REVIEW

Overcoming CDK4/6 inhibitor resistance in ER-positive breast cancer

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Abstract

Three inhibitors of CDK4/6 kinases were recently FDA approved for use in combination with endocrine therapy, and they significantly increase the progression-free survival of patients with advanced estrogen receptor-positive (ER+) breast cancer in the first-line treatment setting. As the new standard of care in some countries, there is the clinical emergence of patients with breast cancer that is both CDK4/6 inhibitor and endocrine therapy resistant. The strategies to combat these cancers with resistance to multiple treatments are not yet defined and represent the next major clinical challenge in ER+ breast cancer. In this review, we discuss how the molecular landscape of endocrine therapy resistance may affect the response to CDK4/6 inhibitors, and how this intersects with biomarkers of intrinsic insensitivity. We identify the handful of pre-clinical models of acquired resistance to CDK4/6 inhibitors and discuss whether the molecular changes in these models are likely to be relevant or modified in the context of endocrine therapy resistance. Finally, we consider the crucial question of how some of these changes are potentially amenable to therapy.

Key Words
- CDK4/6 inhibitors
- estrogen receptor
- breast cancer
- endocrine therapy

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Overview of the cell cycle

Dysregulation of the complex regulatory network that controls cell cycle progression is a hallmark of cancer (Hanahan & Weinberg 2011). A major axis of dysregulation is the gateway to cell cycle entry, which is controlled by the retinoblastoma (Rb) protein. Rb restricts progression from G_1 phase into S phase by binding and suppressing E2F transcription factors. This is overcome by cyclin-dependent kinase 4/6 (CDK4/6) phosphorylation of the Rb protein, which leads to E2F release, and a transcriptional program for proliferation is activated, committing the cell to G_1 exit. Subsequently, there is a cascade of downstream signalling events that ultimately promotes the activity of cyclin E/CDK2 complexes, the phosphorylation of further target proteins and progression into S phase and DNA replication (Kato *et al.* 1993, Burkhart & Sage 2008).

Regulation of CDK4/6 activity is key to the deactivation of the Rb protein. CDK4 and CDK6 become active when CDK4/6 form heterodimers with D-type cyclins, which are upregulated and post-translationally modified in response to mitogenic signalling by the RAS/MAPK and PI3K/AKT/mTOR signal transduction cascades. Entry into the cell cycle is suppressed by two families of CDK inhibitors, INK4 ($p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$) and CIP/KIP ($p21^{Waf1/Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) (Sherr 1996). INK4 proteins selectively inhibit the cyclin D–CDK4/6 complex (Sherr & Roberts 1999, Sheppard & McArthur 2013) to induce cell cycle arrest and senescence

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(Kim & Sharpless 2006). CIP/KIP proteins inhibit both CDK4/6 and CDK2 complexes but have the additional effect of stabilising these complexes and preventing cyclin degradation (Prall *et al*. 1997). In the presence of stable $p16^{INK4A}$-cyclin D-CDK4/6, the binding of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ to CDK2 complexes will reinforce cell cycle arrest (Sherr & Roberts 1999).

Dysregulation of the cyclin-CDK-Rb axis by upregulation of cyclin–CDK activity and/or abrogation of suppressors are features of many tumour types. It is therefore unsurprising that this axis is recognised as a key target for therapeutic intervention (Musgrove *et al*. 2011). Research has focussed on small-molecule inhibition of CDK function and such CDK inhibitors have been designed, developed and trialled in the clinic with increasing success over the last few years.

The development of CDK inhibitors

CDK4 and CDK6 are part of the CDK family of serine/threonine kinases (Peyressatre *et al*. 2015). The initial discovery of cyclin-dependent kinases was in the context of the cell cycle where 'cyclins' were cyclically degraded and includes CDK1, CDK2, CDK4 and CDK6. Since then, further CDK functions have been identified for the transcriptional machinery (CDK7, CDK8, CDK9, CDK12), DNA damage response (CDK12) and in tissue specific functions (CDK5) (Reviewed by Malumbres 2014, Lenjisa *et al*. 2017, Philip *et al*. 2018). Despite these diverse functions, the CDKs are structurally very similar, due to the fact that context-specific cyclins are activated to control each function.

