of 14 out of 15 early-stage breast cancer patients with a confirmed *PIK3CA* mutation found in tumour tissues³⁴. Although *PIK3CA* mutations occur only in approximately 45% of oestrogen-receptor-positive (ER⁺) breast cancers, the study demonstrates that ddPCR analysis is an effective targeted technique for detecting early-stage disease specifically within this population. However, digital PCR platforms can only query for known mutations, so more and more studies have begun to use NGS platforms for ctDNA detection in breast and other cancers. NGS approaches aim to increase the gene panel size and, therefore, increase mutation detection sensitivity. In a pan-cancer study, Bettegowda et al. were able to detect ctDNA in 50% of localised breast cancer cases through a combination of BEAMing and Safe-SeqS³⁵. Currently, NGS panels used for ctDNA detection test a relatively small number of genes/genomic locations and might not include less common genetic

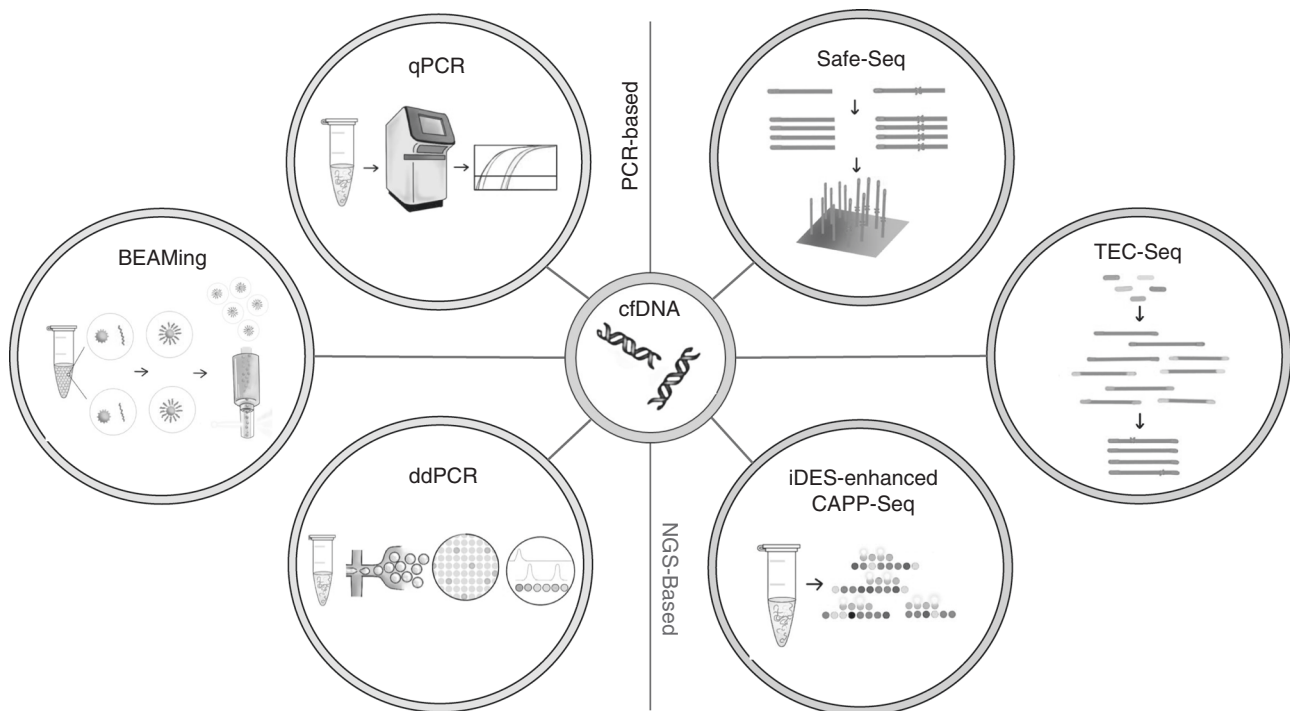


Fig. 2 cfDNA technologies mentioned in this review. Cell-free DNA is primarily analyzed for ctDNA content by PCR-based techniques or NGS-based techniques. In the PCR-based techniques few variants are probed at once whereas in NGS-based techniques many genes are queried in one sequencing run. Each technique has benefits and drawbacks regarding time, money, breadth, sensitivity, and scalability. Images were adapted from literature cited in the text.

