Personalized Medicine and Imaging

Clinical Cancer Research

Cell-Free DNA and Circulating Tumor Cells: Comprehensive Liquid Biopsy Analysis in Advanced Breast Cancer

Giovanna Rossi[1,2], Zhaomei Mu[2], Alfred W. Rademaker[3], Laura K. Austin[4], Kimberly S. Strickland[4], Ricardo Lima Barros Costa[5], Rebecca J. Nagy[6], Vittorina Zagonel[7], Timothy J. Taxter[8], Amir Behdad[8], Firas H. Wehbe[9], Leonidas C. Platanias[10], William J. Gradishar[3], and Massimo Cristofanilli[2]

Abstract

Purpose: Liquid biopsy provides a real-time assessment of metastatic breast cancer (MBC). We evaluated the utility of combining circulating tumor cells (CTC) and circulating tumor DNA (ctDNA) to predict prognosis in MBC.

Experimental Design: We conducted a retrospective study of 91 patients with locally advanced breast cancer and MBC. CTCs were enumerated by CellSearch; the plasma-based assay was performed utilizing Guardant360 and the survival analysis using Kaplan–Meier curves.

Results: Eighty-four patients had stage IV cancer, and 7 patients had no metastases. Eighty patients had CTC analysis: median number 2 (0–5,612). Blood samples [232 of 277 (84%)] had mutations. The average ctDNA fraction was 4.5% (0–88.2%) and number of alterations 3 (0–27); the most commonly mutated genes were *TP53* (52%), *PIK3CA* (40%), and *ERBB2* (20%). At the time of analysis, 36 patients (39.6%) were dead. The median follow-up for CTCs was 9 months; for ctDNA, it was 9.9 months. For CTCs and ctDNA, respectively, progression-free survival (PFS) was 4.2 and 5.2 months and overall survival (OS) was 18.7 and 21.5 months. There was a statistically significant difference in PFS and OS for baseline CTCs < 5 versus CTCs $\geq$ 5 ($P = 0.021$ and $P = 0.0004$, respectively); %ctDNA < 0.5 versus $\geq$ 0.5 ($P = 0.003$ and $P = 0.012$); number of alterations < 2 versus $\geq$ 2 ($P = 0.059$ borderline and $P = 0.0015$). A significant association by Fisher exact test was found between the number of alterations and the %ctDNA in the baseline sample ($P < 0.0001$).

Conclusions: The study demonstrated that liquid biopsy is an effective prognostic tool. *Clin Cancer Res; 24(3); 560–8. ©2017 AACR.*

Introduction

Breast cancer is the most common malignancy among women worldwide and represents the second leading cause of cancer death in American women, exceeded only by lung cancer. Approximately 6% to 10% of new breast cancer cases are initially stage IV (*de novo* metastatic disease), and the number of metastatic recurrences is estimated to range between 20% and 30% of all existing breast tumor cases (1, 2).

The primary goals of metastatic breast cancer (MBC) remain palliation of symptoms and improvement in quality of life with the hope that effective treatments will also produce objective remission and prolongation of disease control ultimately translating in an impact on survival.

The selection of systemic therapeutic strategy considers a combination of molecular and clinical factors, with the objective of using the most effective and least debilitating tailored approach (3).

In particular, in patients with hormone-sensitive tumors, endocrine therapy can provide survivals similar to those obtained with chemotherapy (although with fewer objective responses) and has to be considered as a first choice in the absence clinical evidence of aggressive disease (4–8). Nevertheless, prolonged exposure to endocrine therapy may result in acquired resistance and subsequent progression of disease. Recent evidence showed that activating mutations in the ligand-binding domain of estrogen receptor-α (*ESR1*) occur in approximately 30% of patients exposed to endocrine therapies and those genomic abnormalities may represent the driver of endocrine resistance (9).

One of the major reasons for the failure of cancer systemic therapies is our inability to accurately capture the heterogeneity of breast cancer. In this contest, there is hope that the Precision Medicine—meant as the set of diagnostic tests and resulting treatments targeted to the needs of individual patients—might be one way to address this challenge (10, 11).

[1]Division of Thoracic Oncology, European Institute of Oncology, Milan, Italy. [2]Department of Medicine-Hematology and Oncology, Robert H. Lurie Comprehensive Cancer Center, Feinberg School of Medicine, Northwestern University, Chicago, Illinois. [3]Northwestern University, Feinberg School of Medicine, Chicago, Illinois. [4]Department of Medical Oncology, Thomas Jefferson University Hospital, Philadelphia, Pennsylvania. [5]Department of Breast Oncology, Lee Moffitt Cancer Center, Tampa, Florida. [6]Guardant Health, Inc., Redwood City, California. [7]U.O.C. Oncologia Medica 1, Istituto Oncologico Veneto, IRCCS, Padova, Italy. [8]Department of Pathology, Feinberg School of Medicine, Northwestern University, Chicago, Illinois. [9]Department of Preventive Medicine, Feinberg School of Medicine, Northwestern University, Chicago, Illinois. [10]Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, Illinois.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Massimo Cristofanilli, Robert H. Lurie Medical Research Center, 710 North Fairbanks Court, Chicago, IL 60611. Phone: 312-503-5488; E-mail: massimo.cristofanilli@nm.org

doi: 10.1158/1078-0432.CCR-17-2092

Translational Relevance

The use of blood-based diagnostics, collectively known as liquid biopsy, provides a real-time molecular assessment of metastatic breast cancer (MBC) and holds the promise to help select and monitor treatment efficacy. Circulating tumor cell (CTC) enumeration is already an established prognostic factor in MBC. In this study, we showed that circulating tumor DNA (ctDNA) is consistently detected in MBC using a next-generation sequencing-based method. Moreover, measuring ctDNA levels or number of genomic alterations is associated with strong prognostic value. Furthermore, within the same cohort, we confirmed the prognostic role of both CTCs and ctDNA to associate with the standard prognostic factors (e.g., receptor status). Significantly, the molecular information obtained by ctDNA can be applied to treatment selection and monitoring. The most novel finding from our study is the demonstration that a comprehensive liquid biopsy analysis may represent a tool to assess both tumor burden and molecular features of disease.

Liquid biopsy is a tool of Precision Medicine and provides a real-time assessment of MBC at baseline and recurrence, through the analysis of gene mutations on ctDNA and CTCs.

