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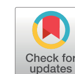
Hot Topic

# Circulating tumor DNA analysis in breast cancer: Is it ready for prime-time?



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A R T I C L E I N F O

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A B S T R A C T

Precision Medicine is becoming the new paradigm in healthcare as it enables better resources allocation, treatment optimization with a potential side-effects reduction and consequent impact on quality of life and survival. This revolution is being catalyzed by liquid biopsy technologies, which provide prognostic and predictive information for advanced cancer patients, without the analytical and procedural drawbacks of tissue-biopsy. In particular, circulating tumor DNA (ctDNA) is gaining momentum as a clinically feasible option capable to capture both spatial and temporal tumor heterogeneity.

Several techniques are currently available for ctDNA extraction and analysis, each with its preferential case scenarios and preanalytical implications which must be taken into consideration to effectively support clinical decision-making and to better highlight its clinical utility.

Aim of this review is to summarize both analytical developments and clinical evidences to offer a comprehensive update on the deployment of ctDNA in breast cancer's (BC) characterization and treatment.

## Introduction: From the traditional biopsy to the new "liquid biopsy" approach

Tumor biopsy is still considered the gold standard for the retrieval of crucial diagnostic, prognostic and predictive information in diagnostic oncology. Notwithstanding the solid evidence supporting traditional biopsy, several caveats often limit this procedure, hindering a precision-medicine approach. The static nature of this technique represents one on the main limits since it is not able to capture both the tumors' inherent biological heterogeneity and the dynamic adaptations caused by anticancer treatments [1]. Moreover, longitudinal or simultaneous multi-site testing is simply not feasible due to the clinical complications associated with seriate tissue sampling and its effects on patients' quality of life (see Figs. 1 and 2).

Currently, liquid biopsy is an attractive approach that aims to overcome such limitations and provide a more accurate representation of disease biology. As body fluids can be easily obtained, liquid biopsy is considered a non-invasive and repeatable test that allows a dynamic assessment of specific molecular markers, capable to intercept the onset of disease recurrence or treatment resistance and potentially predict treatment response and prognosis [2] (see Table 1).

Tumor cells actively release several types of nucleic acids, including DNA, i.e. cell-free DNA (cfDNA) and circulating tumor DNA (ctDNA), microRNAs (miRNAs), non-coding RNA, and microvesicles (such as exosomes) as result of their spread both as single cells, i.e. Circulating Tumor Cells (CTCs) or clusters [3–5]. Liquid biopsies rely on these

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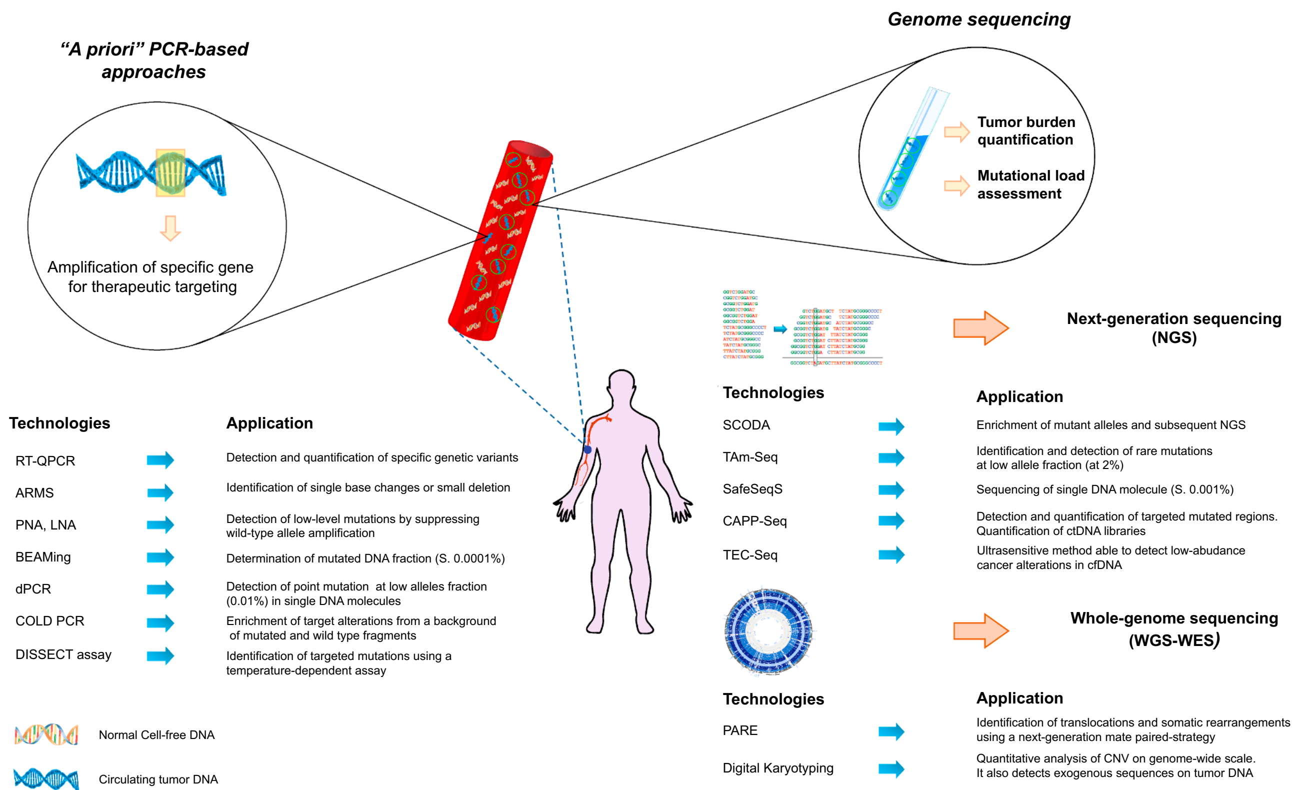


