A review of the use of Next Generation Sequencing methodologies to identify biomarkers of resistance to CDK4/6 inhibitors in ER+/HER2- breast cancer

Alberto Servetto (Conceptualization) (Investigation) (Resources) (Writing - original draft), Fabiana Napolitano (Conceptualization) (Investigation) (Resources) (Writing - original draft), Carmine De Angelis (ce:contributor-role=Writing – review and editing), Pietro De Placido (Investigation) (Resources), Mario Giuliani (Methodology) (Supervision), Grazia Arpino (Methodology) (Supervision), Sabino De Placido (Methodology) (Supervision), Roberto Bianco (Methodology) (Supervision) (Funding acquisition), Luigi Formisano (Conceptualization) (Supervision) (ce:contributor-role=Writing – review and editing) (Funding acquisition)

PII: S1040-8428(20)30327-9
DOI: https://doi.org/10.1016/j.critrevonc.2020.103191
Reference: ONCH 103191
To appear in: Critical Reviews in Oncology / Hematology

Received Date: 11 October 2020
Revised Date: 30 November 2020
Accepted Date: 1 December 2020

A review of the use of Next Generation Sequencing methodologies to identify biomarkers of resistance to CDK4/6 inhibitors in ER+/HER2- breast cancer

Alberto Servetto1,2*, Fabiana Napolitano1*, Carmine De Angelis1,3, Pietro De Placido1, Mario Giuliano1, Grazia Arpino1, Sabino De Placido1, Roberto Bianco15, Luigi Formisano18

1 Department of Clinical Medicine and Surgery, University of Naples Federico II, Naples, Italy
2 Simmons Comprehensive Cancer Center, The University of Texas Southwestern Medical Center, Dallas TX
3 Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, Texas

* The authors contributed equally to the manuscript

*Corresponding authors: Luigi Formisano luigi.formisano1@unina.it, Roberto Bianco robianco@unina.it

Highlights

- CDK4/6 inhibitors revolutionized the treatment of ER+ breast cancer
- NGS methodologies identified several mechanisms of resistance to CDK4/6 inhibitors
- Major limitation of DNA-NGS preclude its routine clinical application
- Genomic alterations are not the only mechanisms of resistance to CDK4/6 inhibitors
- Gene expression analysis will further elucidate this composite scenario
Abstract

The development of cyclin-dependent kinases (CDK) 4 and 6 inhibitors represented a substantial breakthrough in the treatment of estrogen receptor positive (ER+), human epidermal growth factor receptor 2 (HER2) negative metastatic breast cancer. These drugs showed a significant clinical benefit in pivotal clinical trials. However, resistance eventually occurs, leading to disease progression. Next Generation Sequencing methodologies have been employed to investigate predictive biomarkers of response or resistance to CDK4/6 inhibitors. Whole exome and targeted sequencing of solid and liquid biopsies have revealed several possible genomic alterations associated with resistance. Notably, genomic alterations identified by DNA-sequencing did not fully recapitulate the entire landscape of resistance to CDK4/6 inhibitors. Gene expression analysis, such as RNA-Seq methodologies, have provided insights into transcriptional profiles and may need further application. Herein, we report the main findings derived from the use of NGS analysis in the context of resistance to CDK4/6 inhibitors in ER+ breast cancer.

Keywords: CDK4/6 inhibitors, Next Generation Sequencing, breast cancer, precision medicine
1. Introduction

Cyclin-dependent kinases (CDK) 4 and 6 inhibitors, in association with endocrine therapy, represent the gold standard for the treatment of estrogen receptor positive (ER+) human epidermal growth factor receptor 2 negative (HER2-) metastatic breast cancer (BC). Abemaciclib, palbociclib and ribociclib have been tested, in combination with hormone therapy, in phase III randomized clinical trials, demonstrating a clinically significant benefit [1-12] and improved survival compared to endocrine therapy alone [13] (Table 1). Despite undoubted clinical advantages, the debate regarding detection and use of biomarkers predictive of sensitivity or resistance to CDK4/6 inhibitors is still open [14]. Next generation sequencing (NGS) methodologies have been employed for solid and liquid biopsies analysis, to investigate genomic alterations responsible of drug resistance. Furthermore, gene expression analyses have been used in the context of pre- and clinical setting to investigate transcriptional profiles and gene signatures associated with treatment response. However, current NGS methods still present limitations, compromising their routine application in clinical practice. In this review, we report the current knowledge about the mechanisms of resistance to CDK4/6 inhibitors in ER+/HER2- breast cancer, dissecting the different methodologies applied in pre-/clinical studies to investigate the genomic abnormalities and transcriptional reprogramming involved in resistance to these drugs, examining their strengths and weaknesses. Finally, we discuss the possible future directions for both preclinical and clinical research fields.

2. Targeting the cell cycle in ER+ breast cancer

The Retinoblastoma tumor suppressor (Rb), encoded by the RB1 gene, regulates G0/G1-S transition in the cell cycle [15]. During the G0 and early G1 phases, hypophosphorylated Rb binds
E2F transcription factors, inhibiting their activity [16, 17]. Mitogenic signals induce expression of D type cyclins which compete with proteins of the CDKN2 family (CDK inhibitor family 2; also known as INK4 proteins) to bind and consequently activate CDK4 and 6 [18]. Cyclin D-CDK4/6 complex induces phosphorylation of Rb on several serine and threonine residues, promoting its conformational changes leading to E2F release which, in turn, induces the gene expression profile driving S phase entry and cell cycle progression [19-21]. The E-cyclin protein family members Cyclin E1 and Cyclin E2, whose expression is induced by E2F transcription factors, activate CDK1-3 to promote cell cycle progression [18]. Cyclin D-CDK4/6 complex also activates the forkhead box protein M1 (FOXM1) transcription factor, promoting G2/M cell cycle progression [22]. ER+ breast cancer has been shown to be highly dependent on Cyclin D-CDK4/6-Rb axis. First, chromatin immunoprecipitation assays have revealed that estrogen receptor alpha (ERα) binds CCND1 gene enhancer regions [23]. Furthermore, in about 20% of ER+ breast tumors, CCND1 gene locus (chr11q13) is amplified [24, 25]. Comprehensive analysis of cancer cell dependencies identified CDK4 among the top dependent genes in luminal subtype breast cancer cells [26]. Also, RB1 mutations or homozygous deletion of the 13q14.2 cytogenetic band (harboring RB1 gene sequence) are rare and found in only 2-4% of primary and metastatic ER+ BC samples [24, 25, 27]. Thus, the intact and active Cyclin D-CDK4/6/Rb axis provides a valid biological rationale for the use of CDK4/6 inhibitors in ER+ BC.

