ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

ΙΑΤΡΙΚΗ ΣΧΟΛΗ

ΘΕΡΑΠΕΥΤΙΚΗ ΚΛΙΝΙΚΗ ΝΟΣ. ΑΛΕΞΑΝΔΡΑ

ΠΡΟΓΡΑΜΜΑ ΜΕΤΑΠΤΥΧΙΑΚΩΝ ΣΠΟΥΔΩΝ

«ΚΛΙΝΙΚΕΣ ΜΕΛΕΤΕΣ: ΣΧΕΔΙΑΣΜΟΣ ΚΑΙ ΕΚΤΕΛΕΣΗ»

Διευθυντής: Καθηγητής Ευάγγελος Τέρπος

Τίτλος ΜΔΕ: «Ενσωματώνοντας την Ιατρική Ακριβείας στις Κλινικές Μελέτες: το παράδειγμα των προβλεπτικών βιοδεικτών, όπως αυτοί αναλύονται στο κυκλοφορούν καρκινικό DNA, στον καρκίνο του μαστού»

"Precision medicine into clinical trials: the paradigm of circulating tumor DNA-based predictive biomarkers in breast cancer"

Όνομα: Ωραιάνθη Φιστέ

Αρ. Μητρώου: 20180683

Ιδιότητα: Ειδικευόμενη ιατρός Παθολογικής Ογκολογίας, Θεραπευτική Κλινική, ΓΝΑ Αλεξάνδρα

Επιβλέπουσα Καθηγήτρια: κα. Φλώρα Ζαγουρή, Αν. Καθηγήτρια ΕΚΠΑ

ΑΘΗΝΑ 2020

ΕΘΝΙΚΟ ΚΑΙ ΚΑΠΟΔΙΣΤΡΙΑΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ

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<u>Τα Μέλη της Εξεταστικής Επιτροπής</u>

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ΑΘΗΝΑ 2020

CURRICULUM VITAE

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EDUCATION

2017 Oct: Master of Science in Thoracic Oncology, National and Kapodistrian University of Athens (M.Sc. Thesis entitled: "Circulating tumor cells in lung cancer patients")

2014 Jul: Medical Degree, Medical School of National and Kapodistrian University of Athens

2013 Mar -2013 Jul: Erasmus Lifelong Learning Program, Medizinische Universität Innsbruck

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2019 Nov - present: Fellowship in Medical Oncology, Alexandra General Hospital, Athens

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PEER-REVIEWED PUBLICATIONS

1. K. Syrigos, **O. Fiste**, A. Charpidou, D. Grapsa. *Circulating tumor cells count as a predictor of survival in lung cancer*. Crit Rev Oncol Hematol 125, 2018 pp. 60-68doi.org/10.1016/j.critrevonc.2018.03.004

NON PEER-REVIEWED PUBLICATIONS

1. E. Karamitrousis, P. Koliou, P. Baxevanos, A. Mpokas, N. Tsoukalas, **O. Fiste**. *Handbook of Cancer Associated Thrombosis*, on behalf of the Hellenic Society for Medical Oncology (HESMO)
2. Translation in Greek of "*Glioma: Guide for patients*'', based on the ESMO Clinical Practice Guidelines, on behalf of the Hellenic Society for Medical Oncology (HESMO)

PRESENTATIONS IN INTERNATIONAL MEDICAL MEETINGS

1. **O. Fiste**. *Circulating tumor DNA signature*. Invited lecture in 7th Lung Cancer Network ''From the Bench to the Bedside'', 16-18 January 2020, Athens, Greece.
2. **O. Fiste**, D. Grapsa, E. Politaki, I. Stoupis, D. Mavroudis, S. Agelaki, K.N.Syrigos. *Detection of circulating tumor cells is associated with disease burden in patients with advanced non-small cell lung cancer*. *IASLC WCLC 2017*. J Thor Oncol 12(11):S1910, 2017.
3. S. Agelaki, **O. Fiste**, D. Grapsa, E. Politaki, I. Stoupis, D. Mavroudis, K.N.Syrigos. *Prognostic value of circulating tumor cells in non-small cell lung cancer*. *2017 ASCO Annual Meeting*. J Clin Oncol 35, 2017 (suppl; abstr e20520).

SELECTED PRESENTATIONS IN NATIONAL MEDICAL MEETINGS

1. **Fiste O.** *Targeted therapies in colorectal cancer*. Invited lecture in 13th Congress of Oncology in Primary Care ''Developments in Oncology: from research to clinical practice'', Athens, 6-7 December 2020.
2. **Fiste O**., Karampeazis A., Voulgaris E., Xenakis K., Dimos A., Kardara V., Arvanitou E., Stamatogianni E., Mpallasis K., Christofillakis C. *Primary cardiac angiosarcoma: A case report and review of the literature*. 25th Hellenic Society of Clinical Oncology meeting, Athens, 18-20 April 2019.
3. Arvanitou E., Karampeazis A., **Fiste O**., Kardara V., Mpallasis K., Stamatogianni E., Christofyllakis C. *Gynaecomastia as paraneoplasmatic manifestation in a patient with non-small cell lung cancer*. 25th Hellenic Society of Clinical Oncology meeting, Athens, 18-20 April 2019.

GRANTS

2013: State Scholarships Foundation of Greece to attend the Erasmus Program

2009: Eurobank EFG group, as a high-school graduate, who achieved the highest grades in Chios regional unit, for admission to tertiary education

VOLUNTEER

2012 Oct -2013 Oct:	European Medical Students' Association (EMSA) Local Coordinator, HelMSIC ATHENS
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2011 Jul:	First aid provider in Equestrian Games, Special Olympics World Summer Games ATHENS2011

MEDICAL ASSOCIATIONS

- Medical Association of Athens
- Hellenic Group of Young Oncologists (HeGYO)
- Hellenic Society of Geriatric Oncology

LANGUAGES - SKILLS

- Highly proficient in spoken and written English
- Very good command of spoken and written German
- ICH GCP E6 (R2), Global Health Training Center
- Workshop in ‘’Project Management’’ and ‘’Team Dynamics’’, Training Center of Excellence, Yeditepe University Istanbul, 22-28 March 2011
- Computer skills (ECDL Core Microsoft Office, use of PubMED)

Πίνακας περιεχομένων και ευρετήριο πινάκων και σχημάτων

Πρόλογος

Η εκπόνηση της παρούσας μεταπτυχιακής διπλωματικής εργασίας υπήρξε αποτέλεσμα της επιθυμίας να μελετηθεί ο πολλά υποσχόμενος ρόλος των υγρών βιοψιών στο πλαίσιο των ογκολογικών κλινικών μελετών, με σκοπό την ανάπτυξη και εφαρμογή στοχευουσών θεραπειών και εν γένει την εξατομίκευση της θεραπείας του καρκίνου του μαστού.

Πρωτίστως, για την εμπιστοσύνη και την ευκαιρία να συμμετάσχω στο μεταπτυχιακό πρόγραμμα ευχαριστώ τον επιστημονικό υπεύθυνο Καθηγητή κ. Ευάγγελο Τέρπο.

Ιδιαιτέρως εκφράζω την ευγνωμοσύνη μου προς την επιβλέπουσα της διπλωματικής μου, Αν. Καθηγήτρια κα. Φλώρα Ζαγουρή, για τη συστηματική παρακολούθηση της εργασίας, τις υποδείξεις της και κυρίως για τον γόνιμο επιστημονικό διάλογο.