**Fig. 1.** Genome sequencing vs "a priori" techniques: comparison and application summary.

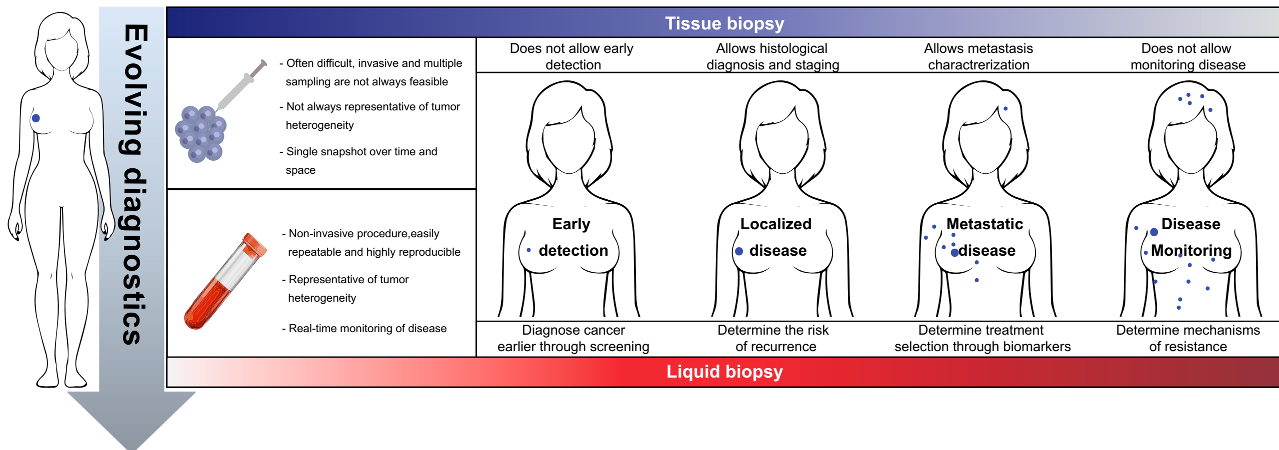


**Fig. 2.** Tissue vs liquid biopsy: advantages according to case scenario.

surrogate sources for a rapid and cost-effective molecular characterization of tumors using minimally invasive biological matrices [6,7].

The development of novel high-throughput technologies has already brought liquid biopsy approaches to the standard treatment of solid tumors, demonstrating their utility for a tissue-free cancer characterization [8].

Aim of this review is to summarize both analytical developments and clinical evidences to offer a comprehensive update on the deployment of ctDNA in breast cancer's (BC) characterization and treatment.

## Biological role of cfDNA and ctDNA

The presence of circulating cell-free DNA was first described in 1948. It can be increased in patients affected by cancer, due to presence of the additional ctDNA fraction, but also in stroke, trauma, myocardial infarction and autoimmune disease [9,10]. Moreover, since normal cells such as leukocytes can actively secrete/release DNA, it is found in small quantities also in healthy subjects [11]. In fact, cfDNA is highly heterogeneous both in size and composition, and can be detected in different body fluids [12].

The mechanism by which cfDNA is released by cells has yet to be fully clarified. Electrophoresis assays demonstrated that most fragments range between 180 and 200 base pairs (bp) and are often associated with histone proteins that form the nucleosome, suggesting that apoptotic cells could be one of the most important source of cfDNA [13,14]. These observations were strongly related to a rapid increase in circulating nucleosomes during anticancer treatments and by a rapid decrease at disease progression, supporting the idea that the quantification of nucleosome bodies can represent an efficient index of responsiveness to the therapy [15].