3. Mechanisms of resistance to CDK4/6 inhibitors

The only established predictive biomarker of response to CDK/6 inhibitors is the expression of estrogen receptor [28]. Recently, pre- and clinical studies identified several mechanisms of resistance to CDK4/6 inhibitors, generally divided in two main groups: alterations of factors
controlling cell-cycle progression and aberrations of tyrosine kinase receptors (RTKs) and PI3K/mTOR signaling pathways (Figure 1).

*RB1* loss-of-function alterations have been characterized by several reports. Condorelli *et al* firstly described the emergence of *RB1* acquired mutations in 3 patients treated with endocrine therapy plus CDK4/6 inhibitors [29]. Next, analysis of paired pre- and post-treatment ctDNA samples collected from 195 patients enrolled in the PALOMA-3 trial, revealed that *RB1* mutations were selectively acquired in the experimental arm (fulvestrant plus palbociclib). All the detected variants were either a gain of a stop codon or frameshift mutations, likely resulting in abolished Rb function [30]. Furthermore, analysis of baseline ctDNA samples from 1534 patients enrolled in the MONALEESA 2, 3 and 7 trials revealed 26 (1.7%) samples harboring *RB1* mutations. In these patients, ribociclib did not improve progression free survival (PFS) [27]. Similarly, Li *et al* demonstrated that *RB1* loss in ER+ BC samples (n=9) was associated with intrinsic resistance to CDK4/6 inhibitors [31]. Finally, Wander *et al* found *RB1* mutations associated with intrinsic or acquired resistant to CDK4/6 inhibitors [32].

Beyond *RB1* alterations, other abnormalities in cell-cycle related genes have been reported. *CDK6* amplification and overexpression have been shown to confer resistance to CDK4/6 inhibitors and to downregulate ER and progesterone receptor (PR), implying an indirect role for CDK6 in inducing resistance to endocrine therapy [33]. Further, overexpression of CDK6 may be consequence of *FAT1* loss-of-functions alterations, through activation of the Hippo pathway [31]. However, *FAT1* alterations are rare. In a cohort of 1,501 ER+ breast cancers, *FAT1* mutations were found in ~2% of primary and about ~6% of metastatic samples and only one third of these alterations were likely associated with abrogated FAT1 function [34]. In the same cohort, rare
Genomic alterations in other members of Hippo Pathway were found, such as \textit{LATS1} truncating mutation (0.3% of cases), \textit{LATS2} homozygous deletion (0.3%) and \textit{YAP1} amplification (0.6%) [31]. However, to date, there is no evidence of acquired alterations of the Hippo pathways members in patients treated with CDK4/6 inhibitors.

Preclinical studies reported that ER+ BC cells early adapt to CDK4/6 inhibition through upregulation of cyclin E2 (CCNE2) and cyclin-dependent kinase 2 (CDK2), promoting cell cycle progression despite the cyclin D-CDK4/6 axis blockade [35]. \textit{CCNE2} gene amplification has been associated with intrinsic or acquired resistance to CDK4/6 inhibitors [32]. Next, gene expression analysis of 302 ER+ BC samples from PALOMA-3 trial revealed that lower Cyclin E1 (\textit{CCNE1}) mRNA levels were associated with better response to palbociclib [36]. This association was confirmed in a preoperative setting, in the cohort of POP (PreOperative Palbociclib) trial [37]. Finally, \textit{AURKA} (Aurora kinase A) gene amplification, mediating cell-cycle progression, has been also correlated with resistance to CDK4/6 inhibitors [32].

Several evidences highlighted the role of RTKs and PI3K/mTOR signaling in resistance to CDK4/6 inhibitors. \textit{FGFR1} amplification has been associated with reduced sensitivity to CDK4/6 inhibitors [38], as well as \textit{FGFR2} amplification and mutations [30, 32, 38, 39]. Hyperactivation of EGFR and ERBB2 have been described in preclinical models of resistance to palbociclib [40]. \textit{KRAS} G12D, \textit{KRAS} Q61L, \textit{HRAS} K117N, \textit{AKT1} mutations and high focal amplification of \textit{NRAS} gene locus were detected in tissue biopsies from patients with intrinsic or acquired resistance to CDK4/6 inhibitors [32]. Notably, 51% of the samples analyzed by Wander et al [32], were collected from patients previously treated with endocrine therapy in the metastatic setting. Since some of the identified alterations have been previously associated with resistance to endocrine
therapy, it is challenging to understand to what extent these genomic abnormalities are involved in resistance to hormone-therapy or, instead, to CDK4/6 inhibitors.

Herrera-Abreu et al reported that PI3K inhibitors have a synergistic antitumor effect with CDK4/6 inhibitors in ER+ BC models carrying activating mutations in the PI3K pathway, inducing cell-cycle arrest in G1 phase and apoptosis [35]. Further, Jansen et al found that 3-phosphoinositide-dependent protein kinase 1 (PDK1) confers acquired resistance to CDK4/6 inhibitors and demonstrated that concomitant blockade of CDK4/6 and PDK1 exerts a synergistic anti-proliferative effect in ER+ BC cells [41]. PDK1, downstream of PI3K, activates AKT phosphorylating its threonine residue 308 [42]. Interestingly, loss of PTEN expression may be acquired after treatment with CDK4/6 inhibitors [43]. Indeed, Costa et al demonstrated that the increased AKT activation due to PTEN loss induces phosphorylation of the threonine 157 of p27, preventing its nuclear accumulation. Reduced p27 nuclear levels cause hyperactivation of CDK2 and promote cyclin D1-CDK4 assembly, thereby enhancing cell-cycle progression.

4. Liquid and tissue biopsies: genotyping tumor DNA

Although solid tumor biopsies are strongly recommended for molecular characterization, they require invasive procedures and may not be readily accessible. Moreover, tissue biopsies may fail in assessing the full mutational landscape of the cancer, due to intra- and inter-lesions heterogeneity [44, 45].

The detection of tumor-derived cell-free DNA (cfDNA) in body fluids, defined circulating tumor DNA (ctDNA), may overcome these limitations [46, 47]. However, liquid biopsy also has major limitations since levels of ctDNA may be extremely low, needing highly sensitive detecting methods. In addition, DNA shedding may vary among tumor subclones and the detection of subclonal alterations may suggest the use of clinically non-meaningful targeted therapies [48].
Emerging sequencing and computational methodologies can outline the clonal evolution and genomic plasticity of tumors. However, these analyses need repeated biopsies from multiple lesions at diagnosis and through time, implying cost-benefit tradeoff and patient’s compliance [49, 50]. Conversely, collection of liquid biopsies over time is a simple, cost-effective and patients’ approved method [30].