Several studies (12–17) have shown that somatic mutations identified in ctDNA are widely representative of the tumor genome and can provide an alternative noninvasive method that overcome many difficulties related to tissue biopsy (e.g., spectrum of mutations limited to a single region of the tumor, serial sampling usually not feasible; ref. 17). ctDNA allows to monitor treatment response in order to avoid ineffective therapies and evaluate the benefit of new drugs. For instance, the detection in ctDNA of mutations in the ligand binding domain of *ESR1* that confer constitutive activity of ER is an emerging predictor of endocrine therapy resistance in MBC (18). It has also been shown that dynamic changes in ctDNA levels closely reflect changes in tumor burden and increases in ctDNA levels often predict the progressive disease several months before the standard imaging. The assessment of ctDNA levels may also be an important indicator of prognosis, but prospective studies in larger cohorts of patients will be needed to validate the role of ctDNA as a prognostic biomarker (10, 19).

CTCs are cancer cells that have been shed or actively migrate into the vasculature from the primary tumor or metastatic lesions and circulate in the bloodstream. They can give rise to metastases from primary or other lesions (seeding hypothesis) in distant organs and being responsible for the vast majority of cancer-related deaths (20, 21). (i) We previously demonstrated the prognostic value of a CTC enumeration $\geq$5 at baseline and follow-up in patients with MBC (22, 23). (ii) The presence of one or more CTCs predicts early recurrence and decrease overall survival (OS) also in chemonaïve patients with non-MBC (24). (iii) Beyond enumeration, there is interest in genotypic and phenotypic characterization of CTCs. Studies on CTCs at single cell level may help revealing the underlying mechanism of tumorigenesis and metastases (25, 26).

In contrast to CTCs, a cutoff of ctDNA that correlates with a worse prognosis has not been identified yet. The goal of our study was precisely to investigate the prognostic value of both CTCs and ctDNA—maximum % (%ctDNA), number of allelic variants (mutations)—in terms of progression-free survival (PFS) and OS in the entire population and in the subgroup of patients with ER^+ disease.

Patients and Methods

The study was conducted at the Thomas Jefferson University Hospital of Philadelphia (PA, USA) and continued at the Robert H. Lurie Comprehensive Cancer Center at Northwestern University of Chicago (IL, USA).

We retrospectively reviewed medical records of patients with locally advanced or MBC treated at Jefferson University Hospital in a period of 3 years, from January 2013 to December 2015, who had longitudinal assessment of their disease by ctDNA or CTC analysis.

A cohort of patients continued their follow-up at Northwestern University from September 2015.

ctDNA analysis was obtained for clinical care and the sample collection began from June 2013.

CTC analysis was evaluated under a prospective clinical trial (a single Institution Investigator Initiated Trial (IIT); protocol number: NU16B06), from February 2013.

The combined data analysis was planned under a retrospective Institutional Review Board (IRB)–approved study that was conducted from September 2015 to March 2016, date of the last follow-up.

Study subjects

Patients with either stage III locally advanced or stage IV MBC; patients who were about to change line of therapy and had at least one blood draw for ctDNA or CTCs were eligible. Exclusion criteria included early-stage breast cancer and patients who did not have a ctDNA analysis as part of their standard clinical care.

By applying these criteria, 91 patients were selected, mostly females (only one male).

Forty-two patients had the first blood draw for CTCs performed at the same time of the first blood draw for ctDNA, whereas 38 patients had them done at different times. In 11 cases, the CTC analysis was not run or the patient refused the test.

The main information collected for each patient was demographic and clinical information about diagnosis, recurrences, treatments (chosen according to the best clinical practice), follow-up; laboratory information about ctDNA and CTCs. A written informed consent was obtained from each participant for both CTC and ctDNA draws.

Methods

The patients had longitudinal assessment of their disease by liquid biopsy.

ctDNA. Guardant Health performed the plasma analysis (Guardant360). The test was designed to analyze ctDNA in 5 to 10 mL of blood. Two 10-mL standard Streck tubes of whole blood were used for each patient. The plasma was stored at room temperature for 24 to 48 hours before the final analysis. We used 5 to 30 ng of ctDNA for the sequencing (sample requirements: > 5 ng cell-free DNA) and the mean amount was 22 ng. First, ctDNA was isolated from plasma using a Qiagen circulating nucleic acid kit, then a panel of >50 cancer genes associated with solid tumors, as reported in the COSMIC (Catalogue of Somatic Mutations in Cancer) database, were sequenced using single-molecule digital

sequencing technology (Supplementary Table S1). Guardant360 sequencing technique is based on a next-generation sequencing (NGS) technology (Guardant Digital Sequencing) with a single-molecule analytical sensitivity and a 99.9999% specificity. It detects various types of alterations, including single nucleotide variants (SNV), insertions/deletions (indels), gene fusions/rearrangements and copy number variations (CNV) present in genes linked to cancer (clinical actionable mutations) with a reportable range of $\geq$0.04%, $\geq$0.02%, $\geq$0.04%, and $\geq$2.12 copies, respectively. Samples were paired-end sequenced on an Illumina Hi-Seq 2500. Normal controls are included with each run. The genomic analysis was performed at a central CLIA (Clinical Laboratory Improvement Amendments)-certified laboratory (Guardant Health; ref. 27).

CTCs. CTCs were enriched and enumerated by CellSearch in 7.5 mL blood samples collected from the patients according to standard protocol (22, 28, 29). Approximately 8 to 10 mL of whole blood was collected into a 10 mL CellSave Preservative Tube containing a cellular fixative (Janssen Diagnostics). Whole blood (7.5 mL) was processed using CellSearch CTC kits, which selects Epithelial Cell Adhesion Molecule (EpCAM)-positive cells by antibody-coated ferrous particles targeting the epithelial cell EpCAM antigen. CTCs were identified by positive staining for both phycoerythrin-conjugated cytokeratins (CK-8, 18, and 19) and double-stranded DNA (DAPI), and CD45-negative staining ($CK^+/DAPI^+/CD45^-$).

Statistical analysis

The statistical analysis has been performed as follows.

ctDNA. After tabulating the number of mutations and quantifications of overall ctDNA detected for every patient at baseline, a logistic regression was performed to identify the best cutoffs that separated the patients who had a disease progression from the patients who had not a progression (for PFS) and patients who died from the living (for OS), for both variables (number of mutations and %ctDNA). For %ctDNA, we initially identified different cutoff points for PFS (1%) and OS (0.7%).