**Table 1**
Clinical studies assessing the impact of ctDNA analysis in breast cancer.

| Study | Type of study | Target | Results |
| --- | --- | --- | --- |
| *Neoadjuvant* | | | |
| Magbanua et al. [76] | Retrospective-prospective analysis of the I-SPY 2 trial | ctDNA levels and exploratory biomarkers | 100% of ctDNA-positive pts. at T3 (n = 5) did not achieve a pCR |
| Sotiriou et al. [75] | Retrospective analysis of the NeoALTTO trial | Copy number aberrations (CNAs) | Amplification of CNA regions on 6q23-24 associated with higher pCR (p = 0.00005 and p = 0.00087)<br>No association with EFS detected |
| Sharma et al. [77] | Prospective | Methylation status of BRCA1, MGMT, GSTP1, Stratifin, and MDR1 | GSTP1 and BRCA1 hypermethylation: independent prognostic factors of disease recurrence [HR 7.6, 95% CI (1.4–44) p = 0.021; HR 6.2, 95% CI (1.1–35.7) p = 0.04] |
| Takahashi et al. [78] | Prospective | RASSF1A DNA methylation after neo-adjuvant CT and 1 year after surgery | Met-ctDNA significantly decreased after NAC in responders (p = 0.006), correlating with residual tumor burden (p = 0.008)<br>Of the 3/7 patients who showed an increase in met-ctDNA at 1 year after surgery developed recurrence |
| *Adjuvant* | | | |
| Fiegl et al. [81] | Observational | RASSF1A DNA methylation 1 year after primary surgery | RR of recurrence 5.1 (p = 0.02); RR of death of 6.9 (p = 0.004) |
| Sharma et al. [80] | Observational | Promoter methylation of BRCA1, MGMT and GSTP1 | GSTP1 and BRCA1 hypermethylation: independent prognostic factors of disease recurrence [HR 7.6, 95% CI (1.4–44.1) p = 0.02; HR 6.2, 95% CI (1.1–35.7) p = 0.04] |
| Fujita et al. [79] | Observational | Promoter methylation of GSTP1, RASSF1A, and RARβ2 | • Met-DNA+: OS rate at 100 months 78 vs. 95% (p = 0.002)<br>• High total DNA: OS rate at 100 months 86 vs. 97% (p = 0.001)<br>• Met-DNA+/high total DNA: OS rate at 100 months 65 vs. 94% (p < 0.001) |
| *Metastatic* | | | |
| Chung et al. [83] | Observational | Genomic alterations | • TP53 (38%), ESR1 (31%) and PIK3CA (31%)<br>• ESR1-altered co-occurring with PIK3CA (35%), FGFR1 (16%), ERBB2 (8%), BRCA1/2 (5%), and AKT1 (4%) |
| AURORA (NCT02102165) | Observational | Molecular aberrations | *ongoing* |
| plasmaMATCH trial (NCT03182634) | IIa | Targetable mutations | *ongoing* with parallel assignment to targeted therapies |
| Fribbens et al. [84] | Prospective-retrospective analysis from the SoFEA and PALOMA 3 trials | ESR1 mutations | SoFEA (fulvestrant vs. exemestane):<br>• ESR1mt: PFS 5.7 vs. 2.6 months [HR 0.52, 95% CI (0.30,0.92), p = 0.02].<br>• ESR1wt: PFS 5.4 vs. 8.0 months [HR 1.07, 95% CI (0.68,1.67), p = 0.77].<br>PALOMA3 (fulvestrant/palbociclib vs. fulvestrant/placebo):<br>• ESR1mt: 9.4 vs. 3.6 months [HR 0.43, 95% CI (0.25, 0.74), p = 0.002].<br>• ESR1wt: 9.5 vs. 5.4 months [HR 0.49, 95% CI (0.35, 0.70), p < 0.001]. |
| Chandarlapaty et al. [85] | Retrospective analysis from the BOLERO-2 trial | ESR1 mutations (Y537S and D538G) | OS:<br>• ESR1wt: 32.1 months [95% CI, 28.09–36.4 months]<br>• ESR1mt: 20.73 months [95% CI, 17.71–28.06 months]<br>• D538G: 25.99 months [95% CI, 19.19–32.36 months]<br>• Y537S: 19.98 months [13.01–29.31 months]<br>• D538G/Y537S: 15.15 months [95% CI, 10.87–27.43 months] |
| Mastoraki et al. [86] | Observational | ESR1 methylation | Lack of response to everolimus + exemestane (p = 0.023, Fisher exact test). |
| Spoerke et al. [118] | Retrospective analysis of the FERGI study | PIK3CA and ESR1 mutations | PFS 3.7 months in fulvestrant ESR1wt vs:<br>• fulvestrant ESR1mt 5.4 [HR 1.056 95% CI (0.618, 1.805) p = 0.9836]<br>• fulvestrant + pictilisib ESR1wt 6.7 [HR 0.700 95% CI (0.430, 1.140) p = 0.1499]<br>• fulvestrant + pictilisib ESR1mt 5.8 [HR 0.925 95% CI (0.527, 1.625) p = 0.7870]<br>PFS 4.4 months in fulvestrant PIK3CAwt vs:<br>• fulvestrant PIK3CAmt 5.4 [HR 0.994 95% CI (0.585, 1.691) p = 0.9836]<br>• fulvestrant + pictilisib PIK3CAwt 8.2 [HR 0.593 95% CI (0.358, 0.982) p = 0.0402]<br>• fulvestrant + pictilisib PIK3CAmt 5.5 [HR 0.991 95% CI (0.590, 1.662) p = 0.9717] |
| Baselga et al. [89] | BELLE-2 phase III trial | PIK3CA mutations | PFS: Buparlisib + fulvestrant vs. Placebo + fulvestrant:<br>• PIK3CAmt: 7.0 vs. 3.2 months [HR 0.58 95% CI (0.41, 0.82) p = 0.001]<br>• PIK3CAwt: 6.8 vs 6.8 months [HR 1.028 95% CI (0.79, 1.30) p = 0.557] |

(*continued on next page*)

**Table 1** (*continued*)

| Study | Type of study | Target | Results |
|---|---|---|---|
| André et al. [90] | Retrospective analysis from the SOLAR-1 trial | PIK3CA mutations | PFS: Fulvestrant + alpelisib vs. Fulvestrant + placebo:<br>● PIK3CAmt: 11.0 vs. 5.7 months [HR 0.65; 95% CI (0.50–0.85), P = 0.00065]<br>● PIK3CAwt: 7.4 vs. 5.6 months [HR 0.85; 95% CI (0.58, 1.25)] |
| Moynahan et al. [92] | Analysis from the BOLERO-2 trial | PIK3CA mutations | PFS: Everolimus vs Placebo:<br>● PIK3CAwt: 7.36 vs. 2.96 months [HR, 0.43 (95% CI, 0.34, 0.56)]<br>● PIK3CAmt: 6.90 vs. 2.69 months [HR 0.37 (95% CI, 0.2, 0.51)] |
| Hortobagyi et al. [96] | MONALEESA-2 phase III trial | PIK3CA or TP53 mutation status, total Rb, Ki67, or p16 protein expression, and CDKN2A, CCND1, or ESR1 mRNA levels | No statistically significant difference in PFS detected |
| O'Leary et al. [97] | Retrospective analysis from the PALOMA-3 study | PIK3CA and ESR1 ctDNA dynamics after 15 days treatment | PIK3CA ctDNA dynamics predicts PFS on palbociclib and fulvestrant [HR 3.94, 95% CI (1.61–9.64), log-rank p = 0.0013] vs. *ESR1* ctDNA dynamics [HR 1.68, 95% CI (0.74–3.82), log-rank p = 0.21] |
| Ma et al. [98] | II | cHER2$^{mut}$ detection | Baseline ctDNA sequencing identified the same *HER2*$^{mut}$ with a sensitivity of 79% (90% CI, 53–%-94%) and a specificity of 100% (90% CI, 91–%-100%) |
| Stover et al. [100] | Retrospective cohort study | Somatic Copy Number Alterations in TNBC (chromosomal gains in drivers NOTCH2, AKT2, and AKT3) | Prespecified cfDNA tumor fraction threshold of ≥ 10%: worse survival (median, 6.4 vs. 15.9 months) [HR 2.14, 95% CI (1.4–3.8), p < 0.001] |
| Vidula et al. [101] | Retrospective | BRCA 1 or 2 mutations | BRCAmt: similar median PFS as compared to non-BRCA mutant BC (HR: 1.17; p = 0.58) |
| Fribbens et al. [107] | Prospective-retrospective analysis from the SoFEA trial | ESR1 and KRAS mutations | ● ESR1 mutations detectable median 6.7 months (95% CI 3.7-NA) before clinical progression and were sub-clonal in 72.2% (13/18) patients<br>● KRAS mutations in 21.2%pts: no impact on PFS and OS |
| Schiavon et al. [109] | Observational | ESR1 mutations | ESR1mt: shorter PFS on subsequent AI-based therapy [HR 3.1; 95% CI (1.9, 23.1) P = 0.0041] |
| Clatot et al. [119] | Retrospective | ESR1 circulating D538G and Y537S/N/C mutations | OS: ESR1mt vs. ESR1wt 15.5 vs. 23.8 months (p = 0.0006)<br>PFS: ESR1mt vs. ESR1wt 5.9 vs 7 months (p = 0.002)<br>After AI failure, no difference in outcome for patients receiving chemotherapy or non-AI ET in ESR1mt and ESR1wt |
| PADA-1 trial (NCT03079011) | III | ESR1 mutations | *Ongoing* with randomization to therapy according to ESR1 ctDNA levels |
| Juric et al. [120] | Ib | PIK3CA mutations | PFS: Alpelisib + fulvestrant:<br>● PIK3CAmt: 9.1 months (95% CI, 6.6–14.6 months)<br>● PIK3CAwt: 4.7 months (95% CI, 1.9–5.6 months)<br>ORR<br>● PIK3CAmt: 29% (95% CI, 17%-–43%)<br>● PIK3CAwt: no objective tumor responses |
| FINESSE trial (NCT02053636) | II | FGFR1 alterations | *Ongoing* with anti-FGDR targeted therapy |