Ολόθερμες ευχαριστίες στα μέλη της τριμελούς επιτροπής, τον Αν. Καθηγητή κ. Ευστάθιο Καστρίτη και την Επ. Καθηγήτρια κα. Μαρία Γαβριατοπούλου για την πολύτιμη καθοδήγηση καθ' όλη τη διάρκεια του μεταπτυχιακού προγράμματος.

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Τέλος, για την ακλόνητη ηθική και έμπρακτη συμπαράσταση ευχαριστώ από καρδιάς την οικογένειά μου.

Στη γενναία μου φίλη, Α.

Ενσωματώνοντας την Ιατρική Ακριβείας στις Κλινικές Μελέτες: το παράδειγμα των προβλεπτικών βιοδεικτών, όπως αυτοί αναλύονται στο κυκλοφορούν καρκινικό DNA, στον καρκίνο του μαστού

Περίληψη

Ο καρκίνος του μαστού αποτελεί την πρώτη σε συχνότητα και δεύτερη σε θνησιμότητα κακοήθεια στις γυναίκες παγκοσμίως. Στην εποχή της Ιατρικής Ακριβείας οι θεραπευτικές αποφάσεις βασίζονται στα πεδία της μεταγραφικής έρευνας: γονιδιωματικής (έκφραση γονιδίων) και πρωτεομικής (έκφραση ορμονικών και HER2 υποδοχέων) ανάλυσης των κυττάρων του όγκου, συνηθέστερα από το αρχικό δείγμα της ιστικής βιοψίας του πρωτοπαθούς όγκου, που δεν αντανακλά απαραίτητα τη δυναμική του μοριακού προφίλ της νόσου. Έτσι, η αδυναμία ελέγχου της γενετικής ετερογένειας και της εξέλιξης της νόσου, σε αληθινό χρόνο, μπορεί να εξηγήσει την αποτυχία της συστηματικής θεραπείας, στις μέρες μας, παρά την ανάπτυξη στοχευουσών θεραπειών. Αντίθετα, η ανάλυση κυκλοφορούντων καρκινικών βιοδεικτών, στο πλαίσιο των υγρών βιοψιών, συμπεριλμβανομένου του κυκλοφορούντος καρκινικού DNA (ctDNA), παρέχει μια εικόνα για το μοριακό προφίλ του πρωτοπαθούς όγκου και των μεταστατικών του εστιών, με μη παρεμβατικό τρόπο. Στη συγκεκριμένη συστηματική ανασκόπηση συνοψίζουμε τα αποτελέσματα πρόσφατα δημοσιευμένων κλινικών δοκιμών που βασίζονται σε αντίστοιχους βιοδείκτες. Είναι επιτακτική η διεξαγωγή καλά σχεδιασμένων, πολυκεντρικών, τυχαιοποιημένων κλινικών μελετών, που χρησιμοποιούν βιοδείκτες βασισμένους σε ctDNA αναλύσεις για τη διαστρωμάτωση των συμμετέχοντων ασθενών, με στόχο τον προσδιορισμό της προβλεπτικής αξίας του ctDNA στην εξατομικευμένη θεραπείων των ασθενών με καρκίνο μαστού.

Λέξεις-κλειδιά: Καρκίνος μαστού, κυκλοφορούν καρκινικό DNA, κλινικές μελέτες, προβλεπτικός βιοδείκτης

Precision medicine into clinical trials: the paradigm of circulating tumor DNA-based predictive biomarkers in breast cancer

Abstract

Breast carcinoma (BC) is the most frequent and the second leading cause of cancer mortality in women worldwide. In the era of precision medicine, therapeutic decisions are mainly based on genomic, transcriptomic (gene expression) and/or proteomic (status of HER2 and hormone receptors) profiling of cells from a single, usually archival, sample of the primary tumour, which may not necessarily represent the current disease status. Thus, the inability to capture tumour genetic heterogeneity and evolution in real-time could explain the failure of systemic therapy, nowadays, despite the advances in targeted treatment modalities. On the contrary, analysis of circulating blood markers in the field of liquid biopsies, including circulating tumour DNA (ctDNA), provides an insight into the dynamic molecular profiling of the primary tumour and its metastases, in a relatively non-invasive way. In this systematic review we summarize the results from recent and ongoing biomarker-driven clinical trials and discuss the quality and limitations of the literature. Further investigation, through the conduct of well-designed, multicenter, randomized, biomarker-stratified clinical trials, is needed to determine the potential predictive value of ctDNA analysis, with respect to tailored, personalized treatment guidance for BC patients.

Keywords: Breast cancer, circulating tumor DNA, clinical trials, predictive biomarker

1. INTRODUCTION

Breast cancer is the most prevalent cancer and the second leading cause of cancer mortality in women (1, 2). Next-generation sequencing (NGS)-based diagnostics have identified around 40 genomic alterations, shedding light into the heterogeneity of this disease (3, 4). Currently, only a few of these somatic alterations have been validated as therapeutic targets, whereas there are 12 targeted therapies[apart from hormonal therapy for hormone receptor (HR)+ disease], effective as signalling blockade, in the adjuvant, neoadjuvant, and metastatic settings.

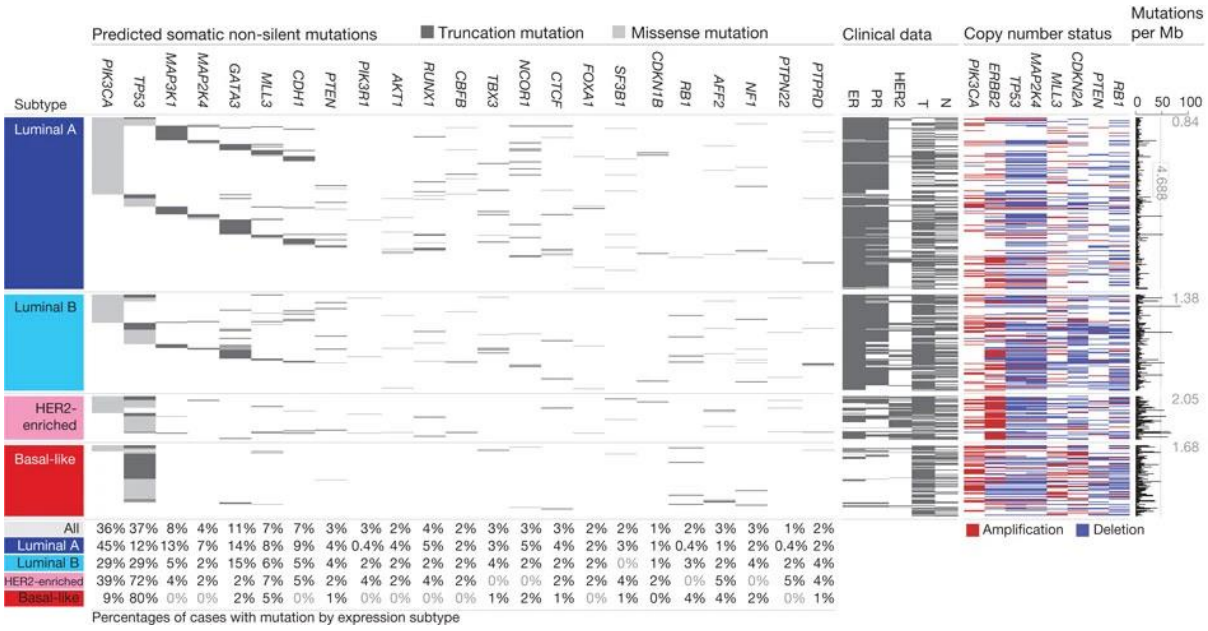

Figure 1. Significantly mutated genes and correlations with genomic and clinical features (Cancer Genome Atlas Network. Nature, 2012)

In particular, trastuzumab (5-7), pertuzumab (8-10), ado-trastuzumab emtansine (11), lapatinib (12) and neratinib (13) are human epidermal growth factor receptor 2 (HER2) inhibitors for the treatment of HER2+ disease, palbociclb (14), ribociclib (15) and abemaciclib (16) are cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors for the treatment ofHR+, HER2- disease, everolimus (17) is a mammalian target of rapamycin (mTOR) inhibitor, also, for the treatment of HR+, HER2- disease, olaparib (18) and talazoparib (19) are poly ADP ribose polymerase (PARP) inhibitors for the treatment of BRCA+ disease, while alpelisib (20) is a phosphoinositide-3-kinase (PI3K) inhibitor for the treatment of PIK3CA+ disease.