variants that contribute to tumour phenotypes. Therefore, there remains a significant need for ctDNA detection technologies that maintain a high sequencing depth across a broad set of cancer genes, with acceptable turnaround times and costs.

ctDNA in presymptomatic detection of cancer. The use of ctDNA in detecting cancer at its earliest, pre-symptomatic, screening stage is also being studied. However, data analysing viral DNA in nasopharyngeal carcinomas suggest that ctDNA detection will require the development of additional technology to achieve the level of sensitivity and specificity necessary for a primary cancer screening test. In a prospectively enrolled clinical trial, qPCR was used to detect circulating Epstein Barr virus (EBV) DNA in asymptomatic patients before the onset of nasopharyngeal carcinoma. Certain nasopharyngeal carcinomas exhibit high levels of EBV DNA and, through gene amplification, DNA detection rates were high in these patients, with 97.1% sensitivity and 98.6% specificity. The authors of this study utilised 50 genomes of EBV, each with approximately 10 PCR target sites for their screen, suggesting that approximately 500 mutations/variants would be required to achieve similar sensitivity and specificity to more traditional mutation-based assays³⁶. This number is currently higher than the number of genes surveyed by most ctDNA NGS and targeted techniques. Although not a study of breast cancer, these data highlight the need for additional cancer biomarkers to achieve ideal screening accuracy.

To overcome this barrier, researchers have explored the inclusion of other analytes, such as protein biomarkers, into ctDNA screening. CancerSEEK (Thrive Earlier Detection) is a technique that uses a panel of cfDNA amplicons and protein biomarkers³⁷. For the detection of early-stage (stages I–III) ovarian and liver cancer, this technique achieved 99% sensitivity in a retrospective analysis; however, a 33% median sensitivity was observed for all breast cancers. It is important to note that the difference in sensitivity may be directly linked to stage. 76% of

ovarian cancer cases studied were in stage III compared to only 30% in breast cancer. This may equate for the observed differences in sensitivity while still maintaining a high specificity for both. In the prospective DETECT-A (a pan-cancer detection study performed in approximately 10,000 women), CancerSEEK in combination with PET-CT was able to increase sensitivity and specificity compared to CancerSEEK alone. Importantly, the use of PET-CT allowed the investigators to identify/locate the tissue of origin from which the cancers arose. However, the results were highly variable depending on the cancer type, and the majority of breast cancers were initially detected by standard of care screening³⁸. To expand these studies to other cancer types, with longer term follow-up, another prospective trial is currently being run (NCT04213326).

Methylation profiling. Methylated ctDNA profiling has gained attention as an alternative detection method for cancer and has the potential advantage of being able to identify the cancer type. Both targeted and untargeted studies have found that methylated gene promoters represent hundreds to thousands of changes in transformed cells and can serve as biomarkers for specific cancers, including breast cancer^{39,40}. Shen et al. performed whole genome methylation analysis in a small cohort of early-stage breast cancer patients using the technique of cell-free Methylated DNA ImmunoPrecipitation sequencing (cfMeDIP-seq) to achieve an ~85% detection rate⁴¹. Currently, large clinical studies led by GRAIL Inc. (Illumina) are ongoing to determine the potential of whole genome methylation profiling for early detection (NCT03934866; NCT03085888; NCT04241796; NCT03372902). Preliminary reports indicate that 60% of early-stage hormone-receptor negative (HR⁻) and 18% of early-stage HR⁺ breast cancers can be detected with 99% specificity⁴². The results of further prospective trials will determine the clinical validity and utility of these pan-cancer screening methods to detect early-stage cancers.

Table 1. Recently completed and ongoing clinical trials using ctDNA detection for breast cancer mentioned in the main text.