On the other hand, cfDNA can also originate from necrotic cells, as proved by large DNA fragments generated by an incomplete and random digestion of genomic DNA or from phagocytosis by macrophages [16].

In cancer patients, ctDNA can derive both from CTCs, for the similarity of CTCs mutational profiles and the ctDNA-detected ones, and from primary or metastatic sites. In some cases, a high amount of ctDNA is not directly related to a high number of CTCs [17].

## *cfDNA quantification is non-cancer specific conditions*

Methods for cfDNA quantification are usually based on fluorometry such as the Picogreen assay or ultraviolet spectrometry such as the Nanodrop, but a standard procedure is currently lacking.

The presence and the amount of cfDNA can be also evaluated through *real-time* qPCR (SYBR green or Taqman assays) for the quantification of ubiquitous gene sequences such as GAPDH-gene, ß-globin-gene, ß-actin gene, hTERT or repetitive elements LINE1, ALU 115, ALU 24 and ALU 247 [18].

A different approach for the detection of circulating nucleosomes is the enzyme-linked immunoassay [19]. In addition to quantification, the integrity of cfDNA also seems to represent an informative marker with respect to tumor burden. In particular, the ALU DNA integrity assay performed in *real-time* qPCR, allows a measurement of the amount of short and long cfDNA fragments on the basis of noncoding repeat sequences. The cfDNA integrity index, defined as the ratio between long and short cfDNA fragments (ALU 247 and ALU 115, respectively) differs between healthy controls and primary breast cancer patients as well as between primary and metastatic breast cancer patients [20,21]. Moreover, cfDNA is not strictly cancer-specific and could be also observed in inflammatory diseases, such as lupus erythematosus, rheumatoid arthritis and thrombotic microangiopathies [22,23]. Due to the low specificity of cfDNA, most of the efforts and expectations have been shifted on circulating DNA fragments directly deriving from tumor cells (i.e. ctDNA) by analyzing tumor-specific mutations, rearrangements and methylation [24]. Due to a DNase impairment and as a consequence of an active ctDNA secretion a fourfold increase of cfDNA, with a ctDNA contribution that varies between 0.01% and ≥90% can be observed, especially in the metastatic setting [9,25].

## *Pre-analytical factors affecting ctDNA analysis*

The achievement of high specificity and sensitivity, contribute to the current status of molecular diagnostics considering ctDNA a promising tool both in early cancer screening and in advanced disease monitoring, including BC [1,26]. On the other hand, different factors can influence the standardization and the quality of cfDNA extraction and ctDNA analysis.

Determining the most appropriate matrix for cfDNA extraction, represents the first crucial step. It has been demonstrated that plasma samples are the best source of cfDNA, since the lysis of white blood cells during the coagulation process generates a large background of DNA fragments, increasing the concentration of genomic DNA about 20-fold in serum [2,27].

Another critical point is represented by the anticoagulant used in the blood collection tubes. Ethylenediaminetetraacetic acid (EDTA) is still considered the best option to ensure the stability and quality of cfDNA, since heparin could inhibit the PCR reaction necessary for ctDNA quantification and analysis [27]. The storage temperature and the time-around-time are two other crucial variables. Blood sample processing within 6 h and preservation at either 4 °C or room temperature do not affect the final concentration of cfDNA, while a longer time of conservation and hemolysis can determine an increase in cfDNA quantity, in particular of the nucleosome fraction levels. [28]. After blood processing, plasma samples can be stored at −80 °C for two weeks, while isolated cfDNA can be stored for a maximum of three months [29]. Parameters that negatively affect cfDNA integrity are repeatedly freeze-thaw cycles both of plasma and enriched cfDNA. Because of the small amount and the highly fragmented nature of the cfDNA, an efficient and reliable isolation is not always easy to perform. Commercially available kits have been optimized to increase cfDNA detection and show different grades of efficiency in terms of extraction rates [30].

The comparison of their performance is highly variable in terms of quality, quantity and contamination of cfDNA.

To date, the inability to obtain consistent and reproducible results is often caused by the lack of standardized procedures, rather than by analytical variabilities [31]. Any future application of ctDNA for diagnostic purposes should be therefore based on defined protocols in order to ensure the reproducibility of the results [2].