We then decided to use a cutoff (0.5%) that allows a comprehensive survival analysis of both PFS and OS, as well as the already established cutoff for CTCs.

CTCs. The established cutoff point (< 5 vs. $\geq$ 5 CTCs; ref. 22) has been used for both the analyses on PFS and OS. Clinical endpoints were PFS and OS. PFS and OS were calculated from the time of the first draw for CTCs or ctDNA until the progression (assessed using RECIST criteria) or death, respectively (22, 23, 28–31).

The survival analysis has been performed using Kaplan–Meier curves, compared using a log-rank test. Separate analyses were done using time from first CTC and time from first %ctDNA. We excluded from the analysis: for ctDNA, patients who were lost at follow up; for CTCs, patients who had not the draw done or it had been done after the progression.

Using these criteria, for the first endpoint (PFS), considering each variable, we examined the following cases: 87 cases for %ctDNA, 90 cases for the number of mutations, 72 cases for the CTCs. Considering the second endpoint (OS): 88 cases (%ctDNA), 91 cases (number of mutations), 80 cases (CTCs), respectively.

The same analysis has been run in the overall population and in the subgroup of patients with ER^+ disease.

Proportional hazards regression was used to assess the role of CTCs and %ctDNA simultaneously on PFS and OS and to determine the effect of each of CTCs or %ctDNA in the presence of age, ER status, and HER2 status. CTCs and %ctDNA were correlated using Spearman correlation.

Results

Patient characteristics

Ninety-one patients with locally advanced breast cancer or MBC were identified. Ninety patients were females and one was a male. Their median age at the first draw for ctDNA was 54 years, with a range of 33 to 78 years. Fifty-eight patients (64%) had an inflammatory breast cancer (IBC). The prevalent tumor subtype was ductal (81.3%) and the majority of the tumors were ER^+HER2^- (43.9%). There were 57 cases ER^+ (of which 17 ER^+HER2^+ and 40 ER^+HER2^-), 25 $HER2^+$ (of which 17 ER^+HER2^+ and 8 ER^-HER2^+) and 26 triple negative (TN). At the time of the baseline sample, 84 (92%) patients were stage IV and 7 (8%) patients had no metastases. Among the patients with metastatic disease, 11 (13%) had bone metastases, 35 (42%) had visceral metastases, and 38 (45%) had both sites of metastases. Most patients (24%) had already received 5 or more lines of treatment for the metastatic disease. A progression has been observed in 76 cases. At the time of analysis, 36 patients (39.6%) were dead, and 55 (60.4%) were currently alive (Table 1).

The median follow-up for CTCs was 9 months (range, 0.3–31.2) and the median follow-up for ctDNA was 9.9 months (range, 0.3–28.1).

ctDNA and CTC samples and treatment planning

A total of 277 blood samples for ctDNA were collected and 232 variant alterations were detected. Sixty-five percent of the patients had serial samples. The average number of alterations detected in each sample was 3 (0–27), and the average ctDNA fraction detected was 4.52 (0–88.20%). The most frequently altered genes were *TP53* (52%), *PIK3CA* (40%), *ERBB2* (20%), *NOTCH1*

Table 1. Patient characteristics at the first draw for ctDNA and distribution of the tumors according to the histopathologic features

Patient characteristics	Patients, *n*
Gender	
Female	90 (99%)
Male	1 (1%)
Clinical type	
IBC	58 (64%)
Non-IBC	33 (36%)
Stage	
Metastatic	84 (92%)
Nonmetastatic	7 (8%)
Sites of metastases	
Bone	11 (13%)
Viscera	35 (42%)
Both	38 (45%)
Histopathologic features	**Tumors, *n***
Infiltrative histology	
Ductal	74 (81.3%)
Lobular	10 (11%)
Other	7 (7.7%)
IHC	
ER^+HER2^+	17 (18.7%)
ER^-HER2^-	26 (28.6%)
ER^+HER2^-	40 (43.9%)
ER^-HER2^+	8 (8.8%)

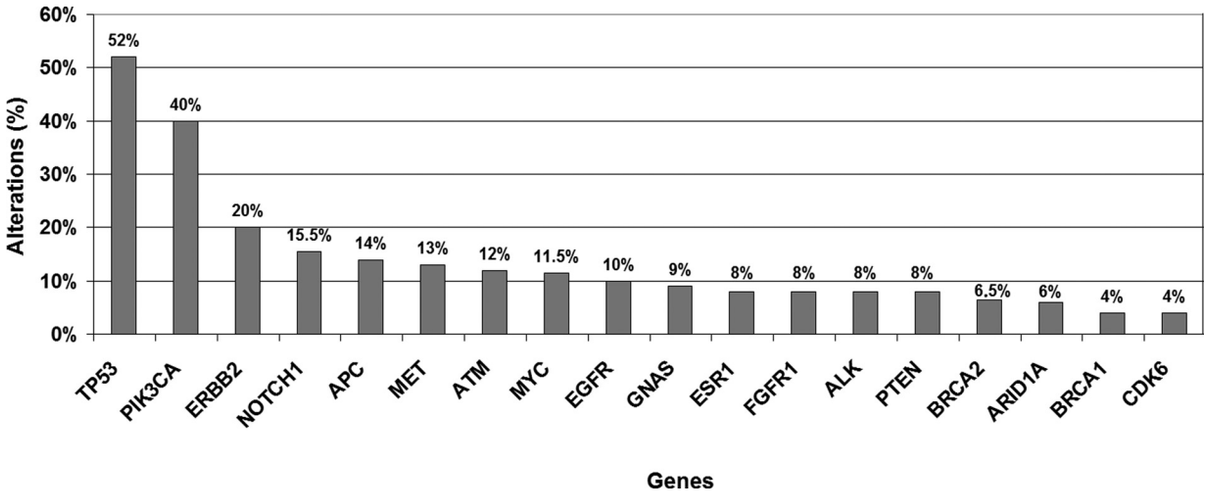

Figure 1.

Distribution of the main genomic alterations in the entire population. To assess the frequency of the alterations within a gene, we considered the total number of alterations of that gene in the total number of samples (277). If a sample showed multiple mutations within the same gene, each mutation was counted.