Study	Trial Number	Purpose
Early disease detection		
ASCEND	NCT04213326	To develop and validate the classification algorithm used by the CancerSEEK cancer screening test by collecting clinically annotated peripheral blood specimens from subjects with cancer and no known cancer
SUMMIT	NCT03934866	To evaluate the performance of cfNA signals measured using high-intensity sequencing (ultra-deep and ultra-broad) for the detection of invasive cancer and identification of tissue of cancer origin using a GRAIL blood test
STRIVE	NCT03085888	To evaluate the performance of the pre-specified GRAIL test to detect invasive cancers (including haematological malignancies) and to refine the predictive algorithms and cut points of the GRAIL test to detect breast and other invasive cancers
PATHFINDER	NCT04241796	To determine the time and level of testing required for diagnostic resolution following a signal detected in a multi-cancer early detection test with stratification of elevated risk groups on the basis of history of smoking, documented genetic cancer predisposition, or personal history of invasive or haematological malignancy
Prognosis: early indications of outcome and recurrence		
c-TRAK-TN	NCT03145961	To assess whether ctDNA screening can be used to detect residual disease following patients standard primary treatment for triple negative breast cancer
Opportunities for treatment of metastatic disease		
plasmaMATCH	NCT03182634	To assess whether ctDNA screening can be used to detect patient subgroups who will be sensitive to targeted therapies and determine the safety and activity of the targeted treatments
EMERALD	NCT03778931	ctDNA utilised for stratification in Phase 3 clinical study comparing the efficacy and safety of elacestrant to the SoC options of fulvestrant or an aromatase inhibitor (AI)
PADA-1	NCT03079011	To evaluate, at the onset of ESR1 mutations in circulating tumour DNA, the efficacy of a change of the hormone therapy
INTERACT	NCT04256941	To assess circulating tumour deoxyribonucleic acid (ctDNA) ESR1 mutant allele fraction (MAF) and kinetics with fulvestrant compared with AI and to correlate ctDNA with cancer antigens (CA) 15-3 tumour marker changes
BYL719 plus letrozole or exemestane for patients with HR-positive locally advanced unresectable or MBC	NCT01870505	ctDNA utilised for descriptive purposes

ctDNA PROGNOSIS: EARLY INDICATIONS OF OUTCOME AND RECURRENCE

As well as investigating the use of liquid biopsy approaches for the detection of early disease, researchers have made strides towards predicting patient outcome through ctDNA testing. This approach could allow those patients who are at high risk to receive additional treatment to prevent recurrence, as well as limiting overtreatment for those at low risk. Other studies aim to detect recurrence as early as possible to implement aggressive therapy (Table 1). If successful, these strategies could prevent suffering associated with unnecessary treatment, as well as the onset of advanced disease.

Prognostication for early disease

The early measurement of ctDNA levels across breast cancer subtypes has demonstrated prognostic ability. This prognostic potential has been particularly well studied in treatment-naïve disease. Rothé et al. found that, in HER2⁺ breast cancers, a lack of detectable *PIK3CA* and *TP53* variants in patient plasma prior to neoadjuvant therapy was associated with a high pCR (pathological complete response) rate⁴³. Similarly, a separate study demonstrated that the prevalence of mutant *PIK3CA* ctDNA pre-surgery was associated with poor relapse-free survival and overall survival, regardless of subtype⁴⁴.

The prognosis of early-stage disease using ctDNA is not limited to the detection of mutational variants in ctDNA. A small study of breast cancer patients demonstrated that detection in ctDNA of the 1q21.3 amplification pre-surgery was prognostic for relapse, regardless of subtype⁴⁵. Additionally, studies involving ctDNA epigenetics determined that the presence of one specific methylated region pre-chemotherapy was a poor prognostic factor, and >70% of patients with this marker relapsed within 5 years⁴⁶. Although these studies focused on prognostication in the pre-treatment setting, other investigators have studied ctDNA during neoadjuvant therapy. Studies in the neoadjuvant setting have shown that the levels of certain ctDNA variants measured quantitatively by ddPCR correlated directly (positively or negatively, depending on the variant) with disease-free survival and overall survival in patients with triple negative breast cancer^{47,48}. McDonald et al. corroborated this result using a specialised sequencing technique⁴⁹. While these studies indicate the power of ctDNA in prognosticating patient outcomes, no prospective clinical trial to date has validated the prognostic potential of ctDNA in early-stage disease. However, the identification and confirmation of high-risk groups through such analyses could lead to more intense treatment in a targeted high-risk population as well as de-escalation of therapy in low risk groups.