## Current technologies for ctDNA analysis

### *Main PCR strategies: Droplet digital PCR (ddPCR) and "Beads, Emulsion, Amplification, Magnetics digital PCR" (BEAMing)*

The deployment of traditional PCR-based liquid-biopsy techniques has been limited by several intrinsic constrains, such as limited sensitivity and the need of "*a priori*" knowledge of the investigated genomic aberrations. Last generation digital PCR (dPCR) methods, have deeply enhanced their sensitivity enabling the detection of point mutations at low allele fractions (0.01% vs 5% of the previous techniques) [20,32–37]. The most common dPCR method is the droplet digital PCR (ddPCR), characterized by the discretization and amplification of each DNA template in single emulsion droplets [38].

The separation of DNA molecules in a large number of compartments through a microfluidic system, enables high throughput analyses by reducing the reaction volume for each compartment to nano/picoliter scale. By creating individual reaction chambers, the cross-contamination between neighboring compartments could be avoided to achieve precise quantification of targets in each sample [39,40].

"Beads, Emulsion, Amplification, Magnetics digital PCR" (BEAMing) is an alternative sensitive approach which provides molecular information about mutations with a frequency of 1 over 10000. It combines emulsion PCR with magnetic beads and flow cytometry for the detection and quantification of target DNA copies. After the amplification step, each droplet contains a bead that is coated with thousands of copies of the single DNA molecule. Then, the beads are magnetically recovered and analyzed within minutes using flow cytometry or optical scanning instruments. In this way, the DNA diversity present in the template population can be accurately characterized and used to determine the fraction of mutated DNA [32,33,41].

### *Other PCR techniques*

Currently, other high-sensitivity PCR assays suitable for ctDNA analysis are employed besides ddPCR and BEAMing. These include *real time* quantitative PCR (RT Q-PCR)-based techniques, such as the Intplex Q-PCR method, the amplification-refractory mutation system (ARMS), the Peptide Nucleic Acid (PNA) or the Locked Nucleic Acid (LNA) clamping PCR, pyrophosphorolysis-activated polymerization (PAP/biPAP), co-amplification at lower denaturation temperature (COLD-PCR), differential strand at critical temperature (DISSECT).

The Inplex assay is a particular Q-PCR-based method where ctDNA can be measured in terms of concentration and presence/absence of mutations through a multi-marker analysis of short fragments [42–44].

Another system useful for single nucleotide polymorphisms (SNPs) identification is ARMS. This assay is based on the use of sequence-specific PCR primers to identify single base changes or small deletion. Since the primers only work when they are complementary to a given DNA sequence, except for a mismatched 3′-terminus, amplification is observed only if the target allele is contained within the sample [45,46].

As an alternative approach, PNA clamping PCR consists of a simple and inexpensive protocol to detect low-level mutations without using fluorescent probes. The PNA/LNA has been used to improve mutation detection by suppressing wild-type allele amplification using a specific sequence that blocks its PCR. A single base mismatch is enough to discriminate amplification of mutant type from wild type. The melting curve analysis identifies single nucleotide polymorphisms or mutations [47,48]. On the basis of specific thermal denaturation of DNA heteroduplexes, the DISSECT assay enriches mutations on target DNA without an enzymatic reaction. The discrimination is purely temperature-dependent [49]. Identification of mutated fragments can also be performed with the COLD PCR protocol. The amplification of heteroduplex DNA is preferentially carried out during PCR thermal cycling. The sequences with the target alterations are enriched from a background of mutated and wild type fragments using a low denaturation temperature [50].

Aside from mutational alterations, ctDNA can be a crucial instrument to measure also epigenetic alterations, such as promoter/enhancers methylation. Methylation-specific PCR (MS-PCR) is the most common technique for gene-specific detection of DNA methylation. To identify methylated CpG sites, a bisulfite conversion is performed as a first step. As a consequence, non-methylated cytosines are converted to uracils, while methylated sites remain unaffected. After conversion, methylated sequences are selectively amplified with methylation-specific primers [51].

### *Targeted deep sequencing*

Targeted deep-sequencing has been used to identify specific genomic regions or new somatic variants in a number of genes simultaneously, through both a pure Next Generation Sequencing (NGS) approach and a combination of PCR and NGS. A hybrid technique allows to circumvent both the sensitivity limits of NGS (1–2%) and the inherent need for "*a priori*" knowledge of the target in PCR. [52]. With this in mind, a technique called synchronous coefficient of drag alteration (SCODA) has been developed [53]. This method firstly enriches the mutant alleles and subsequently analyzes them by means of NGS. [54]. Other approaches of PCR-based targeted deep sequencing are tagged-amplicon deep sequencing (TamSeq), the Safe Sequencing System (SafeSeqS) and CAncer Personalized Profiling by deep Sequencing (CAPP-Seq).

TAm-Seq is useful for *de-novo* identification of rare cancer mutations and is capable to detect cancer-specific alterations at an allele frequency as low as 2% [55]. The SafeSeqS is a sequencing strategy which uses single molecule barcoding before PCR amplification to reduce sequencing error and increase accuracy [1,34,35]. Its theoretical

sensitivity is about 0.001%. This approach allowed to detect single somatic mutations on ctDNA in colorectal cancer patients at different stages, using plasma samples obtained at varied time points [56].

Based on a different principle, CAPP-seq, is focused on the detection and quantification of ctDNA through a probe panel consisting of biotinylated DNA oligonucleotides that target recurrently mutated regions. It is an effective way to enrich and quantify ctDNA libraries with high specificity and ultra-low detection limits [57].

Another ultrasensitive method, called targeted error correction sequencing (TEC-Seq), allow to detect low-abundance sequence alterations using NGS. Based on a panel of 58 cancer-associated genes is used to screen blood samples and analyze the most common cancer alterations in cfDNA [56].

### *Whole-genome sequencing methods*

Novel opportunities for a comprehensive ctDNA profiling are based on detecting genome-wide rearrangements using Whole Genome Sequencing (WGS) and Whole Exome Sequencing (WES) technologies, especially for the characterization of somatic chromosomal aberrations and Copy Number Variations.