As point mutations are easily spotted, detection of copy number alterations (CNA) and fusions need more sophisticated methodologies and very good quality samples, particularly when analyzing liquid biopsies [30]. To date, Whole Exome Sequencing (WES) and targeted sequencing panels are the most used DNA sequencing methodologies to investigate biomarkers predictive of sensitivity or resistance to CDK4/6 inhibitors, in both tissue and liquid biopsies (main findings summarized in Table 2). However, the results reported in literature until now do not fully recapitulate the complex biology of resistance to CDK4/6 inhibitors. Firstly, because of the technical limitations of DNA sequencing methodologies. Further, we believe that new studies comprehensively investigating epigenetic reprogramming, gene expression and proteomics profiles may provide new insights into the composite scenario of the resistance to CDK4/6 inhibitors.

4.1 Whole Exome Sequencing

Murtaza et al first reported the evaluation of WES applied to ctDNA, suggesting its feasibility in cancer research [47]. Later, two main studies have tested the role of WES in the context of CDK4/6 inhibitor resistance. O’Leary et al performed DNA-sequencing of blood samples collected from patients enrolled in the PALOMA-3 trial [30]. They used a novel copy-number and purity targeted sequencing methodology that employs a targeted amplicon panel including about 1000 single-
nucleotide polymorphisms (SNPs) in regions commonly altered in BC. This step allowed to select plasma samples with sufficient tumor purity for exome sequencing. The authors selected 14 patients, enrolled in the fulvestrant plus palbociclib arm, with available paired Day 1 (D1) and End of Treatment (EOT) plasma samples with tumor DNA purity >10% for exome library preparation. Sequencing of pre- and post-treatment samples demonstrated a clear clonal evolution and selection on fulvestrant plus palbociclib in 12/14 patients. APOBEC and mismatch repair signatures were identified as the mutational processes driving the genomic plasticity of the emergent subclones resistant to treatment.

Wander et al performed WES of 59 ER+ BC biopsies from 58 patients receiving CDK4/6 inhibitors [32]. The biopsies were defined “sensitive”, “intrinsic resistant” or “acquired resistant” based on the duration of clinical benefit upon CDK4/6 inhibitors exposure. Genomic alterations of RB1, FGFR2, ERRB2, AKT1, AURKA, KRAS/HRAS/NRAS and CCNE2 genes were identified. Furthermore, exome sequencing of matched pre- and post-treatment samples from 7 patients who acquired resistance to CDK4/6 inhibitors depicted clonal structure and dynamics.

Despite the low number of available pre- and post-treatment liquid/tissue biopsies, these two articles represent the only published reports regarding the use of WES in ER+ breast cancer patients treated with CDK4/6 inhibitors, providing intriguing results with possible implications in designing new clinical trials.

WES methodologies still present some weaknesses which prevent their routine application. First, the detection of genomic alterations, particularly CNAs, is informative when the purity, referring to tumor or ctDNA content, is above 10% [30, 47, 51]. Thus, low tumor burden may challenge the application of WES. A recent systematic review of the use of WES for ctDNA revealed a 50% sensitivity, when using tumor tissue as reference with 31% of overall agreement between cfDNA
and matched tumor tissue [52]. Sensitivity and agreement values resulted increased to 69% and 46%, respectively, when ctDNA content was ≥ 25% [52]. These are important limitations in the context of resistance to CDK4/6 inhibitors, since CNAs, such as loss of RB1, FAT1, and PTEN, amplification of FGFR1 and AURKA, have been clearly associated with resistance to these drugs. Although WES enables detection of alterations across broad genomic areas, overcoming the limitations of targeted sequencing panels, this methodology is costly and only limited depth of sequencing is possible, generally □100x [30, 53-55]. These restrictions may generate significant biases, particularly in the evaluation of presumptive “acquired” alterations detected in post-progression samples. In fact, it may be challenging to determine whether they were already present in the tumor at baseline in a minor undetectable subclone and with too low allele frequency.

4.2 Targeted sequencing

Targeted next generation sequencing allows simultaneous multiple genes analysis, overcoming the defects of the traditional Sanger method, being highly accurate and cost-effective [56].

Targeted sequencing may inquire both tissue and liquid biopsies. Although only a selected number of genes may be interrogated, the analysis is performed with great depth, increasing the ability to detect genomic alterations even with low allele frequency. The Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) assay, a multigene cancer-related panel, now including 468 genes, has a detection limit for low-frequency variants of approximately 2% for hotspot mutations and 5% for non-hotspot mutations, with a coverage depth range of 500x-1000x [57]. Cancer-related panels exist also as commercial tests (Caris Molecular Intelligence, by Caris Life Sciences, 592 genes; FoundationOne CDx, by Foundation Medicine, 324 genes and 2 genetic signatures), with different coverage depths (750x and 500x, respectively). The Guardant360 assay (Guardant Health) is able to detect somatic mutations in 74 genes, CNAs
in 18 genes and fusions in 6 genes, specifically designed for ctDNA analysis [58]. More recently, the platform PredicinePLUS™, using a 180-gene panel based on both ctDNA and ctRNA, revealed high efficiency in detecting genomic alterations, and relative allele frequency, in ctDNA samples from women with metastatic breast cancer [59].

The MSK-IMPACT panel has been employed to investigate CDK4/6 inhibitors resistance in ER+ BC. Firstly, Yang and colleagues investigated potential genomic alterations in 450 genes on MCF-7 cells, made resistant to abemaciclib upon long drug exposure [33]. The panel was unable to detect acquired mutations responsible of drug resistance. However, investigating the expression of the components of the Cyclin D-CDK4/6 axis, the authors found increased CDK6 mRNA levels, with CDK6 amplification confirmed by FISH. This is a clear example of a possible limitation of targeted sequencing panels, since CNAs in genomic loci not included a priori are not detected [60].

To uncover intrinsic genetic alterations associated with resistance to CDK4/6 inhibitors, MSK-IMPACT has been used on 348 ER+/HER2- BC biopsies collected prior to drug exposure [31]. The somatic alterations considered biologically and therapeutically relevant in ER+ BC, as CCND1 amplification, PIK3CA mutations, and ESR1 mutations, had no impact on drug efficacy.

In addition to expected RB1 genetic alterations, FAT1 loss-of-function alterations correlated with shorter PFS (median 2.4 vs 10.2 months in FAT1 altered and wild type tumors, respectively). Other genes included in the Hippo pathway were found altered, including LATS1 and LATS2 mutations, and YAP1 amplification. This study highlighted some important issues when investigating large gene sets. Firstly, the difficulty to assess the role of genetic alterations of tumor suppressor genes whose function and domain have not fully been characterized. Tumor suppressors genes often lack hotspot mutations and more typically present truncating, frameshift and splice site mutations or homozygous deletions. To avoid variants of uncertain significance, the authors applied stringent
criteria in which only likely pathogenic mutations of tumor suppressor genes were included. Consequently, these criteria may exclude other mutations (i.e. missense variants) with a potential biological impact, and/or with a lower recurrence frequency, falling into the “background noise”.