CDK inhibitors are a class of pharmacological agents used to target dysregulated CDK activity in malignant cells. The mechanisms of several first-generation CDK inhibitors have been studied in a variety of cancer types, but few have successfully transitioned to a clinical setting (Asghar *et al*. 2015). A major barrier to the clinical development of first-generation inhibitors was lack of selectivity due to structural similarity between the CDKs (Shapiro 2006, Michaud *et al*. 2010). Compounds such as flavopiridol (CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9, trialled in patients with a range of haematological malignancies and solid tumours) and roscovitine (CDK2, CDK7 and CDK9, trialled in patients with non-small-cell lung cancer (NSCLC) (NCT00372073), triple-negative breast cancer (NCT01333423) and other advanced solid tumours (NCT00999401)) failed clinical trials, demonstrating limited efficacy and considerable toxicity (Lapenna & Giordano 2009). Second-generation pan-CDK inhibitors had greater selectivity across a smaller number of CDKs, and a reduced toxicity profile, but they showed a lesser degree of CDK4/6 inhibitory activity. For example, the pan-CDK inhibitors dinaciclib and SNS-032 target CDK1, CDK2, CDK5, CDK9 and CDK12, and CDK2, CDK7 and CDK9 respectively (Asghar *et al*. 2015). Dinaciclib is currently being trialled on patients with multiple myeloma, NSCLC, melanoma, advanced hematologic, breast and pancreatic malignancies and other solid tumours and has reached phase II clinical trials for some applications. More recently, dinaciclib was shown to be a potent inhibitor of CDK12 with implications for its use in homologous repair-deficient tumour types in combination with PARP inhibitors (Johnson *et al*. 2016), a finding that is now being investigated in patients (NCT01434316).

The relative failure of these first- and second-generation inhibitors led to the search for inhibitors highly selective to individual CDKs and their associated cellular functions. Pharmacological (Tadesse *et al*. 2018, Yin *et al*. 2018) and computer-aided (Kalra *et al*. 2017) approaches are now being employed to design the next generation of CDK inhibitors with higher potency and, crucially, higher specificity – a key attribute for the successful deployment of CDK inhibitors. There has been some progress made in targeting non-cell cycle CDKs such as CDK9 and CDK12 (Johnson *et al*. 2016, Li *et al*. 2017), but the inhibitors that have progressed furthest and have now entered clinical use are those targeted at CDK4/6.

CDK4/6 inhibitors

All CDK4/6 inhibitor compounds are designed by targeting the ATP-binding domains of the proteins (Asghar *et al*. 2015). The pharmaceutical lead compounds that have been translated into clinical use are palbociclib (Ibrance; Pfizer) (Fry *et al*. 2004), ribociclib (Kisqali; Novartis) (Infante *et al*. 2014) and abemaciclib (Verzenio; Eli Lilly) (Patnaik *et al*. 2016). These compounds are highly selective for CDK4/6 over other members of CDK family compared to their historical counterparts. Both palbociclib and ribociclib have >100-fold higher affinity for CDK4 and CDK6 relative to other cell cycle CDKs and CDK9 (Tadesse *et al*. 2017). In contrast, abemaciclib is less selective, with only ~six-fold higher affinity for CDK4/6 than it has for CDK9 and has some activity towards CDK1, CDK2 and CDK5 at higher doses (Gelbert *et al*. 2014, Tripathy *et al*. 2017) (Table 1). Despite being less selective, in a direct comparison, abemaciclib was found to more efficiently pass through the blood–brain barrier than palbociclib (Raub *et al*. 2015), which widens

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Table 1 CDK4/6 inhibitor specificities.

	Palbociclib (Fry *et al.* 2004)	**Ribociclib** (Tripathy *et al.* 2017)	**Abemaciclib** (Gelbert *et al.* 2014)
IC_{50} (nmol/L)			
CDK4-cyclin D1/D3	9–11	10	2
CDK6-cyclin D1/D2/D3	15	39	10
CDK1-cyclin B	>10,000	>100,000	>1000
CDK2-cyclin A/E	>10,000	>50,000	>500
CDK5-p25	>10,000	>40,000	>300
CDK9-cyclin T	Not determined	Not determined	57

IC_{50}, half maximal inhibitory concentration; *in vitro* kinase assay.

its potential application to brain cancers and secondary brain metastases (NCT02308020).

Recent studies using chemoproteomics (Sumi *et al.* 2015), thermal proteome profiling (Miettinen *et al.* 2018) and a mass spectrometry-based competition assay have shown that palbociclib, ribociclib and abemaciclib can inhibit a spectrum of other kinases and that the inhibition profiles of palbociclib and abemaciclib were not similar with the exception of CDK4/6 (Cousins *et al.* 2018). Notably, it was found that in cell line models abemaciclib, but not palbociclib or ribociclib, activated wnt signalling via inhibition of glycogen synthase kinase (GSK) 3B and subsequent stabilisation of β-catenin. GSK3β activity also plays an important role in the regulation of cyclin D family proteins at both the transcriptional and proteomic level such that inhibition of GSK3B is expected to increase the levels of cyclin D (Takahashi-Yanaga & Sasaguri 2008).