Personalized analysis of rearranged ends (PARE) and digital karyotyping, represent the two technologies applicable to liquid biopsy. PARE is a method capable to identify translocations and other somatic rearrangements by taking advantage of a next-generation mate-paired analysis. It is based on short tag pairs to the ends of the template sequences followed by analysis of mate pairs to identify inter- and intra-chromosomal rearrangements [58,59].

Digital karyotyping is a technique used to quantify the CNV in ctDNA on a genome-wide scale. It does not require any prior knowledge of the molecular alteration and its LOD is about 0.001%. It is useful for detecting exogenous sequences in order to understand the depth of the intratumor heterogeneity [60].

## Utility of ctDNA analysis in BC as a basis for personalized medicine

In the recent years, increasing efforts have been made to develop innovative targeted therapies in breast cancer as the detection of tumor-initiating and secondary alterations responsible for treatment resistance and tumor progression is crucial in predicting outcomes and in selecting effective treatments and reduce empiricism. Simultaneous analysis of multiple rare mutations through high-quality genome sequencing technologies provides a *real-time* tool in assessing the development of tumors' genetic alterations [61,62]. Tissue-based next-generation sequencing remains the gold-standard technique to obtain initial information on tumor features; however, liquid biopsy represents an appealing non-invasive alternative for the characterization of the tumor's molecular heterogeneity and its evolving biology. Being still in its infancy, it is crucial to refine its common practice applications to better highlight its clinical utility [63,64].

CTCs have already been demonstrated to independently impact on outcome in prospective trials and, notably, the presence of detectable CTCs is associated with worse disease free survival (DFS) and overall survival (OS) also in patients defined as non-metastatic by conventional means [65]. In the metastatic setting, CTCs enumeration $\geq 5$ at baseline and at any subsequent follow up time point, have also been associated with shorter progression free survival (PFS) and OS [66].

On the other hand, the role of ctDNA levels as a prognostic factor has not been investigated in prospective studies yet, although quantification of tumor-specific alterations in ctDNA has already been associated with tumor burden and possible clinical progression or relapse. High levels of ctDNA are associated with a more aggressive, potentially resistant disease and have been detected both in early and later stages of breast cancer [20,67]. A combined CTCs and cfDNA analysis of 5 patients with CTCs $\geq$ 100/7.5 mL blood showed that cfDNA sequencing is more sensitive in detecting mutation than single CTCs and primary tumor-tissue [68].

Genomic rearrangements responsible for tumors' chromosomal instability in breast cancer were investigated by Leary et al. through PARE. Rearrangements were associated with amplification of the cell cycle regulatory gene cyclin-dependent kinase 6 (*CDK6*) and *ERBB2* (HER2/neu) [69].

Currently, there is an unmet clinical need for biomarkers that could identify patients who are likely responders to targeted therapies or immunotherapy. Chromosomal instability, as reflected by copy number variation in cfDNA and a high mutational tumor burden, influences BC immunogenicity, thus potentially allowing the use of immunotherapy when liquid biopsy supports a hypermutated state of ctDNA. High number of genomic alterations in variants of unknown significance (VUS) and tumor mutational burden have been independently associated to improved rates of stable disease (SD), PFS and OS especially in NSCLC and melanoma; however, few data are currently available for breast cancer patients [70,71]. Moreover, only retrospective studies on small sample sizes have investigated the role of ctDNA as a predictive marker of response to immunotherapy in BC, and further studies are needed to corroborate these findings.

## Minimal residual disease (MRD) and early detection of BC recurrence

Adjuvant chemotherapy and endocrine therapy represent the mainstay strategies for recurrence risk reduction in early breast cancer. As matter of fact, while a large number of patients will benefit from adjuvant therapies, a small subgroup does not and could be therefore exposed to unnecessary severe adverse events. Based on these premises, the identification of useful biomarkers to detect micrometastatic disease is crucial, and ctDNA analysis might play a key role in this scenario. Mostly due to ctDNA undetectable levels in micrometastatic disease, limited data are available on ctDNA in early BC. Few studies have been performed in this setting, using high sensitivity dPCR. In a prospective cohort of 55 women with early breast cancer, who had received neoadjuvant chemotherapy before surgery, detection of ctDNA with serial follow-up plasma samples was able to identify quite accurately patients at risk of distant recurrence [72]. Consistently, post-surgical levels of ctDNA were also found to be quantitatively predictive of both poor prognosis and risk of relapse [73]. Therefore, liquid biopsy is a promising option for detecting driver somatic mutations, and the inherently low ctDNA levels found in early stages represent a challenge for WGS and WES techniques. The CancerSEEK multi-analyte blood test was designed to combine the evaluation of genetic alterations in ctDNA and protein biomarkers in order to identify solid tumors at relatively early stage before distant metastases could occur. CancerSEEK presented a median sensitivity of 73% for stage II, 78% for stage III and 43% for stage I cancers (ranging from 98% in ovarian cancers to 33% in breast cancers) and specificity > 99%. Most importantly, concordance between tumor tissue biopsy and ctDNA, when levels were significant, was evident in 90% of cases among all tumors [74]. However, the implementation of tools like CancerSEEK in larger prospective studies is required for further validation.

## Neo-/adjuvant setting

Because of the curative intent of the neoadjuvant and adjuvant settings, monitoring and detecting minimal disease is of pivotal importance to assess treatment response, and potentially guide therapeutic adaptations. An ancillary ctDNA analysis of the NeoALTTO trial showed that the detection of *PIK3CA* or *TP53* mutations before neoadjuvant therapy was associated with a decreased probability of pCR but not with event-free survival (EFS). On the other hand, ctDNA presence was not significantly associated with pCR or EFS neither at 2 weeks after the treatment start, nor before surgery [75]. Consistent results were reported by a translational sub-study of the I-SPY 2 trial,

where high ctDNA at the pre-neoadjuvant time point was associated with tumor burden, aggressive biology and subtype, while the presence of ctDNA at post treatment time point was associated with low pCR [76].