Targeted sequencing has been also applied to liquid biopsies to investigate the resistance to CDK4/6 inhibitors. Formisano et al [38] analyzed ctDNA from 34 ER+/HER2- BC patients progressing on CDK4/6 inhibitors, through the Guardant360 assay. In addition to PIK3CA (15/34; 44%) and ESR1 (11/34; 32%) mutations, the authors found FGFR genomic alterations occurring in 14/34 (41%) post-progression samples, consisting in FGFR1 (n = 9) and FGFR2 (n = 2) amplification, FGFR1 N546K (n = 1), FGFR2 N549K (n = 1), and FGFR2 V395D (n = 1) mutations. However, the lack of pre-treatment samples prevented from understanding whether these alterations had been acquired during the drug exposure or were already present at baseline.

The authors also analyzed 427 ctDNA pre-treatment samples from patients enrolled in the MONALEESA-2 trial [5], to investigate CNAs of the 8p11.23 genomic locus. This genomic locus, harboring FGFR1 and ZNF703 genes, resulted altered in 5% of cases (20/427 patients). A subgroup analysis showed a reduced clinical benefit for ribociclib in patients with FGFR1/ZNF703 amplification, although non-statistically significant due to small sample size (median PFS 10.61 vs 24.84 months, p=0.075).

Targeted approach has been used by O’Leary et al [30] to detect, on paired pre- and post-treatment ctDNA samples from patients enrolled in the PALOMA-3 trial [4], the landscape of the genomic alterations associated with resistance to fulvestrant plus palbociclib. The panel included all the coding exons of RB1, CDK4, CDK6, CDKN1A, CDKN2B, NF1, exons 5–8 of TP53, and known hotspot mutations in PIK3CA, ESR1, ERBB2, FGFR1/2/3, AKT1, KRAS, NRAS, and HRAS. Two separate libraries were prepared for each sample: Ion Proton led to a median coverage of 2187×
and 3251× for D1 and EOT samples, respectively; Illumina HiSeq to median coverage of 10637× and 8947× for D1 and EOT samples. Totally, paired samples from 184 patients, analyzed by both platforms, met quality requirements. Combining targeted and exome sequencing approaches, the authors identified RB1 mutations as the only alterations selectively acquired in palbociclib plus fulvestrant arm (4.7%). The targeted sequencing panel also revealed enrichment of PIK3CA mutations in the EOT samples, in both arms. However, inquiring D1 samples, digital PCR identified the presence of those “acquired mutations” in 6/18 (33%). These mutations were not detected due to a very low allele frequency, below the limit of detection of the ctDNA sequencing. Similarly, the targeted panel identified positive selection of ESRI Y537S mutations through the treatment, in both arms. However, also in this case, digital PCR detected the “acquired mutation” in 3/17 (17%) D1 samples, with very low allele frequency.

The authors further evaluated CNAs in liquid biopsies. A total of 324 samples, including 163 D1 and 154 paired EOT samples, were sequenced with 1329x coverage, to assess loss of RB1, PTEN, CDKN2A and CNAs in 12 genomic loci commonly gained in breast cancer. Since detection of genomic deletions is particularly challenging in ctDNA sequencing, the authors set a threshold of tumor purity ≥ 20% to capture them. In the 17 paired D1 and EOT samples, no evidence for selection of RB1 loss on treatment was found. Copy numbers of PTEN and CDKN2A were consistent through treatment. Conversely, copy-number gain was assessed in samples with a tumor purity of ≥10%, with no evidence for selection of CNAs at EOT in the 43 paired pre- and post-treatment samples. Interestingly, analyzing the paired samples with tumor content ≥10%, loss of RB1 was identified in 6/37 patients (16.2%) in D1 samples and 14/51 patients (27.4%) of EOT samples. In addition to the non-selection of RB1 loss at EOT (p = 0.30, Fisher’s exact test), it has to be noted that the rate of RB1 genomic loss at baseline is not negligible (16.2%), raising
hypothesis regarding its role in the context of resistance to endocrine therapy and as biomarker of intrinsic resistance to CDK4/6 inhibitors.

Finally, different studies employed different normalization protocols to detect CNAs in ctDNA, which may be influenced by tumor DNA content and the amount of DNA shedding into circulation. To overcome this issue, Formisano et al normalized their results for aneuploidy within each patient sample [38]. Conversely, O’Leary et al employed the OncoCNV package [61], determining specific thresholds for gain and loss and, further, copy number estimates were adjusted for purity within the samples [30].

4.3 Timing of ctDNA analysis

Longitudinal analysis of ctDNA from patients treated with CDK4/6 inhibitors has been employed to investigate predictive biomarkers of sensitivity to treatment. Although the correct timing remains unclear, an early ctDNA analysis checkpoint may uncover the patients who will not benefit from the treatment. O’Leary et al demonstrated how the relative change of PIK3CA mutations levels in ctDNA 15 days after the start of treatment predicts the PFS of patients enrolled in the PALOMA-3 trial [62]. Using a multiplex digital PCR on 73 matched samples, the authors evaluated the ratio of mutation abundance (mutant copies/ml) of cycle 1 day 15 and baseline (circulating DNA ratio, CDR15). The drop in PIK3CA allele frequency at day 15, for both wild-type and mutant PIK3CA alleles, predicted better PFS on palbociclib plus fulvestrant treatment, likely reflecting the fall in ctDNA levels upon drug exposure. Interestingly, baseline PIK3CA ctDNA levels did not predict PFS in the palbociclib plus fulvestrant group. In addition, although ESRI mutant copies were suppressed at day 15, the CDR15 value did not predict PFS. These results highlighted how longitudinal evaluation of truncal PIK3CA mutations, but not subelonal mutations, as ESRI, may predict early response to treatment and long-term outcomes.
The PADA-1 trial inquires the predictive role of the monitoring of *ESR1* mutations in patients treated with CDK4/6 inhibitors [NCT03079011]. Patients with ER+ HER2- metastatic BC were enrolled to receive first line treatment with aromatase inhibitors (AI) plus palbociclib [63]. The authors used a droplet digital PCR (ddPCR) to monitor *ESR1* mutations in plasma. If a rising *ESR1* mutation was detected, but without a concomitant RECIST (Response Evaluation Criteria in Solid Tumors) disease progression, patients were randomized to continue the standard treatment (AI + palbociclib) or to early switch to fulvestrant plus palbociclib. Preliminary results showed that *ESR1* mutation correlated with worse outcome (median PFS 11.0 versus 26.7 months in the *ESR1* mutated versus wild-type group, *p* < 0.001, HR 2.3), but *ESR1* mutations clearance at 4 weeks correlated with better PFS [64]. Final results from the trial will further elucidate the role of longitudinal evaluation of *ESR1* mutations in patients receiving AI + palbociclib.