Detecting minimal residual disease and predicting subsequent relapse

The rate of breast cancer recurrence within 10 years of curative-intent therapy has been shown to be 36.8%⁵⁰. The ability to accurately identify those patients in whom cancer will recur could lead to increased survival through earlier treatment and prevention. Preliminary studies by Beaver et al.³⁴ demonstrated that ddPCR could detect mutant *PIK3CA* ctDNA in some early-stage breast cancer patients after surgery as well as before. In the 29 patient cohort, *PIK3CA* mutations were present in both tumour tissue and pre-surgery blood samples from 14 patients. Post-surgery, 5 of these 14 patients remained mutation-positive, as assessed by ddPCR, suggesting the presence of minimal residual disease (MRD), and raising the possibility that ctDNA abundance post-surgery could identify patients at risk of recurrence. Indeed, one patient with detectable ctDNA post-surgery had triple negative metaplastic breast cancer, a very aggressive form of the disease, and died within two years of her initial diagnosis. Garcia-Murillas et al.⁵¹ used ddPCR to track patient-specific ctDNA mutations in the plasma of patients with early-stage breast cancer. In this study, serial post-surgery ctDNA liquid biopsy identified 12 of 15 relapses and correctly classified 96% of non-relapsing patients. Similarly, Olsson et al. used ddPCR to demonstrate that levels of cancer-specific genetic rearrangements were associated with relapse⁵². Patient panels of 4–6 rearrangements were used to detect ctDNA retrospectively in 93% of patients in whom cancer recurred. Despite advances in ddPCR, however, this approach is limited, as mentioned above, by the number of genetic alterations that can be queried in a biased manner. In this regard, NGS overcomes this limitation owing to its ability to query multiple loci in an unbiased way. Accordingly, studies have leveraged NGS-based approaches in conjunction with liquid biopsies for the detection of ctDNA as a surrogate for MRD. Parsons et al. demonstrated that an NGS-based approach was capable of measuring 488 mutations with 100 times more power than a single mutation ddPCR test *in vitro*⁵³. MRD was detected using a patient-specific version of this assay, and all patients in whom ctDNA was detected 1-year post treatment relapsed (6 of 6). However, many patients still relapsed without the detection of ctDNA at this timepoint, potentially owing to the occurrence of longer disease-free intervals in these patients. In a similar approach by Coombes et al.⁵⁴, 49 breast cancer patients were monitored using ultradeep sequencing of 16 variants to detect ctDNA as an MRD biomarker associated with recurrence. ctDNA was detected in 16 of the 18 patients in whom recurrence occurred, as early as 2 years prior to relapse. These studies indicate that the frequency and duration of monitoring will be critical issues for prospective trials that are monitoring ctDNA as a prognostic marker for MRD and relapse. Additionally, these trials must also address how the detection of ctDNA might influence treatment escalation, which is currently being explored in c-TRAK TN (NCT03145961).

OPPORTUNITIES FOR THE TREATMENT OF METASTATIC DISEASE

The levels of ctDNA have been demonstrated to directly reflect the tumour burden and stage of a patient's cancer²⁶, indicating that ctDNA also has the potential to facilitate real-time tumour surveillance in patients with active metastatic disease. This observation has led researchers to explore the possible clinical usefulness of ctDNA in monitoring the response to therapies in the metastatic setting (Table 1).