The detection and characterization of epigenetic ctDNA alterations is a promising biomarker also in early breast cancer, since the methylation of genes or transcription regulating regions was associated with treatment response and outcome [77–81]. Notably, a drop of the *BRCA1* methylated fraction was associated with tumor burden only in patients responding to the neoadjuvant treatment, while no associations were observed in the non-responder group [77]. Consistent results were observed by analyzing the methylated fraction of *RASSF1A* promoter ($p = 0.006$), which was furthermore found to be more sensitive than CEA and CA15.3 (23%, 8.6% and 7.4%, respectively, $p < 0.05$ [78]. In the adjuvant setting, the methylation of tumor suppressor genes was associated with worse clinical outcome [79–81]. In a cohort of 336 early-stage (I-–II) BC patients, the methylation of *GSTP1, RASSF1A and RARβ2* promoter region before surgery was associated with a significantly worse OS rate at 100 months, with respect to patients where these promoters were all unmethylated (78% vs. 95%; $p = 0.002$) [79]. Consistently, in a prospective study on 100 BCE patients, the hypermethylation of *GSTP1* and *BRCA1* was an independent prognostic factor in terms of disease recurrence (HR:7.6, $p = 0.02$ for *GSTP1* and HR: 6.2, $p = 0.04$ for *BRCA1*), while the detection of *RASSF1A* methylation in samples collected 1 year after surgery was also associated with worse survival (relative risk of recurrence of 5.1, $p = 0.02$ and of death of 6.9, $p = 0.004$) [80,81].

## Detection of predictive ctDNA alterations in the metastatic disease

Liquid biopsy has the potential to overcome several practical issues such as hardly accessible primary and metastatic lesions, patients' refusal to invasive procedures, not feasible serial sampling or inadequate material for molecular analysis [82,83].

With this in mind, several studies, such as the Aiming to Understand the Molecular Aberrations in Metastatic Breast Cancer "AURORA" trial (NCT02102165), the UK Plasma Based Molecular Profiling of Advanced Breast Cancer to Inform Therapeutic CHoices "plasmaMATCH" trial (NCT03182634), and the Study of the Molecular Features of Postmenopausal Women With HR + HER2-negative aBC on First-line Treatment With Ribociclib and Letrozole "BioItaLEE" (NCT03439046), have been designed to further explore the clinical utility and feasibility of this approach.

Acquired *ESR1* missense alterations occur in about 30% of patients who have received prior endocrine therapies (ET) and is associated with an aggressive clinical phenotype and ER-positive breast cancer recurrence. The prospective-retrospective analysis of the SoFEA trial showed a differential impact of *ESR1* mutations according to ET agent (fulvestrant vs. exemestane, HR:0.52; $p = 0.02$) [84]. A secondary analysis of the BOLERO 2 trial on 541 evaluable patients, showed that the presence of *ESR1*$^{D538G}$ and/or *ESR1*$^{Y537S}$ was associated with worse OS (wild-type: 32.1 vs. *ESR1*$^{D538G}$: 25.99 vs. *ESR1*$^{Y537S}$: 19.98 vs. both mutations: 15.15 months). Interestingly, those patients with a *ESR1*$^{D538G}$ mutant MBC experienced a similar PFS benefit to the wild-type counterpart when everolimus was associated to exemestane, while a lack of response to the everolimus/exemestane association was observed as a consequence of *ESR1's* epigenetic silencing [85,86]. Similarly, a retrospective analysis from the EFECT trial demonstrated that the baseline detection of *ESR1* mutations was associated with a shorter time to progression (TTP) (HR:2.03, $p = 0.004$) in the overall study population [87].

Together with *ESR1*, *PIK3CA* is a promising biomarker for the management of MBC. Its mutations can be detected in about 40% of HR + MBC and are associated with a hyper-activation of the PI3K pathway, which stimulates an estrogen-independent growth. Intriguingly, it has been shown that its detection through ctDNA has a greater predictive potential with respect to the archival counterpart [88–91]. On the other hand, *PIK3CA* was not found to be predictive of response to everolimus-based regimens [92].

ET in HR+/HER2-MBC has been recently revolutionized by the introduction of the CDK4/6 inhibitors (i.e. palbociclib, ribociclib and abemaciclib), but a dedicated predictive biomarker is currently lacking [93–96].

The ctDNA dynamics' analysis of the PALOMA-3 study is an example of the manifold ramifications of longitudinal ctDNA assessments [97]. A drop in *PIK3CA* ctDNA levels after 15 days of therapy with fulvestrant and palbociclib strongly predicted PFS (HR 3.94, 95% CI 1.61–9.64, log-rank $p = 0.0013$), while on the other hand, *ESR1* ctDNA trend was not associated with outcome. These apparently counter-intuitive results are linked to the different role of these mutations in treatment response and disease progression.

PIK3CA is commonly mutated in breast cancer and is mainly associated with tumor burden. Its variation, therefore, reflects the dynamic response to treatment. In contrast, ESR1 mutations are commonly sub clonal and thus do not predict outcome overall, but rather highlight the clonal selection induced by therapy [97].

Notably, ctDNA is currently gaining momentum not only in the ET setting. In a phase II study by Ma et al., which aimed to assess clinical benefit rate of neratinib in HER2 mutant (HER2$^{mut}$) non-amplified MBC, ctDNA analysis for HER2$^{mut}$ demonstrated a sensitivity of 79% and a specificity of 100%, when compared to tumor tissue analysis. Interestingly, ctDNA HER2$^{mut}$ variant allele frequency demonstrated to be predictive of response to neratinib, as it decreased in patients responding to the treatment at week 4 and increased upon progression [98]. In triple negative MBC patients, a $\geq 10\%$ ctDNA fraction, computed through the IchorCNA software, was associated with significantly worse outcomes, with a median survival of 6.4 vs. 15.9 months [99,100]. Interestingly, the presence of copy number gain or amplification at specific loci (18q11 and 19p13) was associated with remarkably poorer prognosis, independently from clinico-pathological factors and ctDNA fraction [100]. The *BRCA*-mutant disease represents also an intriguing case scenario, as ctDNA-based assessment of somatic *BRCA* mutations could potentially expand the cohort of patients treatable with poly ADP-ribose polymerase (PARP) inhibitors and platinum salts [101,102]. Finally, Micro Satellite Instability (MSI) and Loss of heterozygosity (LOH) are being studied through PCR and sequencing approaches, as promising markers of response for targeted agents and immunotherapy [103].