5. Gene expression profiling

Despite the constant advances, there is a large fraction of unknown mechanisms of resistance to CDK4/6 inhibitors. The limited sequencing depth of WES and the use of “restricted” targeted panels may represent possible reasons of low detection of genomic alterations. Moreover, the involvement of epigenetic and transcription profiles alterations in the context of resistance to CDK4/6 inhibitors may be underestimated.

RNA-Seq methodologies may provide further information regarding gene expression profiles associated with resistance to CDK4/6 inhibitors. Turner *et al* performed mRNA profiling on formalin-fixed paraffin-embedded (FFPE) tumor samples from 302 patients enrolled in the PALOMA-3 trial [65], employing a “targeted” RNA-Seq panel (EdgeSeq Oncology BM Panel - HTG Molecular Diagnostics, Tucson, AZ) assessing 2,534 cancer-related genes [36]. The analysis revealed that palbociclib was less effective in patients with high Cyclin E1 mRNA levels (median
PFS: palbociclib arm, 7.6 vs 14.1 months; placebo arm, 4.0 vs 4.8 months, respectively; interaction \( p=0.00238 \). Cyclin E1 mRNA levels were even more predictive in metastatic biopsies, compared to primary tumor samples. Interestingly, CDK4, CDK6, Cyclin D1 and Rb expression levels did not affect palbociclib efficacy, suggesting that they are not valid predictive biomarkers of response to CDK4/6 inhibitors. A pan-cancer cell lines analysis revealed that genomic alterations inducing activation of D-type cyclins were associated with higher sensitivity to abemaciclib [66]. However, Cyclin D1 mRNA and protein levels have not been correlated with sensitivity to CDK4/6 inhibitors in ER+ breast cancer patients, suggesting that Cyclin D1 levels are not representative of active Cyclin D-CDK4/6-RB1 axis [36, 67].

Similarly, gene expression analysis in the NeoPalAna trial revealed significant upregulation of Cyclin E1, Cyclin D3 and CDKN2B mRNA expression levels in primary stage II-III ER+ breast cancers samples resistant to preoperative treatment with palbociclib and anastrozole [68]. Since these gene are transcriptionally regulated by E2F1, these results suggest the role of hyperactivation of signalling pathways promoting E2F transcriptional activity in tumors resistant to CDK4/6 blockade [69-71]. Furthermore, gene expression profile of tumors resistant to preoperative treatment with anastrozole and abemaciclib, from patients enrolled in the NeoMonarch trial, revealed lower Rb and higher Cyclin E1 mRNA expression levels. Also, inflammatory response and interferon gamma gene signatures (including PDL1 pathway) were enriched in resistant tumors [72].

The NanoString 230-gene nCounter® GX Human Cancer Reference panel was employed to perform a biomarker analysis of 391 tumor samples from patients enrolled in the MONALEESA-2 trial [73]. Ribociclib efficacy resulted consistent across all subgroups, even greater in the subgroup with higher Estrogen Receptor and lower RTKs expression levels [73]. Furthermore,
Formisano et al revealed that high FGFR1 mRNA expression in tumor samples from patients treated with ribociclib plus letrozole in the MONALEESA-2 clinical trial was associated with worse PFS (high vs low FGFR1 mRNA, 22.2 months vs not reached, $p=0.01$), further suggesting that FGFR1 overexpression is associated with early progression after treatment with CDK4/6 inhibitors [38].

One caveat of analyses evaluating prognostic/predictive role of biomarkers is the selection of the exact cut-off to define the level of mRNA or protein expression [74]. Generally, patients are dichotomized in “low” vs “high expression” based on 50% cut-off, or “presence” versus “absence” criteria. However, specific cut-offs should be determined for each biomarker, employing accurate statistical methods to optimize the cut-off values for predictive analysis [75-78].

The detection of gene expression profiles predicting sensitivity to CDK4/6 inhibitors might have future research and clinical application. Guerrero-Zotano et al found that E2F4 is a significant transcription factor involved in resistance to neoadjuvant endocrine therapy in ER+ breast cancer patients. Further, they identified a 24-gene E2F4 target gene signature associated with resistance to neoadjuvant endocrine therapy and, concurrently, predicting sensitivity to palbociclib in vitro [79]. Also, high E2F4 signature activity correlated with response to palbociclib in a cohort of patients enrolled in the POP trial [37]. Further prospective validation of this signature is needed, but it may be useful to identify patients to be treated with CDK4/6 inhibitors in neo/adjuvant setting.

However, bulk RNA-Seq methods are inadequate to dissect the features of tumor and non-tumor compartments, responsible for intra-tumor heterogeneity, and detect the subclones responsible for adaption to drug exposure and following disease progression [80, 81]. Single cell RNA-
Sequencing methodologies may overcome this weakness, but they still present technical and economic limits which prevent their wide application in preclinical research [82].

Routinely performing RNA-Seq on tumor biopsies in clinical practice may be unattainable. Furthermore, the use of gene expression assays, both in clinical practice and translational research, is generally limited to fresh-frozen (FF) bio-specimens, due to the chemical artifacts induced by FFPE samples that may affect the quality of the RNA and degrade it over time [83-85]. However, the definition of specific gene signatures predicting sensitivity or resistance to CDK4/6 inhibitors may push towards the development of targeted gene expression assays which can be helpful to select the patients to be treated, or not, with these drugs. After all, the experiences of multi-gene signatures tests, such as Oncotype Dx, MammaPrint and Prosigna, demonstrated that these strategies can become common practice in the management of ER+ BC patients [86-89].

6. Challenges and future directions

Despite our increasing knowledge regarding the clinical efficacy of CDK4/6 inhibitors, it is still unclear how the current biological awareness of mechanisms of resistance should be applied in regular clinical practice, to impact the clinician’s choices. The use of DNA-NGS methodologies provide indispensable information for the best management of ER+ BC patients, both at baseline or after progression on first line treatment with endocrine therapy plus CDK4/6 inhibitors. Based on the current awareness of the genomic alterations associated with resistance to CDK4/6 inhibitors, the use of DNA-NGS methodologies may allow the identification of up to ~65% of potentially resistant tumors [32]. Nevertheless, other than \textit{RB1} and \textit{FAT1} loss-of-function alterations, occurring in less than 5% of patients with treatment naïve metastatic ER+/HER2-breast cancer, the current evidences do not support the notion that the other genomic abnormalities associated with resistance to CDK4/6 inhibitors represent an absolute contraindication to start
treatment with these drugs. However, identification of specific genomic abnormalities, at diagnosis or progression after treatment, would be helpful to suggest enrollment in clinical trials.