Despite therapeutic advance in personalized medicine strategies, metastatic breast cancer remains an incurable disease, with a 5-year survival rate of approximately 25% [21, 22]. Breast cancer's plasticity, over time and under treatment pressure, represents the greatest challenge in its therapeutics, due to disease recurrence and drug resistance (23, 24).

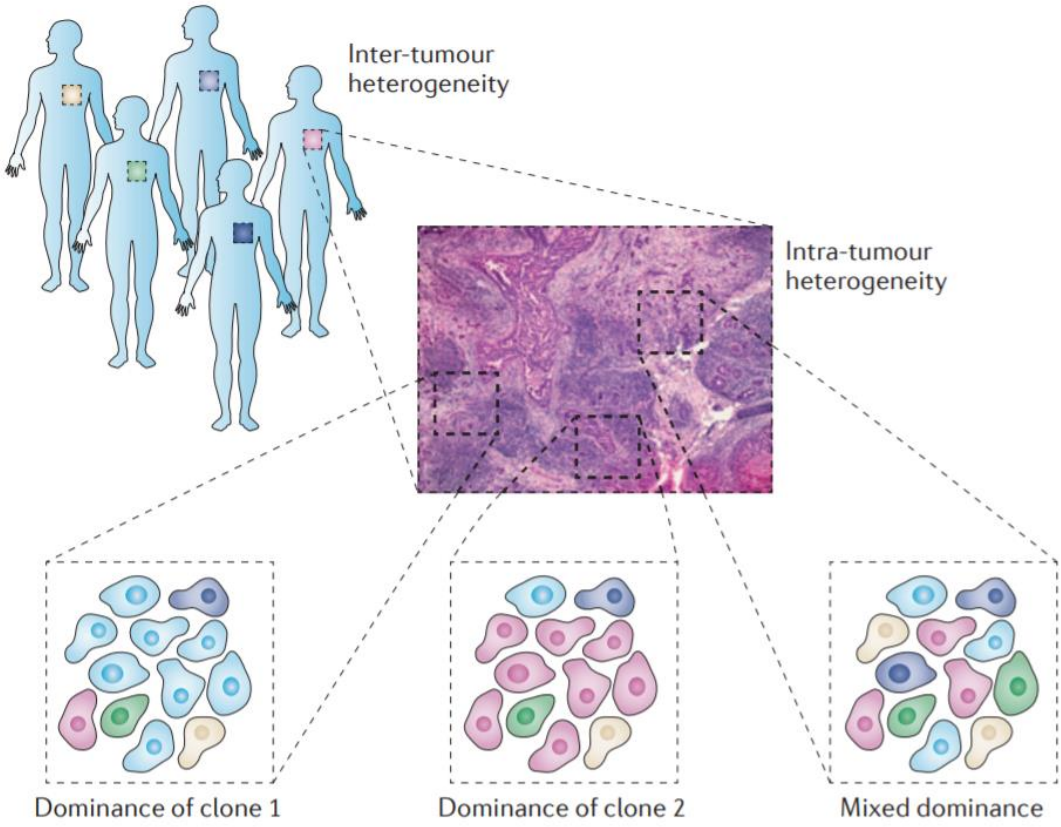

Figure 2. Tumor heterogeneity in diagnostics (Marusyk A., et al. Nature, 2012)

Thus, both American and European guidelines recommend reassessment of biomarkers, like HR and HER2 status, if feasible, in the metastatic setting (25).

Unfortunately, tissue biopsies are fraught with several caveats; they are invasive, patient-unfriendly procedures, not always feasible either because of patient's condition and comorbidities or because of tumor's accessibility, and they don't permit longitudinal monitoring of tumor (26-30).Thus, the ideal approach to address the diverse molecular profile of breast tumors would be a minimally invasive method that could capture the entire genetic make-up of the tumor, in 'real-time', during the course of treatment.

Currently, analysis of circulating blood biomarkers, like circulating tumor DNA (ctDNA), under the umbrella-term of 'liquid biopsies', offer an attractive approach to evaluate patient's entire tumor burden, in a non-invasive, convenient, repetitive, dynamic, and cost-effective way (31-37).

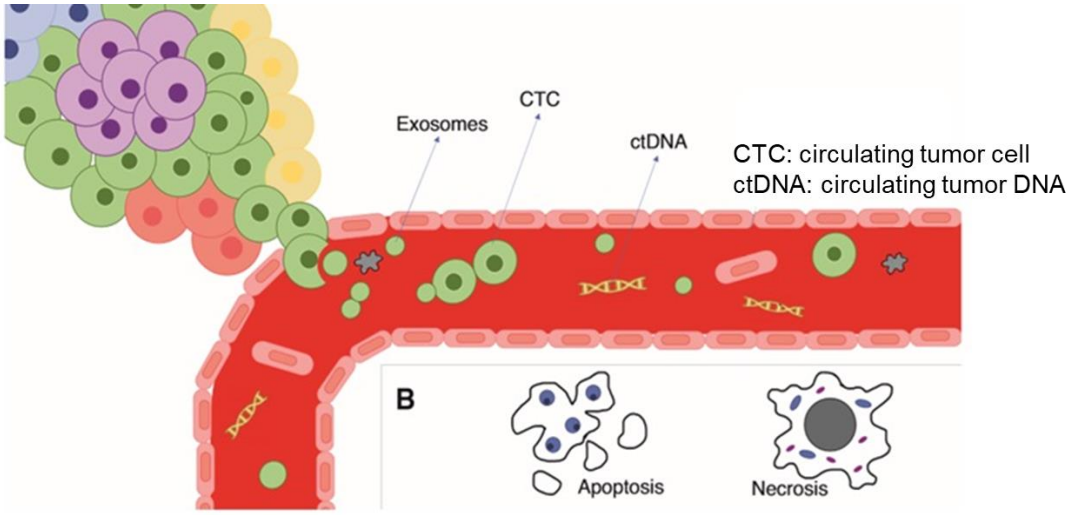

Figure 3. Origin and different types of the liquid biopsy approach (Leers MPG. Clin Chem Lab Med, 2019)

Several studies have evaluated the emerging role of ctDNA in monitoring treatment response or resistance and in predicting early relapse (38-49). Nevertheless, studies investigating the potential capacity of serial ctDNA monitoring for treatment guidance are still scarce, small-scale, and lack a strict clinically-centered protocol. We, therefore, performed a systematic review of the published literature of recent and ongoing clinical trials, which incorporate ctDNA-based predictive biomarkers, in breast cancer patients, to assess the potential of ctDNA in optimizing disease management.

2. MATERIALS AND METHODS

2.1 Search strategy and study identification

A systematic review of published literature was conducted, to assess the predictive value of ctDNA analysis in the setting of clinical trials in breast cancer patients.

All eligible studies were identified by a search in www.clinicaltrials.gov, MEDLINE/PubMed database and Cochrane Database of Systematic Reviews (CDSR) for the period up to August 31, 2019.