Monitoring and prognosticating late-stage disease progression
Serial plasma monitoring in patients with metastatic breast cancer gives clinicians the opportunity to quickly determine the response (if any) to current treatment. Multiple studies in metastatic breast

cancer have demonstrated that a direct correlation exists between the levels of tumour-specific ctDNA and changes in diagnostic imaging seen in response to treatment. Although most of these studies primarily track the frequency of commonly mutated genes such as *PIK3CA* and *TP53*^{55–57}, other alterations have also been tracked and yielded similar results⁴⁵. Furthermore, these studies have demonstrated a link between the persistence of mutations in the ctDNA during ongoing therapy and poor outcome. According to additional studies, ctDNA dynamics during the early stages of treatment might also predict the clonal composition of the tumour upon progression. In the PALOMA-3 Study, a Phase 3 study of combination CDK4/6 inhibitor and fulvestrant, results suggest that early ctDNA dynamics demonstrate divergent response of tumour sub clones to treatment⁵⁸. Recently, our group demonstrated that using cfDNA, we could accurately track non-random sub clonal dynamics in heterogeneous tumours that significantly impacted the survival of cells⁵⁹. The ability to identify these variants early-on could significantly impact the treatment course and potential response upon relapse. It is important to note, however, that such techniques are not yet useful for clinical management, as discordant responses between ctDNA and scans have been documented⁶⁰, possibly due, in part, to tumour heterogeneity and the subclonal nature of metastatic breast cancer. For this method of surveillance to become clinically relevant, assay sensitivity, subclonal dynamics, and the emergence of resistance mutations need further study and development.

Treatment resistance and iterative drug choice

Despite the existence of effective treatments for early-stage and late-stage breast cancer, the majority of metastatic breast cancers will develop resistance. Many mechanisms of resistance exist, including those mediated by microenvironmental and metabolic factors, but the acquisition of mutations is an established mechanism in breast cancer^{61,62}. The potential for ctDNA, accessible in plasma samples throughout most of the body, to contain subclonal mutations makes it an ideal tool for revealing somatic mutations in the metastatic setting. The surveillance of newly arising mutations provides a method for monitoring progression and drug resistance in real time. This approach could allow clinicians to adjust treatment courses and might improve patient outcome. Although many alterations responsible for therapeutic resistance have been identified in breast cancer, few studies have identified alternative treatment courses to overcome resistance based on these specific mutations. Currently, clinical trials are making use of ctDNA to address these needs (NCT03182634; NCT03778931; NCT02102165). For example, an early assessment of plasmaMATCH (NCT03182634) showed that the detection in ctDNA of targetable mutations could lead to effective therapy in pre-treated populations. In this study, mutations in *AKT1* and *ERBB2* were targeted with capivasertib and neratinib, respectively, leading to confirmed responses in four out of 18 and five out of 20 patients⁶³. Although other arms of this study did not show similar responses, these results indicate the potential for mutation-guided treatment in late-stage, pre-treated breast cancer patients. Below, we will briefly discuss two prevalent mechanisms of resistance relevant to metastatic breast cancer: *ESR1* mutations against hormone therapy, and various downstream mutations against PI3K α inhibition.

ESR1 mutations. Resistance to endocrine therapy can evolve through multiple mechanisms, including the acquisition of mutations in *ESR1*, which encodes ER α . In 2013, Toy et al. and Robinson et al. were among the first to establish that *ESR1* mutations constitute a major mechanism of resistance to endocrine therapy in patients with HR⁺ metastatic breast cancer^{64,65}, occurring in approximately 30–40% of cases, particularly after treatment with aromatase inhibitors. Studies using ctDNA have shown that *ESR1*-mutant cells are rare in primary