*RB1* loss-of-function alterations, in addition to *FAT1* truncating mutations or deep deletion, represent the only biomarker commonly accepted to contraindicate treatment with CDK4/6 inhibitors, since they clearly affect the pharmacodynamic properties of these drugs, impairing their clinical benefit [27, 31]. To date, there is no prospective evaluation of continuing CDK4/6 inhibitors, switching the endocrine therapy backbone from AI to fulvestrant, beyond progression, but this treatment strategy is being evaluated in specific trials (PALMIRA, NCT03809988; MAINTAIN, NCT02632045). Interestingly, recent studies reported a synthetic lethal relationship between *RB1* mutations and inhibition of Aurora kinases A and B [90, 91]. Several clinical trials are currently testing the safety and efficacy of Aurora kinase inhibitors in solid malignancies (NCT03955939, NCT03092934) and they might represent a future treatment option for *RB1* mutant ER+ BC.

FGFR signaling alterations have been identified as mechanisms of resistance to CDK4/6 inhibitors, leading to the phase Ib clinical trial NCT03238196, testing the effect of the triple combination of fulvestrant, palbociclib and erdafitinib, a potent and specific FGFR1-4 TKI [92], in patients with ER+/HER2-/FGFR1-4-amplified breast cancer. Although previous studies demonstrated involvement of FGFR1 gene amplification in resistance to endocrine therapy [93, 94] and the PI3K inhibitor alpelisib [95], recent evidences reported that FGFR1-amplified breast cancer retains sensitivity to TORC1 inhibitors [96]. Thus, in patients progressing on CDK4/6 inhibitors and with tumors harboring FGFR1 amplification, the combination of exemestane and everolimus [97] may be considered a valuable treatment strategy.
Since the inhibition of PI3K/AKT pathway has been shown to impair the early adaptation of ER+ BC to treatment with CDK4/6 inhibitors [35], the triple combination of hormone therapy, CDK4/6 and PI3K inhibitors is currently being tested in clinical trials (NCT01872260, NCT02088684). Notably, only few patients (n=20, 5.9%) enrolled in the SOLAR-1 trial, testing the effect of combination of fulvestrant and the α-specific PI3K inhibitor alpelisib in PIK3CA mutant ER+ BC, had previously received CDK4/6 inhibitors [98].

Homozygous deletion or truncating mutations of *PTEN* have been described in 6% of primary ER+ breast cancer patients [24] and the rate of these alterations may be even higher in patients recurring after adjuvant endocrine therapy. Loss of PTEN has been described as mediator of cross-resistance to CDK4/6 and PI3K inhibitors [43, 99]. It would be important to assess the genomic status of *PTEN*, or its expression by IHC, at diagnosis of metastatic ER+/HER2- breast cancer, for prognostic evaluation of sensitivity to CDK4/6 inhibitors. Furthermore, assessment of PTEN status at progression on first line treatment could help to evaluate the potential benefit of the use of alpelisib as second line therapy. However, it has to be noted that the results of the pre- and clinical studies reported so far, are not enough to prevent starting a standard first line treatment with antiestrogens plus CDK4/6 inhibitors in patients with newly diagnosed *PTEN* altered ER+/HER2-metastatic breast cancer.

An increase in establishment of biological models collected directly from patients, such as patient derived organoid/xenografts from primary or metastatic tumor biopsies [100], or circulating tumor cells [101], as recently reported, may further help the characterization of biological mechanisms associated with drug resistance, with possible future impacts on personalized medicine.

The effect of epigenetic remodeling has been extensively investigated in ER+ BC, demonstrating its significant effect on resistance to endocrine therapy [102, 103]. In this context, the use of Whole
Genome Sequencing (WGS), in association with chromosome conformation capture (3C) methodology (Hi-C), have highlighted the role of single-nucleotide variants (SNVs) occurring in enhancer regions, in remodeling the landscape of transcription factors-bound enhancers, eventually promoting endocrine resistant phenotype [104]. Recent evidences also demonstrated that treatment with CDK4/6 inhibitors induces the enhancer landscape of ER+ breast cancer, through activation of the activator protein-1 (AP-1) transcriptional activity [105], suggesting that these mechanisms of early adaptation may ultimately induce drug resistance... These studies further support the notion that a future extensive use of WGS and 3C-based methods may provide new insights into the biological effects of CDK4/6 inhibitors.

**Conflict of interest statement:** CDA is a consultant/advisory board member for Novartis, GSK, Eli Lilly, and Pfizer. He has received research support from Novartis (to institution). GA and SDP declare honoraria from Roche, Pfizer, AstraZeneca, Novartis, Celgene, Eli Lilly, Amgen, and Eisai. MG is a consultant/advisory board member for Novartis, Pfizer, Lilly, Celgene, Eisai, Roche, AstraZeneca, Teva. He has received travel grants from Pfizer, Lilly, Roche, Amgen, Celgene and research funding from Roche, Novartis and AstraZeneca. LF has received research funding from Lilly. RB is Advisory Board for Boeringer Ingellheim, Pfizer, Astrazeneca, Novartis. AS, FN and PDP have no conflict of interest to declare.

**Authors Contribution**

Conceptualization, AS, FN and LF; investigation resources, AS, FN and PDP; methodology, MG, GA, SDP and RB; supervision, MG, GA, SDP, RB and LF; writing - original draft, AS and FN; writing – review & editing, CDA and LF; funding acquisition, LF and RB.

All authors have approved the final article.
Conflict of interest statement: the authors have no conflict of interest to declare.

Funding: This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) (Investigator Grant IG2018-21339 to R.B.; My First Grant MFGA2018-21505 to L.F.).

References


Author Biography

Alberto Servetto short biography:
Alberto Servetto is an MD, PhD student at University of Naples Federico II. Dr. Servetto obtained his MD degree summa cum laude at the University of Naples Federico II. He completed the residency program in Medical Oncology at the same Institution. He is currently attending a PhD program in Advanced Biomedical and Surgical Therapies. After completing his clinical training, in September 2017 Dr. Servetto joined the laboratory of Dr. Carlos L. Arteaga at Vanderbilt University (Nashville, TN) for 6 months and then at the University of Texas Southwestern Medical Center (Dallas, TX), as visiting researcher. His current main research area is the discovery of mechanisms of resistance to endocrine therapy and CDK4/6 inhibitors in ER+ breast cancer.

Fabiana Napolitano short biography:
Fabiana Napolitano, MD, PhD student. Currently completing her Residency at "Federico II" University of Naples. Her current areas of focus are mechanisms of resistance to targeted therapy and translational research.