Clinical Trials incorporating ctDNA analysis, as source of potential predictive biomarkers, in patients with breast cancer were considered for inclusion. To create a search strategy, medical subject heading (MeSH) terms (breast, cancer, neoplasm, carcinoma, clinical trial, ctDNA, cfDNA, predictive, biomarker) were used in addition with Boolean search terms (AND, OR).

2.2 Study eligibility

Eligible for inclusion were considered all randomised and non-randomised clinical trials carried out in adult patients (≥18 years old), irrespective of gender, with breast cancer, reporting results of ctDNA analysis and its correlation with treatment efficacy. Abstracts presented in conferences were also included.

Language restrictions were applied (only articles published in English were considered eligible). Animal studies, book chapters, observational study designs, commentaries, case reports, reviews, meta-analyses and studies not in cancer patients were also excluded.

2.3 Data extraction and quality assessment

Data extraction was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) guidelines.

The following data were extracted from each clinical trial: clinical trial name and ID number, status, first author, year of publication, setting (primary or advanced breast cancer), line of therapy (neoadjuvant, adjuvant, and 1^{st} or 2^{nd} line for metastatic setting etc.), pathological subtype/hormonal status, allocation of study (randomized, non-randomized), intervention model (sequential-, parallel-, single group- assignment), masking, phase, treatment modalities (intervention and control arm regimens), number of patients enrolled in biomarker sub-study, primary endpoint, ctDNA sequencing technique, results.

3. RESULTS

Our search strategy retrieved initially 64clinical trials, which were screened at title and abstract (if it was available) using the study inclusion criteria. Of these, 43 were unpublished, 1 was observational study, and 20 (containing data on 6502 patients) were finally eligible for the systematic review. The aforementioned stages of the study design and article selection process are illustrated in Figure 4.

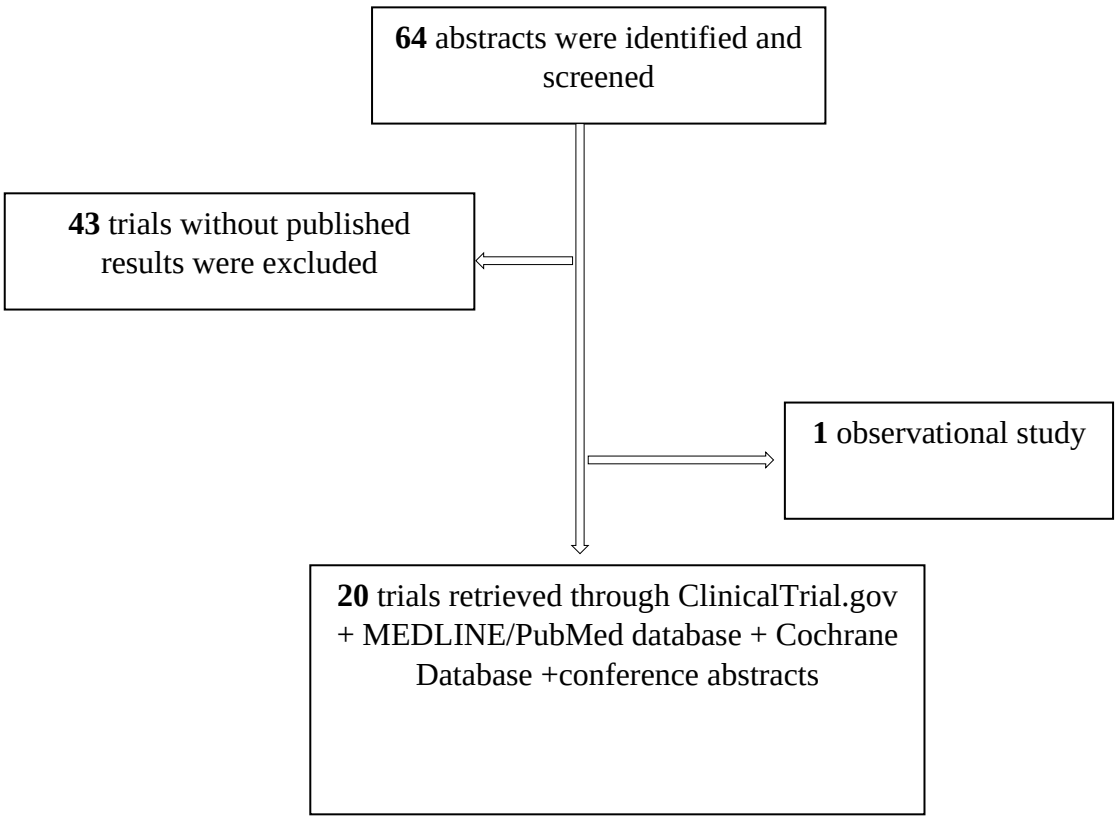

Figure 4. Schematic chart of search strategy

The trials were designed, implemented, and reported in accordance with the International Conference on Harmonization Guidelines for Good Clinical Practice; applicable local regulations along with the ethical principles of the Declaration of Helsinki were observed. The trials also received Institutional Review Board and Independent Ethics Committee approval prior to initiation at study sites.

Across the 20 trials, containing data from 6,502 patients, 4 (20%) were completed, 11 (55%) were Phase III, 11 (55%) were double-blind, whereas 4 (20%) were non-randomized. Moreover, our review included 1 adaptive-designed clinical trial and 2 Basket trials. 4 (20%) trials included patients with HER2 subtype BC, 2 (10%) with triple negative BC (TNBC), and 2 (10%) evaluated the predictive role of ctDNA in the neo-adjuvant setting. PFS was the primary endpoint in the vast majority of the trials (14/20, 70%), while PCR-based methods were used in 50% of the included trials.

Characteristics of studies are presented in Table 2.

Preliminary results from PAlbociclib and Curculating Tumor DNA for ESR1 Mutation Detection (PADA-1) trial demonstrated that ESR1mut detection is uncommon in untreated aromatase inhibitor (AI)-sensitive, ER+, HER2- metastatic breast cancer patients (detection rate of 2.1% at baseline) and is related to prior AI exposure in the adjuvant setting (4.9% with AI use vs 0% without AI use, Yates Chi2: p=0.009). Remarkably, 1-month use of AI and palbociclib, the first CDK4/6 inhibitor approved as an anticancer regimen, led to undetectable ESR1mut in 13 among the 17 patients with ESR1mut detected at baseline (50).

In the PALOMA-3 study, which compared the combination of palbociclib plus fulvestrant to placebo plus fulvestrant, in patients with HR+, HER2- advanced breast cancer, progressing on prior endocrine therapy, changes in PIK3CA ctDNA dynamics upon 15 days treatment predicted response to targeted therapy in combination with fulvestrant (HR 3.94, 95% CI 1.61-9.64, log-rank p = 0.0013), while ESR1 ctDNA levels change was less predictive on PFS on palbociclib plus fulvestrant (14, 44). Detection of PIK3CA and ESR2 mutations in plasma ctDNA samples, compared with their detection in archived tissue samples, has been associated with significantly improved PFS and response to abemaciclib (another selective CDK4/6 inhibitor) plus fulvestrant, in postmenopausal women with HR+, HER2- advanced breast cancer, progressing on prior endocrine therapy (51, 52).