breast cancer but undergo subclonal expansion when tumours are exposed to endocrine therapy, further supporting their role in treatment resistance^{66–70}. The utilisation of ctDNA in clinical studies has allowed clinicians to better understand metastatic disease and provide better options for standard of care therapy. The combined use of endocrine therapy and CDK4/6 inhibitors has recently been established as a standard of care for patients with HR⁺ metastatic breast cancer^{71–73}. Results from the PALOMA-3 study revealed early ctDNA dynamics may provide a biomarker for CDK4/6 inhibitors and, as previously mentioned, may demonstrate divergent response of subclones to treatment⁵⁸. Furthermore, ctDNA analysis from the PALOMA-3 trial revealed that treatment with fulvestrant alone and/or in combination with palbociclib led to the emergence of the *ESR1* Y537S mutation, which confers constitutive activation of the ER (and, consequently, resistance to treatment)⁷⁴. The PADA-1 trial, which evaluated the utility of ctDNA to monitor the onset of *ESR1* mutations after treatment with a combination endocrine therapy and CDK4/6 inhibitor, found that the benefit is short-lived in metastatic breast cancer patients with the *ESR1* mutation compared with those with wild-type tumours⁷⁵. Finally, a combined analysis of the SoFEA and EFACT trials of patients with metastatic breast cancer who progressed on non-steroidal aromatase inhibitors showed that patients with ctDNA-detected *ESR1* mutations showed increased overall survival and progression-free survival when treated with fulvestrant instead of exemestane⁷⁶. This treatment strategy continues to be probed in the clinic through studies such as INTERACT (NCT04256941). Additionally, orally bioavailable selective ER degraders with similar mechanisms of action to fulvestrant are under development and have the potential to improve quality of life and outcome^{77,78}.

PI3K α inhibition. *PIK3CA* is the most commonly mutated gene in breast cancer and can be selected for during treatment. Mutations in this gene are often truncal driver mutations, with the same mutation present at the beginning of therapy and at relapse⁵⁸. However, *PIK3CA* mutations can be dynamic throughout treatment, indicating that screening and rescreening using ctDNA could be important for treatments that target these mutations in particular⁷⁹. In 2019, the PI3K α inhibitor alpelisib was FDA approved in combination with hormone therapy to target HR⁺ *PIK3CA* mutant metastatic breast cancer⁸⁰, concurrent with the approval of the Therascreen *PIK3CA* RGQ PCR kit as a companion diagnostic to detect mutations in tumours and the blood for patients conscripting onto alpelisib treatment. Unfortunately, a significant number of patients develop resistance to alpelisib. Loss of *PTEN* is one of the most well validated genetic resistance mechanisms that arises in response to PI3K α inhibition^{81,82}, but the ability to analyse ctDNA has been instrumental in identifying additional putative resistance mutations. A 2020 analysis of ctDNA pre- and post-progression on alpelisib in combination with aromatase inhibitors found that resistance mutations converged on genes downstream of the PI3K and mitogen-activated protein kinase (MAPK) pathways⁸². Therapies against many of these targets have already been developed but have yet to be extensively tested in this setting.

Thus, by using ctDNA for tumour surveillance in metastatic disease, the detection of resistance mutations might facilitate clinical decision making prior to clinical/radiographic progression while on therapy. This would ideally lead to an earlier change in therapy, with improved and personalised treatment outcomes for patients with metastatic breast cancer.

CONCLUSIONS

The detection and analysis of ctDNA from liquid biopsies has the potential to revolutionise clinical oncology. Methods for the use of ctDNA in early detection, patient stratification, therapy selection and response monitoring are currently being investigated, and

provide the opportunity for a significant paradigm shift in the clinical setting. However, large-scale clinical trials to determine the clinical utility in tumour surveillance and to address previous limitations with sensitivity and duration of follow-up are still required. The effective application of ctDNA for late-stage disease monitoring, enrolment onto trials or off-label use of targeted therapies still requires an intimate knowledge of clonal dynamics, mutation function and drug efficacy. Despite these challenges, however, the potential for ctDNA to guide clinical decision making remains high, and the prospect of using liquid biopsy approaches in the provision of personalised care for patients with breast cancer continues to fuel laboratory research and future clinical trials.

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AUTHOR CONTRIBUTIONS

B.A.D., S.C. and B.H.P. conceived the design and wrote the manuscript.

ADDITIONAL INFORMATION

Ethics approval and consent to participate Not applicable.

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Competing interests B.H.P. is a paid consultant for Jackson Labs, Casdin Capital, Pathovax, Sermonix and is a paid scientific advisory board member for Celcuity Inc. Under separate licensing agreements between Horizon Discovery, Ltd and The Johns Hopkins University, S.C. and B.H.P. are entitled to a share of royalties received by the University on sales of products. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies. B.A.D. declares no potential conflicts of interest.

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