(15.5%), *APC* (14%), and *MET* (13%). The distribution of the main genomic alterations in the whole population is shown in Fig. 1.

In particular, there were 32 *ERBB2* genomic abnormalities that consisted of base substitutions alterations and 25 gene amplifications. Among the 12 patients who harbored *HER2* amplification in ctDNA (in one sample at least), 2 patients had a $HER2^{-}$ disease. All 2 patients received an anti-HER2 treatment based on the liquid biopsy results, achieving a stable disease for 5 and 3 months, respectively.

ESR1 mutation was detected in 11 cases and *PIK3CA* mutation in 22 cases. Interestingly, 4 patients harbored both mutations. Among the 22 patients who harbored *PIK3CA* mutations in one sample at least, 8 patients were started on combination treatment with everolimus (Afinitor) based on the ctDNA findings.

The most common *ESR1* mutations were detected in known hotspots: Y537S (6/11, 55%), D538G (4/11, 36%), and Y537N (3/11, 27%). Four patients carried polyclonal *ESR1* mutations, of which 1 patient harbored 4 *ESR1* mutations (polyclonal). At the time of mutation detection, 10 patients had already failed at least 1 line of endocrine therapy (average 2; range, 1–5) (Supplementary Table S2). After the mutation detection, 5 patients were on endocrine therapy and 4 patients were started on/continued chemotherapy. *ESR1* mutation disappeared in 2 patients (fulvestrant–palbociclib and chemotherapy, respectively) who achieved stable disease as best response.

Among the 4 patients who harbored both *ESR1* and *PIK3CA* mutations, the median PFS was 5.8 and 7.5 months and median OS was 18.6 and 21.7 months for the CTCs and ctDNA, respectively.

Considering the whole population, 16 patients were initiated on a targeted therapy based on ctDNA test (Supplementary Table S3).

Forty-seven patients had NGS in the tissue, and a concordance with the mutations in ctDNA was found in 24 patients (51%).

Eighty (88%) patients had CTC analysis done. A total of 251 samples for CTCs were collected. The median number of CTCs in each sample was 2 (range, 0–5612). Of 80 patients with CTC values, 68% (54 patients) had multiple samples (median, 3; average, 4; range, 2–10).

The median number of CTCs in the baseline sample was 17.5 among the patients who harbored both *ESR1* and *PIK3CA* mutations versus 1 for the remaining 76 patients.

Univariate analysis

The cutoff that has been identified for both PFS and OS was 0.5 for %ctDNA and 2 for the number of mutations.

First endpoint: PFS

Median PFS was 5.2 months from the first blood draw for ctDNA and 4.2 months from the first draw for CTCs. In the entire population, a statistically significant difference in PFS by log-rank test was found between patients with:

- %ctDNA < 0.5 versus ≥ 0.5 ($P = 0.003$; Fig. 2 1.a);
- CTCs < 5 versus CTCs ≥ 5 ($P = 0.021$; Fig. 2 1.c).

The difference in PFS between patients with number of mutations < 2 versus ≥ 2 was borderline ($P = 0.059$; Fig. 2 1.b).

In the subgroup of 57 patients with ER^{+} disease, median PFS was 6.3 months from the first blood draw for ctDNA and 4.4 months from the first draw for CTCs. A statistically significant difference in PFS by log-rank test was found between patients with:

- %ctDNA < 0.5 versus ≥ 0.5 ($P = 0.002$);
- number of mutations < 2 versus ≥ 2 ($P = 0.018$);
- CTCs < 5 versus CTCs ≥ 5 ($P = 0.006$).

Second endpoint: OS

Median OS was 21.5 months from the first blood draw for ctDNA and 18.7 months from the first draw for CTCs. In the entire population, a statistically significant difference in OS by log-rank test was found between:

- %ctDNA < 0.5 versus ≥ 0.5 ($P = 0.012$; Fig. 2 2.a);
- number of mutations < 2 versus ≥ 2 ($P = 0.0015$; Fig. 2 2.b);
- CTCs < 5 versus CTCs ≥ 5 ($P = 0.0004$; Fig. 2 2.c).

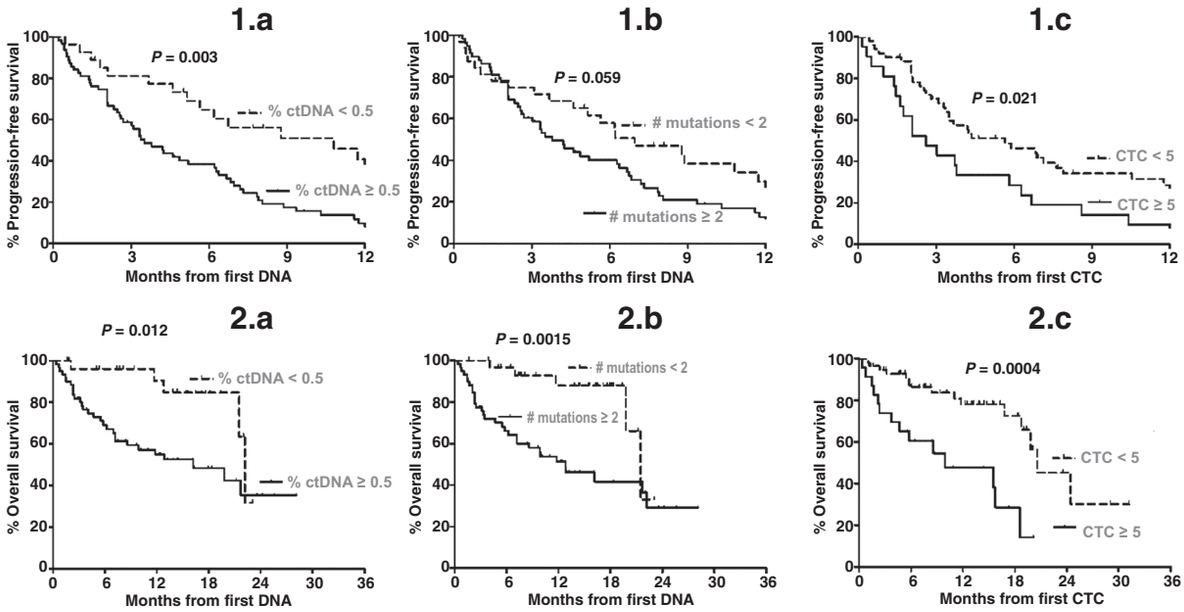

Figure 2.