On the contrary, ctDNA sequencing from 494 patients enrolled in the randomized MONALEESA-2 trial of letrozole ± ribociclib, showed a consistent PFS benefit for the combination of endocrine therapy plus CDK4/6 inhibitor, regardless of the baseline status of ctDNA biomarkers (PIK3CA, TP53, ZNF703/FGFR1, ESR1) (15, 53). Consistent treatment benefit was observed for fulvestrant and ribociclib, irrespective of baseline ctDNA alteration status (PIK3CA, ESR1, TP53, CDH1, FGFR1/ZNF703/WHSC1L1) in Phase III MONALEESA-3 study (54, 55).

In BELLE-2, which evaluated the combination of the panPI3 kinase inhibitor buparlisib with fulvestrant in patients with refractory to AI, HR+, HER2- advanced breast cancer, the presence of PIK3CA mutations in ctDNA corresponded to improved PFS in the buparlisib arm (7.0 vs 3.2 months; HR=0.58; 95% CI 0.41-0.82; 1-sided nominal p=0.001) (56, 57).Clinical benefit of the addition of buparlisib to fulvestrant in HR+, HER2- advanced breast cancer patients, with prior use of mTOR inhibitors, has also been observed in the randomized phase III BELLE-3 trial, even if this benefit was irrespective of PIK3CA status in ctDNA (58). Both, BELLE-2

and BELLE-3 highlighted the potential of PIK3CA mutational status in plasma ctDNA as predictive biomarker for benefit of buparlisib treatment, in this subset of breast cancer patients, whereas the discordance in PIK3CA status between tumor tissue and ctDNA samples (76.7% in BELLE-2 vs 84.8% in BELLE-3) underline the need for an optimal standardized assay.

In a single group assignment, Phase I/II trial the combination of alpelisib and nab-paclitaxel resulted in increased PFS in HER2- advanced breast cancer patients, harbouring ctDNA PIK3CA mutations (59).

A subsidiary analysis of the BOLERO-2 trial on 550 ER+ advanced breast cancer patients, demonstrated that the addition of everolimus to exemestane prolonged PFS, irrespective of cfDNA PIK3CA mutation status (HR=0.43 and 0.37 respectively) (17, 60).

Furthermore, the ongoing POSEIDON trial and Neratinib HER Mutation Basket Study (SUMMIT) support the predictive value of early evaluation of ctDNA changes, before radiologic treatment response (61, 62).

The translational sub-study of the ongoing I-SPY 2 trial demonstrated the significance of serial monitoring of ctDNA in predicting response to neo-adjuvant treatment (63). ctDNA analysis of the NeoALTTO trial demonstrated that the detection of PIK3CA and/or TP53 mutations, in the baseline (before neo-adjuvant therapy) plasma sample was correlated with lower rates of pathological complete response, whereas persistent ctDNA detection both at baseline and after 14 days of neo-adjuvant therapy was significantly associated with the lowest rate of pathological complete response (64, 65).

In open-label WJOG6110B/ELTOP trial, whereas patients with HER2+ advanced breast cancer, were randomized to receive either lapatinib and capecitabine or trastuzumab and capecitabine, PIK3CA mutations in both tissue and ctDNA samples associated with shorter PFS, regardless of the treatment arm (66).The presence of concomitant genetic alterations of HER2, PI3K/AKT/mTOR pathway and TP53 in ctDNA analysis was significantly correlated with worse PFS, compared to ≤1 genetic alteration, in the open-label, Phase I BLTN-Ic trial, of the combination of pyrotinib plus capecitabine in HER2+ advanced breast cancer patients (67).

Dynamic ctDNA analysis of plasma samples from phase I/II trial BEECH, whereas patients with ER+ metastatic breast cancer randomized to either paclitaxel plus AKT inhibitor capivasertib or paclitaxel plus placebo, predicted long-term outcome (PFS of 11.1 months in patients with suppressed ctDNA at 21 days vs 6.4 months in patients with high levels of ctDNA, HR=0.20; 95% CI 0.083-0.50; $p<0.0001$), thus serving as a surrogate for PFS (68).

The double-blind, Phase II LOTUS trial, comparing the combination of ipatasertib plus paclitaxel with paclitaxel monotherapy in triple negative advanced breast cancer patients, demonstrated the predictive value of dynamic evaluation of ctDNA in evaluating both objective response and PFS, consistently in both arms (69, 70).

As part of the phase II, INSPIRE basket trial, a secondary analysis of ctDNA at baseline and before the initiation of 3rd cycle of the single-agent immune checkpoint inhibitor pembrolizumab in 10 triple negative metastatic breast cancer patients, strongly correlated with PFS, OS and overall clinical response rate (71, 72).

In the 1st comprehensive genomic analysis of ctDNA of premenopausal patients with ER+ and/or PR+, HER2- advanced breast cancer, the combination of the CDK 4/6 inhibitor ribociclib and NSAI or tamoxifen and goserelin resulted in PFS benefit, irrespective of the baseline genetic landscape status (73, 74).

Based on the results of SOLAR-1, FDA approved, on May 24, 2019, the use of PIK3CA selective inhibitor alpelisib in combination with fulvestrant for the treatment of men and postmenopausal women, with HR+, HER2-, PIK3CA-mutated advanced breast cancer, following disease progression on or after an endocrine-based regimen. In particular, the combination of alpelisib and fulvestrant resulted in significant prolongation of PFS (HR 0.55; 95% CI 0.39-0.79; n=186) in patients with ctDNA PIK3CA mutant status. Concurrently, FDA also approved the companion diagnostic test PIK3CA RGQ PCR kit to detect the PIK3CA mutation in a tissue and/or a liquid biopsy. Thus, the assessment of PIK3CA mutations in ctDNA became the first liquid biopsy to be used in the clinical setting for breast cancer patients (20, 75).

4. DISCUSSION

Research into understanding breast cancer's complexity, both at cellular and molecular level, and development of targeted therapies underline the urgent need of conducting novel biomarker-driven clinical trials, with the ultimate goal of optimizing disease management (76, 77). The traditional process of drug research and development, where investigational drugs were evaluated for safety and optimal dosing scheme in Phase I, for early signs of efficacy in Phase II, and for confirmation of efficacy, effectiveness and safety in Phase III, gradually fades out. Over the last decade, novel clinical trial designs have found their way into clinical research, in order not only to streamline but also to expedite drug development (78).

Master Protocol (MAPs) use a single, biomarker-driven, trial design and protocol to concurrently evaluate multiple drugs and/or diseases (79), and include:

(a) basket trials, which enrol patients based on the presence of a specific biomarker (e.g. mutation), regardless of histology, to identify efficacy of a biomarker-specific, thus targeted, therapy (80, 81),

(b) umbrella trials and,

(c) adaptive platform trials, where patients who share the same cancer histology are allocated to different arms, based on their biomarker status (e.g. mutation), in order to evaluate new investigational agents matches to biomarker-derived cohorts (82).

The main difference between umbrella and platform trials is that the last incorporate more adaptions, during the trial, based on efficacy results of interim analyses, by permitting in a flexible way the addition or exclusion of new treatment modalities (82).