Carmine De Angelis short biography:
Carmine De Angelis is an Assistant Professor of Medical Oncology at the Department of Clinical Medicine and Surgery of University of Naples Federico II and an Adjunct Professor at the Lester and Sue Smith Breast Center of Baylor College of Medicine. Dr. De Angelis obtained an M.D. degree at the University of Naples Federico II. Subsequently, he completed the residency program in medical oncology and the PhD program in Advanced Medical-Surgical Therapies at the University of Naples Federico II. After completing his clinical training, he joined the laboratory of Dr. Kent Osborne and Dr. Schiff at Baylor College of Medicine in Houston (TX), where he successfully completed his post-doctoral research training. Dr. De Angelis’s research focus on understanding mechanisms of endocrine and anti-HER2 resistance and on the development and optimization of innovative therapies for women with breast cancer.

Pietro De Placido short biography:
Pietro De Placido, MD is currently completing his Residency at "Federico II" University of Naples.

Mario Giuliano short biography:
Mario Giuliano is an Associate Professor in Tenure Track of Medical Oncology at the Department of Clinical Medicine and Surgery of University of Naples Federico II and an
Adjunct Professor at the Lester and Sue Smith Breast Center of Baylor College of Medicine. In 2004, Dr. Giuliano obtained an M.D. degree summa cum laude and special mention for the academic career at the University of Naples Federico II. He completed the residency program in Medical Oncology at the same Institution and subsequently he attended a PhD program in Clinical Immunology and Oncology at the Second University of Naples. After completing his clinical training, he completed a 6-month research experience as visiting scientist at the Department of Hematopathology at the University of Texas MD Anderson Cancer Center. Moreover, in April 2010, he joined the laboratory of Dr. Kent Osborne and Dr. Schiff at Baylor College of Medicine in Houston (TX), where he completed his preclinical research training as post-doctoral associate. Dr. Giuliano has experience on phase II and III clinical trials and basic/translational research projects in breast cancer and rare tumors.

Grazia Arpino short biography:
Professor Grazia Arpino completed the clinical fellowship in Medical Oncology and the PhD in Molecular Oncology at the University of Naples Federico II. From 2001 till 2009 she worked at Baylor College of Medicine in Houston Texas in Dr Kent Osborne department and laboratory as postdoctoral fellow and faculty. Currently she is professor in Oncology at the University of Naples Federico II, she works in the University Hospital Federico II where she is the head of the unit developing novel predictive and prognostic molecular markers in breast cancer. Her research interests mainly focus on mechanisms of endocrine and anti-HER2 resistance development and optimization of adjuvant therapies strategies in pre and post menopausal women with breast cancer. Professor Arpino is involved as principal investigator in many clinical trials testing novel therapeutic approaches to overcome the resistance development.

Sabino De Placido short biography:
Prof De Placido is Full Professor in Medical Oncology at the Department of Clinical Medicine and Surgery of University of Naples Federico II. Prof. De Placido obtained his MD degree summa cum laude at the University of Naples Federico II. After completing the residency program first in Internal Medicine and the in Medical Oncology, he continued his carrier as Assistant Professor. From 2006 to 2013, Prof De Placido has been Director of the Residency Program in Medical Oncology of University of Federico II, in Naples. Since 2013 Prof De Placido is part of the Academic Senate and takes part as Coordinator in the Medicine and Surgery Degree Program.

Roberto Bianco short biography:
Roberto Bianco is an Associate Professor in Medical Oncology at the Department of Clinical Medicine and Surgery of University of Naples Federico II. Prof. Bianco obtained his MD degree summa cum laude at the University of Naples Federico II. He completed the residency program in Medical Oncology at the same Institution and subsequently he attended a PhD program in Molecular Oncology at Chieti University. After completing his preclinical research training as post-doctoral associate, he continued his research career as Assistant Professor. Since 2016, Prof Bianco has been Director of the Interdepartmental Center for Clinical and Translational Research (CIRCET). Since 2018 he has been Director of the Residency Program in Medical Oncology of University of Federico II, in Naples. Prof Bianco has experience on phase II and III clinical trials and basic/translational research projects in lung and pancreatic cancer.

Luigi Formisano short biography:
Luigi Formisano is an Assistant Professor in Medical Oncology at the Department of Clinical Medicine and Surgery of University of Naples Federico II. Dr. Formisano obtained his MD degree summa cum laude at the University of Naples Federico II. He completed the residency program in Medical Oncology at the same Institution and subsequently he attended a PhD program in Biomedical and Surgery Advanced Therapies. After completing his clinical training, he completed a 6-month research experience as visiting scientist at the Department of Medicine at Vanderbilt University (Nashville, USA). Moreover, in April 2014, he joined the laboratory of Dr. Carlos L. Arteaga at Vanderbilt University (Nashville, TN), where he completed his preclinical research training as post-doctoral associate. Dr. Formisano has experience on phase II and III clinical trials and basic/translational research projects in melanoma, breast, and pancreatic cancer.
Figure 1. Mechanisms driving resistance to CDK4/6 inhibitors. Schematic representation of signaling pathways involved in resistance to CDK4/6 inhibitors in ER+ breast cancer. FAT1=FAT atypical cadherin 1; MST1/2=Macrophage stimulating 1/2; LATS1/2=Large tumor suppressor kinase 1/2; YAP=Yes1 associated transcriptional regulator; TAZ=Tafazzin; CDK1/2/4/6=Cyclin dependent kinase 1/2/4/6; EGFR=Epidermal growth factor receptor; FGFR=Fibroblast growth factor receptor; HER2=erb-b2 receptor tyrosine kinase 2; PTEN=Phosphatase and tensin homolog; PI3K=Phosphatidylinositol 3-kinase; PDK1=Pyruvate dehydrogenase kinase 1; AKT=Protein kinase B; TSC1/2=TSC complex subunit 1/2; mTORC1=Mammalian target of rapamycin complex 1; RAS=Ras GTPase; Raf-1 proto-oncogene; MEK=Mitogen-activated protein kinase kinase; MAPK=Mitogen activated protein kinase; Cyclin-dependent kinase inhibitor P27;
Rb=Retinoblastoma tumor suppressor protein; CycB/D/E=Cyclin-B/D/E; E2F=E2 transcription factor 1; AURKA=Aurora kinase A.
<table>
<thead>
<tr>
<th>Trial Name</th>
<th>Phase</th>
<th>Description of treatment arms</th>
<th>PFS</th>
<th>OS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Combination with AI or tamoxifen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MONALEESA-7(^{a})</td>
<td>III</td>
<td>Ribociclib + Goserelin + AI/tamoxifen vs Placebo + Goserelin + AI/tamoxifen</td>
<td>23.8 months vs 13.0 months HR 0.553</td>
<td>70.2% vs 46.0%, HR 0.712 (estimated OS at 42 months)</td>
<td>Tripathy et al Im et al</td>
</tr>
<tr>
<td>PALOMA-2(^{b})</td>
<td>III</td>
<td>Palbociclib + Letrozole vs Letrozole</td>
<td>27.6 months vs 14.5 months HR 0.563</td>
<td>N/A</td>
<td>Finn et al</td>
</tr>
<tr>
<td>MONALEESA-2(^{c})</td>
<td>III</td>
<td>Ribociclib + Letrozole vs Placebo + Letrozole</td>
<td>25.3 months vs 16.0 months HR 0.568</td>
<td>N/A</td>
<td>Hortobagyi et al</td>
</tr>
<tr>
<td>MONARCH-3(^{b})</td>
<td>III</td>
<td>Abemaciclib + AI vs Placebo + AI</td>
<td>28.18 months vs 14.76 months HR 0.54</td>
<td>N/A</td>
<td>Goetz et al</td>
</tr>
<tr>
<td><strong>Combination with fulvestrant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PALOMA-3(^{d})</td>
<td>III</td>
<td>Palbociclib + Fulvestrant vs Placebo + Fulvestrant</td>
<td>9.5 months vs 4.6 months HR 0.46</td>
<td>34.9 months vs 28.0 months HR 0.81 (median OS)</td>
<td>Turner et al Turner et al</td>
</tr>
<tr>
<td>MONALEESA-3(^{e})</td>
<td>III</td>
<td>Ribociclib + Fulvestrant vs Placebo + Fulvestrant</td>
<td>20.5 months vs 12.8 months HR 0.593</td>
<td>57.8% vs 45.9% HR 0.72 (estimated OS at 42 months)</td>
<td>Slamon et al Slamon et al</td>
</tr>
</tbody>
</table>
Table 1. Results from the main clinical trial testing the effect of CDK4/6 inhibitors in ER+/HER2-negative breast cancer.