The curves show the association between PFS or OS and, respectively, %ctDNA [**1.a,** <0.5, 18 progressions in 27 patients (=18/27 progressions); ≥0.5, 55/60 progressions; **2.a,** <0.5, 5 deaths in 27 patients (5/27 deaths); ≥0.5, 29/61], number of mutations in ctDNA (**1.b,** <2, 23/32 progressions; ≥2, 53/58 progressions; **2.b,** <2, 5/32 deaths; ≥2, 31/59 deaths), and number of CTCs (**1.c,** <5, 40/51 progressions; ≥5, 21/21 progressions; **2.c,** <5, 15/57 deaths; ≥5, 14/23 deaths) in the entire population.

In the subgroup of patients with ER^+ disease, median OS was 21.7 months from the first blood draw for ctDNA and 18.7 months from the first draw for CTCs. A statistically significant difference in OS by log-rank test was found between patients with:

- number of mutations < 2 versus ≥ 2 ($P = 0.016$);
- CTCs < 5 versus CTCs ≥ 5 ($P = 0.013$).

No significant difference in OS was observed between patients with %ctDNA < 0.5 versus ≥ 0.5 ($P = 0.098$).

In the subgroup of patients with ER^+ disease (57 patients) we also performed the analysis of follow-up ctDNA, considering the serial samples and the most commonly altered pathways.

Five genes were analyzed: *ESR1*, *PIK3CA*, *PTEN*, *AKT1*, and *MAP2*.

For each gene: if the gene was mutated at some time during the follow-up, it was counted as positive; if there was no mutation during the follow-up, it was counted as negative.

Considering the follow-up samples, there were 11 patients who harbored *ESR1* mutation, 22 patients who harbored *PIK3CA* mutation, 7 patients had *PTEN* mutation, 2 patients had *AKT1* mutation, and 2 patients had *MAP2* mutation in ctDNA.

Each gene was analyzed separately, but no significant difference in OS was observed between the patients with or without the mutation in ctDNA in the follow-up blood collection.

The median OS was 21.5 months in the subgroup of patients with *ESR1* mutation versus 22.2 months in the $ESR1^-$ patients ($P = 0.97$ by log-rank test); 21.7 months in $PIK3CA^+$ patients versus *PIK3CA*- patients (median OS not attained, $P = 0.40$); 22.2 months in $PTEN^+$ patients versus 21.5 months in $PTEN^-$ patients ($P = 0.33$); 12.8 months in $AKT1^+$ patients versus 21.7 months in $AKT1^-$ patients ($P = 0.67$); 22.2 months in $MAP2^+$ patients versus 21.5 months in $MAP2^-$ patients ($P = 0.71$).

Multivariate analysis

First endpoint: PFS. For time from the first blood draw for ctDNA, a multivariate analysis of PFS indicated that %ctDNA remained statistically significant ($P = 0.021$) while CTCs were not significant ($P = 0.22$). For time from the first blood draw for CTCs, a multivariate analysis of PFS indicated that both %ctDNA and CTCs were not statistically significant ($P = 0.06$ for each).

For time from the first blood draw for ctDNA, a multivariate analysis of PFS which included %ctDNA, age, ER status, and HER2 status indicated that %ctDNA remained statistically significant ($P = 0.03$). When we included in this analysis an indicator of whether the CTCs and %ctDNA were observed on the same day or not, %ctDNA was not statistically significant ($P = 0.31$).

For time from the first blood draw for CTCs, a multivariate analysis of PFS which included CTCs, age, ER status, and HER2 status indicated that CTCs was not statistically significant ($P = 0.078$). When we included in this analysis an indicator of whether the CTCs and %ctDNA were observed on the same day or not, CTCs were statistically significant ($P = 0.024$).

Second endpoint: OS. For time from the first blood draw for ctDNA, a multivariate analysis of OS indicated that CTCs were significant ($P = 0.009$) while %ctDNA was not significant ($P = 0.14$).

For time from the first blood draw for CTCs, a multivariate analysis of OS indicated that CTCs were significant ($P = 0.002$) while %ctDNA was not significant ($P = 0.10$).

For time from the first blood draw for ctDNA, a multivariate analysis of OS which included %ctDNA, age, ER status, and HER2 status indicated that %ctDNA was not statistically significant ($P = 0.10$). When we included in this analysis an indicator of whether

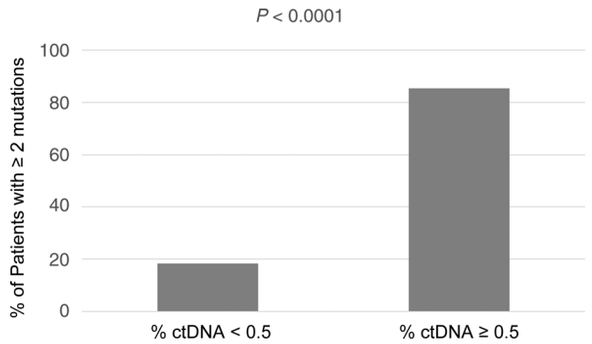

Figure 3.
The histograms show the distribution of the patients with ≥2 mutations in ctDNA at baseline according to the tumor burden (%ctDNA < 0.5 vs. ≥ 0.5).

the CTCs and %ctDNA were observed on the same day or not, %ctDNA was not statistically significant ($P = 0.14$).

For time from the first blood draw for CTCs, a multivariate analysis of OS which included CTCs, age, ER status, and HER2 status indicated that CTCs were statistically significant ($P = 0.006$). When we included in this analysis an indicator of whether the CTCs and %ctDNA were observed on the same day or not, CTCs remained statistically significant ($P = 0.004$).

Secondary objectives

Interestingly, a statistically significant association by Fisher exact test was observed between the number of alterations and the %ctDNA detected in the baseline sample (% of patients with mutations ≥ 2 was 19% when %ctDNA < 0.5%, versus 85% when %ctDNA ≥ 0.5%; $P < 0.0001$; Fig. 3). That is to say, higher values of %ctDNA correlate with higher number of mutations (mutations < 2 and %ctDNA < 0.5%, mutations ≥ 2 and %ctDNA ≥ 0.5%; $P < 0.0001$).