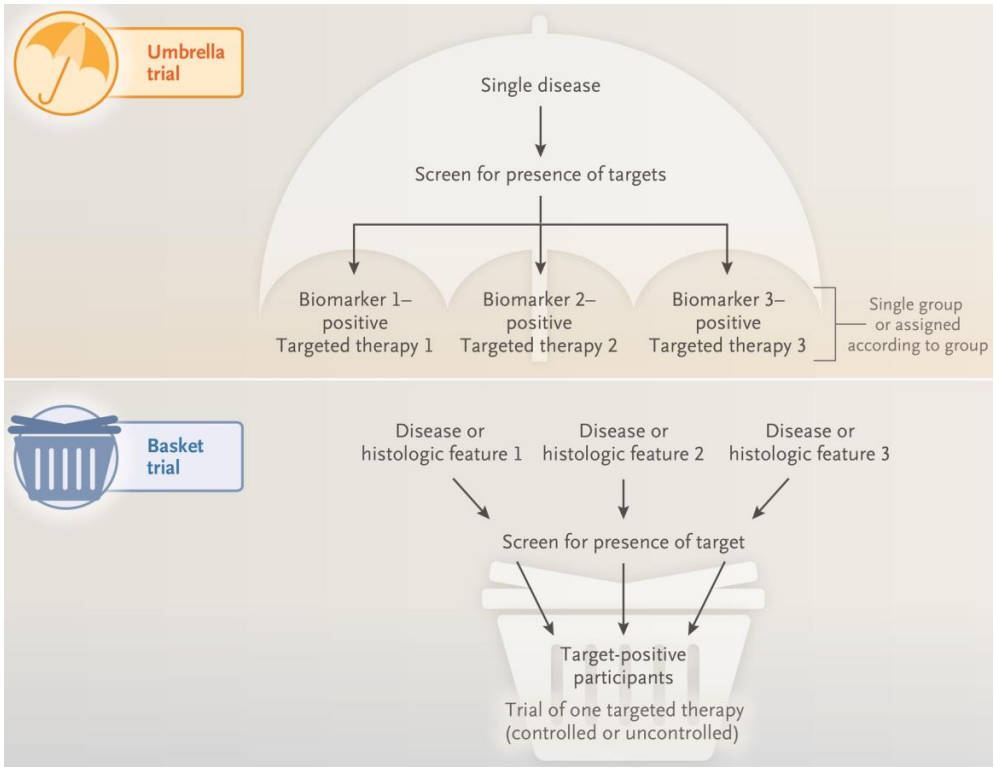

Figure 4. Umbrella trial and Basket trial (Drazen JM. et al. NEJM, 2017)

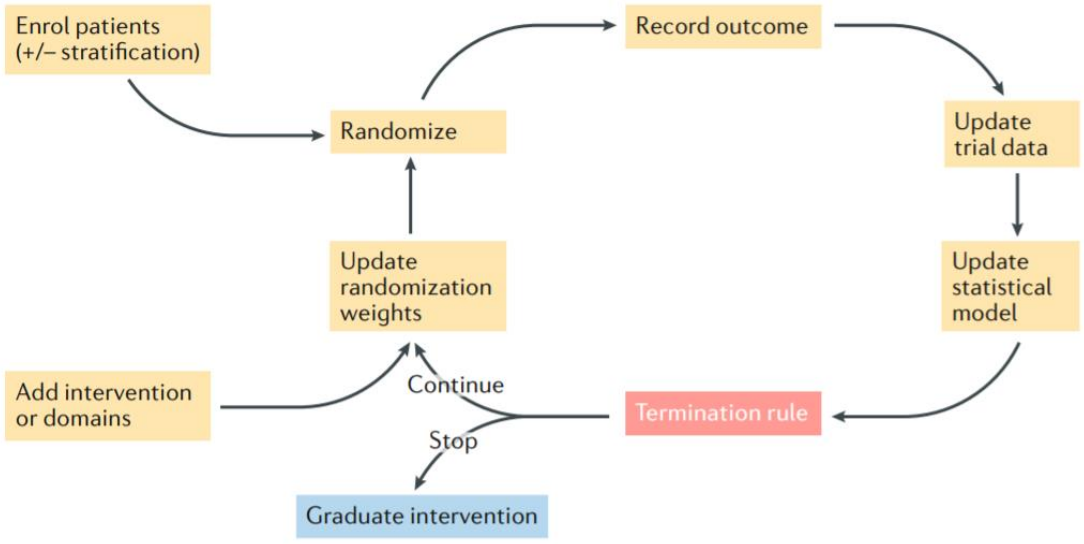

Figure 5. General operational flow of an adaptive platform trial (The Adaptive Platform Trials Coalition. Nat Rev Drug Discov, 2019)

MAPs could offer the patient-centric approach into the field of clinical trials, by enrolling the right patient in the optimum treatment arm. Moreover, they could reduce costs by terminating unsuccessful programs quite early, and evaluating several treatment combinations or competing drugs. Furthermore, MAPs could test multiple clinical hypotheses in parallel, thus are of value in complex disease areas.

Among the hurdles to overcome when implementing these innovative trial designs are the quality and timeliness of the screening technology platforms, used to stratify enrolled patients, and the different regulatory standards across countries. Also, both the choice of primary endpoint (e.g. OS, PFS, ORR) and the probable use of a comparator arm should be taken into consideration, when designing these novel clinical trials.

Establishing biomarker-stratified clinical-trial design frameworks in the context of spatial and temporal heterogeneity is challenging because the traditional use of archival tissue samples may not be reflective of the dynamic genomic status of the tumor, especially in the metastatic setting (83, 84). Such hurdle could potentially be overcome through the incorporation of ctDNA analyses, for the longitudinal evaluation of predictive biomarkers. Overall, results emerged from the clinical trials presented in this systematic review highlight the importance of dynamic ctDNA monitoring in the era of precision medicine; measurement of ctDNA provides representative data of spatiotemporal tracking of mutational landscape of both primary tumour and metastases, thus serving as a sensitive biomarker for both monitoring tumor progression and evaluating treatment response (38, 83, 85, 86).

Nowadays, digital PCR (dPCR)- and next generation sequencing (NGS)-based methods are most frequently used to detect ctDNA in a background of wildtype DNA (87-89). Despite the wide variety in the number of available technologies for ctDNA analysis, only 2 companion diagnostic kits are FDA-approved: cobas EGFR Mutations Test v2 for detection of EGFR mutations in NSCLC, and therascreen® PIK3CA RGQ PCR Kit for detection of PIK3CA mutations in advanced or metastatic breast cancer (90).

Standardization challenges for integration of ctDNA analysis into routine clinical practice include:

(a) biological variability (thus tumor heterogeneity),

(b) pre-analytical variability (e.g. specialized collecting tubes to prevent leukocyte lysis, optimal time period between blood-draw and sample processing, centrifugation conditions, quantification methods) (91-93), and

(c) analytical variability (an ideal technology should be accurate, highly sensitive and specific, robust, and cost-effective) (94).

To accelerate the development and establishment of liquid biopsies in clinical practice, consortium of researchers from academia, industry, regulatory agencies and public, both in United States (BloodPAC) (95), and Europe (Cancer-ID) (96) have been developed.

Facilitating randomized, well-controlled, multicenter, prospective clinical trials with extensive cohorts of patients and standardized ctDNA analysis techniques will allow not only the reproducibility but also the comparison of their clinical results, thus contributing to the evidence-based introduction of ctDNA, from laboratory perspective, into routine oncology practice in the near future.

5. CONCLUSION

In conclusion, it can be said that the majority of published results from both recent and ongoing biomarker-driven clinical trials in breast cancer patients seem to concur that ctDNA profiling may significantly correlate with response to targeted therapies, thus indicating its potential as a non-invasive predictive biomarker, both in adjuvant, neo-adjuvant, and metastatic setting.

The incorporation of ctDNA analysis into sophisticated, biomarker-driven clinical trials, with adequate statistical power and sufficient sample sizes, remains the most reliable way to demonstrate not only the analytical and clinical validity, but also the clinical utility of ctDNA as liquid biopsy, in tailoring decision-making in breast cancer patients.