## Treatment response monitoring in the metastatic setting

The ctDNA levels are associated with tumor burden, therefore longitudinal plasma-based assessments may represent an indirect measure of treatment response and could potentially predict long-term clinical outcome.

The inhibition of specific genetic and molecular drivers is generally clinically effective translating in objective response or benefit, although at some point target therapies may cease to obtain successful responses. Drug resistance is most likely due to the heterogeneous mechanisms underlying cancer development and sub-clones arising spontaneously or as a consequence of selection-pressure over time.

Being released into the bloodstream from multiple tumor regions, ctDNA reflects both intra-tumor heterogeneity and clonal evolution. By potentially detecting secondary alterations responsible for treatment resistance months earlier than traditional instrumental assessments, longitudinal assessment of ctDNA could offer a *real time* opportunity to optimize treatment options and clinical decision making in breast cancer.

Moreover, ctDNA dynamics not only has a significant prognostic impact but also has higher sensitivity with respect to tumor markers, and is capable to anticipate imaging-based disease progression [104,105].

A multiparametric longitudinal assessment, therefore, could enable the clinician to keep track of both the overall tumor burden and acquired mutations with intriguing perspectives on a clinical decision-making standpoint.

Deep-sequencing analysis of 70 cancer genes of 21,807 patients with > 50 treated, advanced cancer types was conducted to explore quantitatively and qualitatively the evolution of actionable resistance alterations having arisen along targeted therapy. *ESR1*$^{L536/Y537/D538}$ was the most common in patients with MBC compared with early stage, treatment naïve tumor tissue, likely reflecting therapy pressure by aromatase inhibitors (AI) [106].

Acquired *ESR1* mutations at first relapse are about 5% but range from 30% to 50% in patients who previously received AI in the first-line metastatic setting. [107,108]. As recently demonstrated, these mutations are sub-clonal in 72.2% of patients and often associated with the onset of other mutations such as RAS [107]. These results were confirmed in the SoFEA trial, where KRAS mutations were detected in 21.2% of patients, although no impact on PFS or OS was demonstrated [84].

Consistently, *ESR1* mutations were associated to shorter PFS when AIs were administered beyond disease progression (HR = 3.7; p = 0.008) or as maintenance therapy after chemotherapy (HR = 3.1; p = 0.0041) [109].

The ongoing PAlbociclib and Circulating Tumor DNA for *ESR1* Mutation Detection "PADA-1" trial (NCT03079011) was designed to assess whether switching the ET backbone from AI to fulvestrant, maintaining palbociclib after the onset of ctDNA *ESR1* mutations, could translate into a benefit for MBC patients. Preliminary results showed a detection rate of 2.1% at baseline, with an allelic frequency ranging from 0.3% to 47% (median = 3.5%). Notably, among the 17 patients with baseline *ESR1* mutations, only 4 had detectable ESR1 mutations after 1 month of therapy [110].

The *ESR1* p-D538G mutation determines ligand-independent activation of ERα and it is acquired in patients who have received aromatase inhibitors. The *p-E380Q* mutation has been observed to remain sensitive to antiestrogens [68]. The use of ctDNA to detect *ESR1* mutations and predict future resistance have been validated by Chu et al [67]; however, the sensitivity of detection of *ESR1* mutations through ctDNA varies among different studies ranging from 57% to 75% [109,111], probably due to sample size and different techniques (digital PCR in the first and ultra-high-sensitivity multiplex digital PCR assay in the latter).

Recently, a ligand-independent and hyperactive *ESR1* fusion protein was detected in recurrent BC through a ctDNA-based assay; suggesting additional secondary resistance mechanisms to endocrine therapies [112]. IchonCNA highlighted alterations in *ESR1* (D538G and L536P) in a MBC patient previously treated with aromatase inhibitors at $t_1$ (0.12 and 0.45 cancer cell fraction) while their clonal fractions resulted inverted at $t_2$ (0.73 and 0.12) after 51 days of therapy with a selective estrogen receptor degrader (SERD). This clonal shift may indicate that different *ESR1* mutations could show different response to SERDs [99].

It has been also showed that higher doses of fulvestrant and tamoxifen or more potent ER antagonist, are able to fully antagonize mutant ER signaling, suggesting that effective strategies to overcome resistance are possible and knowing the causal mutation could be of pivotal importance [113,114].

The onset of genetic alterations after CDK 4/6 inhibition is currently under the spotlight. The emergence of somatic RB1 mutations was reported in 3 patients after exposure to palbociclib or ribociclib and consistent data were presented in an extended ctDNA analysis of 194 paired PALOMA-3 plasma samples using a custom 87-gene NGS assay [115,116].

Notably, the FGFR1 pathway was also investigated both in endocrine and CDK4/6 inhibition resistance. It has been shown that FGFR1 amplifications or activating mutations were present in 29% of post-progression specimens, suggesting that a FGRF1 alterations could have a role both as biomarker and therapeutic target [117].

## Conclusions

Several techniques are comprised under the umbrella-term of "liquid biopsy", each with its strengths and peculiarities. Among them, ctDNA sequencing analysis is a crucial option to assess time-dependent variables, such as tumor mutational burden and molecular features. Therefore, ctDNA could be used to detect and characterize early stage disease but also to longitudinally monitor tumors' genomic profile and detect the emergence of genetic alterations in the advanced setting before clinical symptoms or radiological evidence of progression. As a result, ctDNA analysis may guide clinical decision-making and through the integration with other solid and liquid biopsy techniques, will ultimately lead to a growingly cancer care personalization.

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- Giuseppe Buono has no conflict of interests to declare.
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