The Spearman correlation coefficient between %ctDNA and the number of CTCs was 0.37, $P < 0.001$. Seventy-seven patients had both CTCs and ctDNA. In the 57 patients with CTCs < 5, 20 had %ctDNA < 0.5 and 37 had %ctDNA ≥ 0.5. In the 20 patients with CTCs ≥ 5, 4 had %ctDNA < 0.5 and 16 had %ctDNA ≥ 0.5.

There was a higher correlation between CTCs and %ctDNA when they were measured on the same day ($R = 0.42$, $P = 0.016$) than when they were measured on different days ($R = 0.26$, $P = 0.12$). CTCs were similar between the two groups, but %ctDNA was higher on the days when both were made.

Discussion

ctDNA and CTCs have been promoted as minimally invasive biomarkers useful for a real-time monitoring of tumor heterogeneity and predict for clinical behavior. However, challenges still remain in the use of these methodologies to guide clinical practice.

Whereas CTCs and their prognostic impact have been widely studied, the association between ctDNA levels and prognosis in large cohorts of patients has not been extensively investigated.

In the current study, we demonstrated the role of both CTCs and ctDNA as prognostic factors in MBC, to associate with the standard prognostic factors such as the ER/PgR/HER2 status.

The results of our study confirm that CTCs levels at baseline are predictors of both PFS and OS and confirm that the detection of 5 cells per 7.5 mL of blood is the best cutoff point to stratify the patients' prognosis (22, 32). In MBC, whereas the prognostic power of CTCs increases according to the number of cells, quantification of tumor-specific mutations in ctDNA has been shown to correlate significantly with tumor burden (33).

In the literature (33), increasing levels of ctDNA—treated as a continuous time-dependent variable—were associated with inferior OS using a different NGS panel. In particular, Dawson and colleagues (33) evaluated individual mutations (*TP53* and *PI3KCA*) that while frequent are certainly less sensitive and accurate than using NGS with a panel of more than 50 genes.

Nevertheless, irrespective of NGS platform and gene panel, a cutoff that identifies the patients more likely to relapse or die of their disease has not been established yet. In our population, we observed a higher rate of progression (92% vs. 67%) and death (47.5% vs. 18.5%) in the subgroup of patients with %ctDNA ≥ 0.5 at baseline compared with the subgroup with %ctDNA < 0.5. A statistically significant difference in both PFS ($P = 0.003$) and OS ($P = 0.012$) was found between the two groups, suggesting 0.5% as a possible cutoff to stratify the patients.

Our findings are particularly interesting because they were obtained using an NGS technology (digital sequencing), which is more accurate than standard sequencing methods and has the advantage of allowing the assessment of a comprehensive panel of genomic alterations at the very low concentration typical of ctDNA (27). In addition to the evaluation of a larger number of loci simultaneously, NGS technology provides high-quality sequencing of each molecule of ctDNA, overcoming the systematic biases of digital PCR (especially overestimation due to nonspecific amplification; refs. 21, 34).

Regarding the frequency of alterations in ctDNA, we confirmed the evidence in the literature. *PIK3CA* mutations are very frequent (20%–40%) genetic alterations in MBC, particularly in node-negative, ER^+ and $HER2^-$ breast cancer, and are generally related to a good clinical outcome with a lower recurrence and mortality rate (35). Nevertheless, tissue-based NGS had already demonstrated that at least 33% of TN breast cancer have deregulated PI3K/AKT pathways, making these pathways an attractive target for pharmacologic treatment (e.g., everolimus) and highlighting the importance of mutation profiling for individualized therapies (36).

In our population, maybe because enriched in IBC (64% of the patients), with a rate of 40% in the entire population and of 31% in the TN subgroup, PIK3CA/mTOR pathway (altered in 22 patients, including 12 patients with IBC) appears the second most altered pathway, immediately after TP53 (52% and 61%, in the entire population and TN subgroup respectively). Indeed, the mutations in the *TP53* gene are relevant in breast carcinogenesis, with a frequency up to 83% in basal-like tumors (35). Among the patients with $HER2^+$ disease, HER2 alteration is the second most frequent.

ESR1 mutation is another important alteration, with implications on treatment selection (37, 38). It occurs in approximately 30% of patients exposed to endocrine therapies and may represent the driver of the endocrine resistance (3, 9). It was observed that patients with *ESR1* mutations had improved PFS after taking fulvestrant compared with exemestane (SoFEA trial); moreover, the adding of palbociclib to fulvestrant improved PFS compared with fulvestrant plus placebo (PALOMA III trial) (38). Among the 57 patients with ER^+ disease, we identified 11 patients (19%) harboring *ESR1* mutation in ctDNA.

The correlation between the number of mutations in ctDNA and the outcome has not been explored yet. In our population, we observed a higher rate of progression (91% vs. 72%) and death (52.5% vs. 16%) among the patients with a number of mutations ≥ 2 in the baseline sample compared with the subgroup with <2 mutations. Using this cutoff, we found a borderline difference between the 2 groups in PFS ($P = 0.059$), whereas in OS the difference was statistically significant ($P = 0.0015$).

When we performed the analysis in the subgroup with ER^+ disease using the same cutoffs, we came to conclusions similar to those that we reported for the entire population, except for few minor differences. The difference in the rate of progression (91% vs. 65%) between the patients with a number of mutations ≥ 2 in the baseline sample compared with the subgroup with <2 mutations was statistically significant ($P = 0.018$). The difference in the rate of death (46% vs. 26%) between the patients with %ctDNA $\geq$ 0.5 in the baseline sample compared with the subgroup with %ctDNA < 0.5 was not statistically significant within this cohort of patients.

Overall, based on our study, we can assume that patients with a greater burden of disease, are at higher risk of disease progression and have a poor outcome, therefore requiring attention to treatment selection based on both, subtype-specific guidelines and molecularly driven treatments when appropriate.

It has been showed that ctDNA can capture the majority of mutations found in tissue biopsy and the concordance is 72.5–100%, according to the different techniques that were used and the timing of tissue biopsy (contemporaneous tissue and blood draw or sampling at different times; ref. 39).

The mutational tumor burden can be related to the immunogenicity of breast cancer. TN, luminal B-like, or $HER2^+$ breast cancer have a high mutational burden and can be considered immunogenic (40). Thus, our results could support the use of immunotherapy in these molecular subtypes, especially when liquid biopsy confirms the great burden of disease (%ctDNA ≥ 0.5 and number of mutations ≥ 2).