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7. TABLES

Table 1. Tissue biopsy versus Liquid biopsy comparison

Tissue Biopsy	**ctDNA analysis**
Invasive, uncomfortable procedure	Minimally invasive (blood draw)
Variable biopsy risks	Always accessible
Difficulties in serial testing, tissue quantity	Real-time, longitudinal monitoring
Histology and cellular phenotype	Molecular phenotype
Selection bias	Tumor heterogeneity
Validated tissue processing	Non-validated handling procedures
Time consuming	Rapid purification
Gold standard	Evolving clinical utility

Table 2. Characteristics of clinical trials incorporating ctDNA-based predictive biomarkers

Clinical trial (Name/ID number)	**Status**	**Design**	**Intervention model**	**Setting**	**Population characteristics**	**Intervention vs Control arm**	**Enrollment**(biomarker analysis)	**Patients (%) with detectable ctDNA**	**Endpoints**	**ctDNA sequencing technique**	**Concordance of tissue and plasma samples**	**Results**
PADA-1/ NCT03079011	Active, not recruiting	Open label, randomized, phase III	Sequential assignment	1st line (metastatic setting)	ER+, HER2-, postmenopausal female, ECOG PS: 0-2	Palbociclib + Aromatase Inhibitors (AI) vs Palbociclib + Fulvestrant	803	17/803 (2.1%)	Safety, Efficacy	Droplet Digital PCR-based assay		76.47% of patients had undetectable ESR1m after 1 month of palbociclib + AI therapy
SOLAR-1/ NCT02437318	Active, not recruiting	Triple blind, randomized (1:1), phase III	Parallel assignment	2nd line (metastatic setting)	PIK3CA-mutant, HR+, HER2-, male or postmenopausal female, 1 prior line of endocrine therapy, ECOG PS:0-1	Alpelisib + Fulvestrant vs Placebo + Fulvestrant	549	186/549 (33.87%)	PFS	Assay developed by Qiagen	94.7%	PFS of 3.7 months for tissue PIK3CAm and of 10.9 months for ctDNA PIK3CAm. Treatment benefit, with the combination of Alpelisib and Fulvestrant, ın

												PFS for patients with ctDNA PIK3CAm, irrespective of prior treatment for advanced breast cancer and/or prior CDK4/6 inhibitors use.
MONALEESA-2/ NCT01958021	Active, not recruiting	Double blind, randomized (1:1), phase III	Parallel assignment	1st line (metastatic setting)	HR+, HER2-, postmenopausal female, ECOG PS:0-1	Ribociclib + Letrozole vs Placebo + Letrozole	494	427/494 (86%)	PFS	Next-generation sequencing		≥1 ctDNA genomic alteration: PIK3CA (33%), TP53 (12%), ZNF703/FGFR1 (5%), ESR1 (4%), and in genes involved in RTK signaling (12%). Treatment benefit, with the combination of ribociclib and letrozole,

												irrespective of ctDNA genetic alterations at baseline.
MONALEESA-3/ NCT02422615	Active, not recruiting	Double blind, randomized (2:1), phase III	Parallel assignment	≤2nd line (metastatic setting)	HR+, HER2-, postmenopausal female, ≤1 prior line of endocrine therapy ECOG PS:0-1	Ribociclib + Fulvestrant vs Placebo + Fulvestrant	600	124/600 (20.66%) for PIK3CAm	PFS	Next-generation sequencing		ctDNA genomic alterations: PIK3CA (35%), ESR1 (14%), TP53 (18%), CDH1 (12%), FGFR1/ZNF703/ WHSC1L1 (11%), cell cycle-related (CCC) genes (16%), genes involved in RTK signaling (20%) and genes involved in the MAPK pathway (10%). Treatment benefit, with the combination of ribociclin and

												fulvestrant, irrespective of ctDNA genetic alterations; shorter PFS was correlated with altered genetic status.
BELLE-2/ NCT01610284	Completed	Double blind, randomized (1:1), phase III	Parallel assignment	2nd line (metastatic setting)	HR+, HER2-, postmenopausal female, AI-refractory disease	Buparlisib + Fulvestrant vs Placebo + Fulvestrant	587	200/587 (34%)	PFS	Sanger sequencing	77%	64 of 307 (21%) patients with PIK3CAwt tumour tissue had PIK3CAm ctDNA, indicating evolution between initial diagnosis and the present time. ctDNA PIK3CAm corresponded to improved median PFS in the buparlisib arm

												(7.0 vs 3.2 months; HR=0.58; 95% CI 0.41-0.82; 1-sided nominal p=0.001).
BELLE-3/ NCT01633060	Terminated	Double blind, randomized (2:1), phase III	Parallel assignment	≥2nd line (metastatic setting)	HR+, HER2-, postmenopausal female, prior treatment with AI, progression to the combination of mTORi and endocrine therapy, ECOG PS:0-2	Buparlisib + Fulvestrant vs Placebo + Fulvestrant	348	135/348 (39%)	PFS	Inostics BEAMing assay.	83%	Treatment benefit, with the combination of buparlisib and fulvestrant, irrespective of ctDNA PIK3CA mutational status (PFS of 4.2 vs 1.6 months; HR=0.46; 95% CI 0.29-0.73; p=0.00031 for PIK3CAm and 3.9 vs 2.7 months; HR=0.73; 95% CI 0.53-1.00; p=0.026 for PIK3CAwt).

PALOMA-3/ NCT01942135	Active, not recruiting	Double blind, randomized (2:1), phase III	Parallel assignment	2nd line (metastatic setting)	HR+, HER2-, female of any menopausal status, progression to prior adjuvant or metastatic endocrine therapy, ECOG PS:0-1	Palbociclib + Fulvestrant vs Placebo + Fulvestrant	455	100/455 (22%) for PIK3CAm and 114/445 (25.6%) for ESR1m	PFS	ddPCR-based assay		Both PIK3CA mutant copies and wild-type allele and ESR1 mutant copies and wild-type allele were significantly lower in the Palbociclib treatment group (Wilcoxon signed-rank test, $p<0.0001$). Early ctDNA PIK3CA dynamics (after 2 weeks of therapy) were predictive on response to palbociclib and fulvestrant,
WJOG6110B/ ELTOP/	Completed	Open label, randomized	Parallel assignment	≥1st line (metastatic setting)	HER2+, female, prior use of taxanes,	Lapatinib + Capecitabine vs	35	8/35 (23%)	PFS	ddPCR-based assay	85%	PIK3CAm in both tissue and plasma samples correlated

UMIN000005219		(1:1), phase II			progression on trastuzumab-containing regimens, ECOG PS:0-2	Trastuzumab + Capecitabine						with shorter PFS, irrespective of the treatment arm. Especially, for ctDNA PIK3CAwt PFS was 8.2 months and 4.9 months for the lapatinib arm and for the trastuzumab arm, respectively (HR=0.38; 95% CI 0.16-0.93; p=0.035), whereas for ctDNA PIK3CAm PFS was 4.1 months and 6.1 months for the lapatinib arm and for the trastuzumab arm, respectively (HR=0.60; 95%