<table>
<thead>
<tr>
<th>Study</th>
<th>Phase</th>
<th>Monotherapy Option</th>
<th>Median PFS</th>
<th>Median OS</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MONARCH-2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>III</td>
<td>Abemaciclib + Fulvestrant vs Placebo + Fulvestrant</td>
<td>16.4 months vs 9.3 months</td>
<td>46.7 months vs 37.3 months</td>
<td>HR 0.553 vs 0.757 (median OS)</td>
</tr>
<tr>
<td>MONARCH-1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>II</td>
<td>Abemaciclib monotherapy</td>
<td>6.0 months</td>
<td>17.7 months</td>
<td>Dickler et al</td>
</tr>
</tbody>
</table>

HR PFS: Hazard Ratio for Progression Free Survival; HR OS: Hazard Ratio for Overall Survival; AI: non-steroidal aromatase inhibitors; N/A: data not available.

*a* Premenopausal and perimenopausal women with HR-positive and HER2-negative advanced breast cancer (locoregionally or metastatic disease) were enrolled. Previous endocrine therapy for advanced disease was not allowed. No more than one previous line of chemotherapy for advanced disease was allowed. Adjuvant or neoadjuvant endocrine therapy was permitted.

*b* Postmenopausal women with HR-positive and HER2-negative advanced breast cancer were enrolled. Previous systemic treatment for advanced breast cancer was not allowed. Adjuvant or neoadjuvant endocrine therapy was permitted if disease-free interval >12 months from therapy completion.

*c* Postmenopausal women with HR-positive and HER2-negative advanced breast cancer (recurrent or metastatic de novo) were enrolled. Previous systemic treatment for advanced breast cancer was not allowed. Previous (neo)adjuvant therapy was permitted, if disease-free interval >12 months from therapy completion.
Women with HR-positive and HER2-negative, regardless of their menopausal status, with advanced breast cancer who had disease progression or relapse during a previous (neo)adjuvant endocrine therapy were enrolled. Premenopausal or perimenopausal patients were required to receive concurrent goserelin for at least 4 weeks before the start of the trial.

Men and postmenopausal women with HR-positive and HER2-negative advanced breast cancer (locoregionally or metastatic disease) were enrolled. Patients should have not previously received treatment for advanced disease, or progressed after previous endocrine therapy for advanced disease, or relapsed during or within 12 months after completion of adjuvant or neoadjuvant endocrine therapy.

Postmenopausal women with HR-positive HER2-negative metastatic breast cancer were enrolled. Patients must have progressed on or after prior endocrine therapy and had prior treatment with at least two chemotherapy regimens; ≥1, but no more than two of which, had been administered in the metastatic setting (one regimen must have included a taxane either in the adjuvant setting or metastatic disease setting).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Sequencing method</th>
<th>Findings</th>
</tr>
</thead>
</table>
| O’Leary et al     | WES – ctDNA                                 | • Clonal evolution on fulvestrant plus palbociclib in 12/14 matched D1 and EOT samples from patients enrolled in the PALOMA-3 trial.  
• APOBEC mutational signature associated with clonal evolution. |
| Wander et al      | WES – tissue biopsy                        | • Genomic alterations in RB1, FGFR2, ERBB2, AKT1, AURKA, KRAS/HRAS/NRAS, CCNE2 associated with resistance to CDK4/6 inhibitors.  
• Evolutionary analysis to determine clonal structure and dynamics in 7 matched pre- and post-treatment samples. |
| Li et al          | Targeted Sequencing (MSK-IMPACT) – tissue biopsy | • RB1 loss-of-functions alterations, FAT1 loss-of-function alterations, LATS1 and LATS2 mutations associated with de novo resistance to CDK4/6 inhibitors. |
| Formisano et al   | Targeted sequencing (Guardant 360) – ctDNA   | • FGFR1-amp (n=9), FGFR2-amp (n=2), FGFR1 N546K (n=1), FGFR2 (n=1) and FGFR2 V395D (n=1) mutations in 14/34 (41%) post-progression samples from patients treated with CDK4/6 inhibitors. |
| Formisano et al   | Targeted sequencing – ctDNA                 | • FGFR1/ZNF703 amplification in 20/427 (4.7%) pre-treatment ctDNA samples associated with reduced clinical benefit from ribociclib from patients enrolled in the MONALEESA-2 trial. |
| O’Leary et al     | Targeted sequencing – ctDNA                 | • RB1 mutations selectively acquired in the palbociclib plus fulvestrant arm from the PALOMA-3 trial (6/127, 4.7%, matched pre- and post-treatment samples). |
| Turner et al      | “Targeted” RNA-Seq (EdgeSeq Oncology BM Panle) – tumor biopsy | • High CCNE1 mRNA expression levels predictive of worse response to palbociclib, in 302 pre-treatment samples from the PALOMA-3 trial. |
| Ma et al          | Microarray gene expression analysis – tumor biopsy | • High CCNE1, CCND3 and CDKN2B mRNA expression levels associated with resistance to palbociclib in the NeoPalAna trial. |

Table 2. Next Generation Sequencing methodologies applied on tissue and/or liquid biopsies and most meaningful findings.