In the multivariate analysis, we confirmed the independent prognostic value of CTCs in OS ($P = 0.006$), whereas ctDNA results an independent prognostic factor of PFS. This finding may suggest a possible use of ctDNA in catching the progression and the relapses.

Moreover, we found a directly proportional correlation between %ctDNA and the number of CTCs (Spearman correlation coefficient +0.37, $P < 0.001$)—higher when they were measured on the same day—proving the utility of a comprehensive liquid biopsy analysis.

Second, we observed a statistically significant association between the number of alterations and the %ctDNA detected in the baseline sample. ctDNA levels have been shown to increase with the stage of the disease (10) and have a dynamic range and a correlation with changes in tumor burden (41). ctDNA mutations may reflect a transition to a more aggressive disease, and the increasing number of mutations might be a mechanism of resistance to the treatments (41, 42) that enhances with the progression of the disease.

Our findings show the prognostic role of ctDNA that should be added to the established CTCs prognostic value in a comprehensive liquid biopsy analysis. Moreover, we performed an observational analysis correlating specific mutations to outcome. No difference was found in the survival of patients with ER^+ endocrine-resistant disease ($ESR1^+$) versus the patients ER^+ESR1^-.

Furthermore, the follow-up ctDNA analysis performed on 57 patients with ER^+ disease considering 5 genes (*ESR1*, *PIK3CA*, *PTEN*, *AKT1*, and *MAP2*) did not result in any prognostic information, probably due to the small number of patients.

Our study, despite its retrospective nature, was conceived with a prospective plan that allowed us to use the information from both CTCs and ctDNA to select treatment strategies ("historical prospective study"). However, there are several limitations to this study primarily due to its retrospective nature. In particular, we have to point out that in the individual patient, the first blood draw for CTCs has not always been performed at the same time of the first blood draw for ctDNA. For this reason, we calculated two different PFS and OS, one from the first draw for ctDNA, the other from the first draw for CTCs. Moreover, the patients did not have the same number of samples (both for CTCs and ctDNA). Therefore, we included in the analysis only the baseline draw for the individual patient. This has allowed us to standardize our population, but at the same time it may have resulted in a loss of follow-up information.

However, our results showed that liquid biopsy represents an effective tool in predicting the risk of disease relapse and patient outcome. The most novel finding be represented by the demonstrating that quantification of ctDNA may offer a tool to assess both, tumor burden and molecular features of disease. After validation, these findings may lead to modify the clinical practice, allowing the physician to select the patients that benefit from an intensification/change of treatment.

Glossary

- **%ctDNA:** The number of mutant molecules over the total number molecules at a given genomic position.
- **Maximum %ctDNA:** The maximum mutant allele fraction (MAF) in each sample. This is the variable we evaluate in our study. We used the abbreviation "%ctDNA" intending the "maximum %ctDNA."
- **Genomic alterations:** Genomic variants, including point mutations, copy-number variations, insertions/deletions, gene fusions/rearrangements. Using Guardant360, we looked for all these actionable somatic alterations. We used the term "mutations" intending "genomic alterations."

Disclosure of Potential Conflicts of Interest

L.K. Austin reports receiving speakers bureau honoraria from Guardant Health. R.L.B. Costa reports receiving commercial research grants from Bristol-Myers Squibb. M. Cristofanilli reports receiving speakers bureau honoraria from Pfizer and is a consultant/advisory board member for Vortex. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: G. Rossi, M. Cristofanilli
Development of methodology: M. Cristofanilli
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Mu, L.K. Austin, K.S. Strickland, R.J. Nagy, A. Behdad, W.J. Gradishar, M. Cristofanilli

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Rossi, A.W. Rademaker, R.L.B. Costa, R.J. Nagy, V. Zagonel, A. Behdad, F.H. Wehbe, L.C. Platanias, W.J. Gradishar, M. Cristofanilli

Writing, review, and/or revision of the manuscript: G. Rossi, A.W. Rademaker, K.S. Strickland, R.L.B. Costa, R.J. Nagy, T.J. Taxter, A. Behdad, F.H. Wehbe, L.C. Platanias, W.J. Gradishar, M. Cristofanilli

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.J. Nagy, F.H. Wehbe

Study supervision: M. Cristofanilli

Other (collection and organization of the data in a database): G. Rossi

Acknowledgments

The work was supported by the Lynn Sage Cancer Research Foundation as part of the Lurie Cancer Center Breast OncoSET Program (M. Cristofanilli).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 21, 2017; revised November 1, 2017; accepted November 20, 2017; published OnlineFirst November 27, 2017.