												CI 0.11-3.13; p=0.54).
POSEIDON/ NCT02285179	Recruiting	Double blind, randomized (1:1), phase Ib (3+3 design)	Parallel assignment	≥2nd line (metastatic setting)	HR+, HER2-, female of any menopausal status, prior endocrine therapy, ≤5 chemotherapy lines in the metastatic setting	Taselisib + Tamoxifen vs Placebo + Tamoxifen	22		PFS	dPCR/ tagged amplicon deep-sequencing		ctDNA PIK3CA dynamics were predictive on response to taselisib and tamoxifen, before radiologic treatment response.
SUMMIT/ NCT03433274	Recruiting	Open label, Non-randomized, phase II	Single group assignment	BASKET trial: Colon, lung, breast, bladder cancer, fibromellar carcinoma, Any line of therapy	HER2+ or EGFR+ or HER3+	Neratinib	381		Clinical benefit rate	70-gene digital sequencing assay	93.5%	Early ctDNA HER2 dynamics were predictive on response to neratinib; ctDNA HER2mut frequency decreased in 9 of 11 paired samples, at week 4, followed by an

												increase upon radiographical disease progression at week 8.
BEECH/ NCT01625286	Active, not recruiting	Double blind, randomized (1:1), phase I/II	Parallel assignment	1st line (metastatic setting)	ER+, HER2-, WHO PS:0-1	Capivasertib + Paclitaxel vs Placebo + Paclitaxel	148		Dose-limiting toxicity events, PFS	ddPCR-based assay for ctDNA quantification . Roche cobas PIK3CA assay for PIK3CAmut identification		Early ctDNA dynamics were predictive on PFS irrespective of treatment arm (median PFS was 11.1 months in patients with decreased ctDNA levels at week 4, and 6.4 months in patients with higher ctDNA levels; HR=0.20; 95% CI 0.083-0.50; p<0.0001).
I-SPY 2/ NCT01042379	Recruiting	Open label, randomized , phase II	Parallel assignment	Locally advanced breast	Any tumor ER/PgR/HER2 status, female,	AMG 386 ± Trastuzumab/ AMG 479 +	84		Pathologic complete response	Mutational profiles derived from		Early ctDNA dynamics were predictive on

		(adaptive design)		cancer (Stage II, III), Neoadjuvant setting	no prior cytotoxic regimens, ECOG PS:0-1	Metformin/ MK-2206 ± Trastuzumab/ T-DM1 + Pertuzumab/ Ganetespib/ ABT-888/ Neratinib/ PLX3397/ Pembrolizumab/ Talazoparib + Irinotecan/ Patritumab ± Trastuzumab/ SGN-LIV1A/ Duvalumab + Olaparib/ SD-101 + Pembrolizumab/ Tucatinib vs Standard therapy/ Pertuzumab + Trastuzumab			(pCR) after the use of experimental agents	pretreatment tumor biopsy and germline DNA whole exome sequencing were used to design personalized assays		response to neoadjuvant treatment.

MONARCH 2/ NCT02107703	Recruiting	Double blind, randomized (2:1), phase III	Parallel assignment	2nd line (metastatic setting)	HR+, HER2-, postmenopausal female, ECOG PS:0-1	Abemaciclib + Fulvestrant vs Placebo + Fulvestrant	334	96/238 (40.3%) for PIK3CAm and 190/295 (64.4%) for ESR1m	PFS	ddPCR-based assay	62.8% for PIK3CAm and 37.1% for ESR1m	ctDNA mutational status associates with improved PFS and response to abemaciclib and fulvestrant arm. For ctDNA PIK3CAm PFS was 15 months and 5.7 months for the abemaciclib arm and for the control arm, respectively (HR=0.46; 95% CI 0.27-0.78), whereas for ctDNA ESR1m PFS was 21.9 months and 10.3 months for the abemaciclib arm and for the control arm, respectively

												(HR=0.49; 95% CI 0.33-0.73).
LOTUS/ NCT02162719	Active, not recruiting	Double blind, randomized (1:1), phase II	Parallel assignment	1st line (metastatic setting)	Triple negative, female of any menopausal status, ECOG PS:0-1	Ipatasertib + Paclitaxel vs Placebo + Paclitaxel	88		PFS	FoundationACT assay (plasma samples) and FoundationOne genomic profiling (tumor tissue samples)		ctDNA dynamics were predictive on PFS and objective response irrespective of treatment arm.
NCT02379247	Active, not recruiting	Open label, non-randomized, phase I/II	Single group assignment	≥2nd line (metastatic setting)	HER2-, female, prior chemotherapy for metastatic disease, ECOG PS≥2	Alpelisib + Nab-paclitaxel	42	17/42 (40%)	Recommended phase II dose, objective response rate, PFS	Next-generation sequencing	70%	PFS of 13 months for ctDNA PIK3CAm and 7 months for ctDNA PIK3CAwt (HR=0.39; p=0.03).

INSPIRE/ NCT02644369	Active, not recruiting	Open label, non-randomized, phase II	Single group assignment	BASKET trial: Squamous cell Ca of the head and neck, TNBC, high-grade serous ovarian cancer, Melanoma, mixed advanced solid tumors, Any line of therapy	Triple negative, male or female, ECOG PS:0-1	Pembrolizumab	10 (mTNBC)		Changes in genomic and immune biomarkers that will be measured in blood and tumor pre-treatment, on-treatment and at progression	Single cell suspensions were pooled for exome/RNA sequencing, flow cytometry for immunophenotyping.		Early ctDNA dynamics were predictive on PFS, OS and overall clinical RR
BOLERO-2/ NCT00863655	Completed	Double blind, randomized (2:1), phase III	Parallel assignment	2nd line (metastatic setting)	ER+, postmenopausal female, disease refractory to NSAI, recurrence or	Everolimus + Exemestane vs Placebo + Exemestane	550	238/550 (43.3%)	PFS	ddPCR-based assay	70.4%	Treatment benefit, with the combination of everolimus and exemestane, irrespective of

					progression on or after the last systemic therapy							ctDNA PIK3CA status (HR=0.43 for PIK3CAwt tumors and 0.37 for PIK3CAm tumors).
BLTN-Ic/ NCT02361112	Completed	Open label, non-randomized, phase I	Single group assignment	2nd line (metastatic setting)	HER2+, male or female of any menopausal status, no previous treatment of capecitabine during the past 1 year, ECOG PS:0-1	Pyrotinib + Capecitabine	28		MTD			Median PFS of 15.8 months for ≥2 ctDNA genetic alterations of HER2, PI3K/AKT/mTOR pathway and TP53 and of 26.2 months for ≤1 ctDNA genetic alteration (p=0.006).
Neo ALLTO/ NCT00553358	Active, not recruiting	Open label, randomized (2:2:2), phase III	Parallel assignment	Primary invasive breast cancer,	HER2+, female, invasive breast cancer >2cm	Lapatinib + Paclitaxel + Trastuzumab vs	455		Number of participants with pCR at	Next-generation sequencing		ctDNA PIK3CAm and/or TP53m detection at baseline and at

				Neoadjuvant setting	diameter, ECOG PS:0-1	Paclitaxel + Trastuzumab			the time of surgery			serial plasma samples was predictive of low rates of pathological response
MONALEESA-7/ NCT02278120	Active, not recruiting	Double blind, randomized (1:1), phase III	Parallel assignment	1st line (metastatic setting)	ER+ and/or PR+, HER2-, premenopausal or perimenopausal female, ECOG PS: ≤1,	Ribociclib + Tamoxifen/ Letrozole/ Anastrazole + Goserelin vs Placebo + Tamoxifen/ Letrozole/ Anastrazole + Goserelin	489		PFS	Next-generation sequencing		Treatment benefit, with the combination of ribociclib and NSAI or tamoxifen and goserelin, irrespective of ctDNA mutational status at baseline.

Table 3. NGS-based versus PCR-based methods comparison

NGS-based methods	**PCR-based methods**
Comprehensive detection of both known and unknown mutations	Detection of a limited number of known mutations
	High specificity
	High sensitivity
Expensive	Cost-effective
Longer time to process and analyse results	Rapid genotyping
Ratios of mutant to wild type quantities	Absolute quantification of mutant and wild type copies
	Difficulty in identifying gene fusions and copy variations
Bioinformatics skills needed	No bioinformatics expertise required