References

1. NCI SEER data analysis 2000–2005.
2. O'Shaughnessy J. Extending survival with chemotherapy in metastatic breast cancer. Oncologist 2005;10:20–9.
3. Toss A, Cristofanilli M. Molecular characterization and targeted therapeutic approaches in breast cancer. Breast Cancer Res 2015;17:60,015-0560-9.
4. Nabholtz JM, Buzdar A, Pollak M, Harwin W, Burton G, Mangalik A, et al. Anastrozole is superior to tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: results of a North American multicenter randomized trial. Arimidex Study Group. J Clin Oncol 2000; 18:3758–67.
5. Mouridsen H, Gershanovich M, Sun Y, Perez-Carrion R, Boni C, Monnier A, et al. Phase III study of letrozole versus tamoxifen as first-line therapy of advanced breast cancer in postmenopausal women: analysis of survival and update of efficacy from the International Letrozole Breast Cancer Group. J Clin Oncol 2003;21:2101–9.
6. Paridaens RJ, Dirix LY, Beex LV, Nooij M, Cameron DA, Cufer T, et al. Phase III study comparing exemestane with tamoxifen as first-line hormonal treatment of metastatic breast cancer in postmenopausal women: the European organisation for research and treatment of cancer breast cancer cooperative group. J Clin Oncol 2008;26:4883–90.
7. Bergh J, Jonsson PE, Lidbrink EK, Trudeau M, Eiermann W, Brattstrom D, et al. FACT: an open-label randomized phase III study of fulvestrant and anastrozole in combination compared with anastrozole alone as first-line therapy for patients with receptor-positive postmenopausal breast cancer. J Clin Oncol 2012;30:1919–25.
8. Johnston SR, Kilburn LS, Ellis P, Dodwell D, Cameron D, Hayward L, et al. Fulvestrant plus anastrozole or placebo versus exemestane alone after progression on non-steroidal aromatase inhibitors in postmenopausal patients with hormone-receptor-positive locally advanced or metastatic breast cancer (SoFEA): a composite, multicentre, phase 3 randomised trial. Lancet Oncol 2013;14:989–98.
9. Chu D, Paoletti C, Gersch C, VanDenBerg DA, Zabransky DJ, Cochran RL, et al. ESR1 mutations in circulating plasma tumor DNA from metastatic breast cancer patients. Clin Cancer Res 2016;22:993–9.
10. Ignatiadis M, Dawson SJ. Circulating tumor cells and circulating tumor DNA for precision medicine: dream or reality? Ann Oncol 2014;25: 2304–13.
11. Jameson JL, Longo DL. Precision medicine–personalized, problematic, and promising. N Engl J Med 2015;372:2229–34.
12. Board RE, Wardley AM, Dixon JM, Armstrong AC, Howell S, Renshaw L, et al. Detection of PIK3CA mutations in circulating free DNA in patients with breast cancer. Breast Cancer Res Treat 2010;120:461–7.
13. Higgins MJ, Jelovac D, Barnathan E, Blair B, Slater S, Powers P, et al. Detection of tumor PIK3CA status in metastatic breast cancer using peripheral blood. Clin Cancer Res 2012;18:3462–9.
14. Murtaza M, Dawson SJ, Tsui DW, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–12.
15. Thierry AR, Mouliere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med 2014;20:430–5.
16. De Mattos-Arruda L, Weigelt B, Cortes J, Won HH, Ng CK, Nuciforo P, et al. Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. Ann Oncol 2014;25:1729–35.
17. Rothé F, Laes JF, Lambrechts D, Smeets D, Vincent D, Maetens M, et al. Plasma circulating tumor DNA as an alternative to metastatic biopsies for mutational analysis in breast cancer. Ann Oncol 2014;25:1959–65.
18. Canzoniero JV, Park BH. Use of cell free DNA in breast oncology. Biochim Biophys Acta 2016;1865:266–74.
19. De Mattos-Arruda L, Caldas C. Cell-free circulating tumour DNA as a liquid biopsy in breast cancer. Mol Oncol 2016;10:464–74.
20. Wechsler J. General. In: Wechsler J editor. Circulating tumor cells from solid cancers. Montpellier (France): Sauramps Medical; 2015. p. 13–14.
21. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Discov 2014;4: 650–61.
22. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 2004;351:781–91.
23. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 2006;12:4218–24.
24. Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, et al. Circulating tumour cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 2012;13:688–95.
25. A multicenter, phase III study to compare standard therapy ± lapatinib in HER2-ve MBC-patients with HER2+ve CTCs (DETECT III, trial NCT01619111).
26. Peeters DJ, De Laere B, Van den Eynden GG, Van Laere SJ, Rothé F, Ignatiadis M, et al. Semiautomated isolation and molecular characterisation of single or highly purified tumour cells from CellSearch enriched blood samples using dielectrophoretic cell sorting. Br J Cancer 2013; 108:1358–67.
27. Lanman RB, Mortimer SA, Zill OA, Sebisanovic D, Lopez R, Blau S, et al. Analytical and clinical validation of a digital sequencing panel for quantitative, highly accurate evaluation of cell-free circulating tumor DNA. PLoS One 2015;10:e0140712.
28. Bidard FC, Peeters DJ, Fehm T, Nole F, Gisbert-Criado R, Mavroudis D, et al. Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data. Lancet Oncol 2014;15:406–14.
29. Budd GT, Cristofanilli M, Ellis MJ, Stopeck A, Borden E, Miller MC, et al. Circulating tumor cells versus imaging—predicting overall survival in metastatic breast cancer. Clin Cancer Res 2006;12:6403–9.
30. Zhang L, Riethdorf S, Wu G, Wang T, Yang K, Peng G, et al. Meta-analysis of the prognostic value of circulating tumor cells in breast cancer. Clin Cancer Res 2012;18:5701–10.
31. Lv Q, Gong L, Zhang T, Ye J, Chai L, Ni C, et al. Prognostic value of circulating tumor cells in metastatic breast cancer: a systemic review and meta-analysis. Clin Transl Oncol 2016;18:322–30.
32. Madic J, Kiialainen A, Bidard FC, Birzele F, Ramey G, Leroy Q, et al. Circulating tumor DNA and circulating tumor cells in metastatic triple negative breast cancer patients. Int J Cancer 2015;136:2158–65.
33. Dawson SJ, Tsui DW, Murtaza M, Biggs H, Rueda OM, Chin SF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368:1199–209.
34. Huggett JF, Cowen S, Foy CA. Considerations for digital PCR as an accurate molecular diagnostic tool. Clin Chem 2015;61:79–88.

35. Massihnia D, Perez A, Bazan V, Bronte G, Castiglia M, Fanale D, et al. A headlight on liquid biopsies: a challenging tool for breast cancer management. Tumour Biol 2016;37:4263–73.
36. Cossu-Rocca P, Orru S, Muroni MR, Sanges F, Sotgiu G, Ena S, et al. Analysis of PIK3CA mutations and activation pathways in triple negative breast cancer. PLoS One 2015;10:e0141763.
37. Angus L, Beije N, Jager A, Martens JW, Sleijfer S. ESR1 mutations: moving towards guiding treatment decision-making in metastatic breast cancer patients. Cancer Treat Rev 2017;52:33–40.
38. Fribbens C, O'Leary B, Kilburn L, Hrebien S, Garcia-Murillas I, Beaney M, et al. Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer. J Clin Oncol 2016;34:2961–8.
39. Chu D, Park BH. Liquid biopsy: unlocking the potentials of cell-free DNA. Virchows Arch 2017 May 2. [Epub ahead of print].
40. Criscitiello C, Curigliano G. Immunotherapy of breast cancer. Prog Tumor Res 2015;42:30–43.
41. Alix-Panabières C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. Cancer Discov 2016;6:479–91.
42. Bardelli A, Pantel K. Liquid biopsies, what we do not know (yet). Cancer Cell 2017;31:172–179.