The clinical relevance of the off-target kinase inhibitory activity for each of these drugs has yet to be fully assessed (Chen *et al.* 2016). The increased potency of abemaciclib toward CDK9 (reported in some, but not all studies) (Gong *et al.* 2017) correlates with a gastrointestinal toxicity profile specifically in those patients (Shohdy *et al.* 2017). Conversely, abemaciclib allows for continuous dosing, whereas palbociclib and ribociclib require a break in treatment of 1 week in four in order to allow for neutrophil recovery. All three inhibitors are associated with toxicities resulting in loss of haematocytes (Kassem *et al.* 2018), consistent with a role for CDK6 in the activation of haematopoetic stem cells (Scheicher *et al.* 2015). Toxicities associated with all three drugs are, however, considered to be manageable and reversible (Spring *et al.* 2017).

This family of compounds have found a natural home in the treatment of advanced ER+ breast cancer (Lapenna & Giordano 2009, Musgrove *et al.* 2011). ER+ breast cancer is by far the most common subtype of breast cancer, representing approximately 70% of breast cancers in women. Estrogen is mitogenic and drives cell proliferation, partly through the increase in levels of cyclin D1 and CDK4/6 activity (Filmus *et al.* 1994).

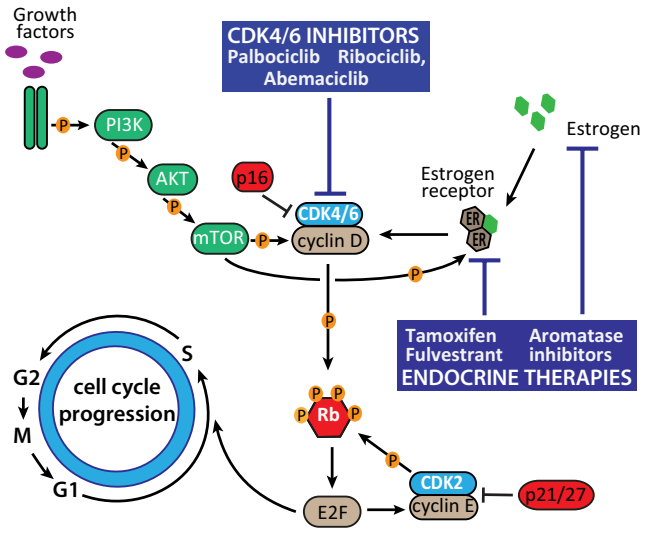

Figure 1
Regulation of cell cycle in ER+ breast cancer. The key pathways in promoting entry into the cell cycle in ER+ breast cancer and the nodes to which current therapies are targeted.

Clinical development of CDK4/6 inhibitors in endocrine-resistant breast cancer

Endocrine therapy is the bedrock of systemic therapy for ER+ breast cancer. Adjuvant therapy comprises 5–10 years or more of ER-directed endocrine therapy such as tamoxifen and aromatase inhibitors to inhibit ER-driven activation of cell cycle progression. Approximately 50% of patients with early stage ER+ breast cancer obtain benefit from adjuvant endocrine therapy, resulting in a reduction in breast cancer mortality by approximately 40% (Early Breast Cancer Trialists' Collaborative Group 2015). However, resistance to endocrine therapy leading to early stage ER+ breast cancer recurrence is not uncommon and inevitable in the setting of advanced disease. Importantly, the majority of these cancers have a perturbed but essentially intact Rb axis downstream of the mechanism of endocrine resistance, making them highly suitable for CDK4/6 inhibitor treatment (Ertel *et al.* 2010) (Fig. 1).

Early preclinical breast cancer studies demonstrated that palbociclib preferentially inhibited the proliferation of ER+ in contrast to ER– breast cancer models *in vitro*

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(Finn *et al.* 2009). Phase III clinical trials have shown that the combination therapy of CDK4/6 inhibitors and endocrine therapy for advanced ER+ breast cancer, improves progression free survival (PFS) when compared to endocrine therapy alone; overall survival data are yet to be published. Therefore, the combination of CDK4/6 inhibitor and endocrine therapy is now standard of care as first-line therapy for advanced ER+ breast carcinoma in many countries.

Phase III trials of all three CDK4/6 inhibitors in the first-line setting in combination with non-steroidal aromatase inhibitors and in the second-line setting in combination with fulvestrant has been completed. Palbociclib was the first CDK4/6 inhibitor approved by the FDA in February 2015. It was granted accelerated approval in combination with letrozole for the first-line treatment of advanced ER+ HER2− breast cancer due to the phase II PALOMA-1 trial (Finn *et al.* 2015). The follow-up phase III PALOMA-2 trial demonstrated that palbociclib and letrozole improved PFS from 14.5 to 24.8 months, when compared to letrozole alone (hazard ratio 0.58; 95% CI 0.46–0.72, $P<0.001$) (Finn *et al.* 2016). It also received FDA approval in February 2016 for a second indication, the treatment of advanced ER+ HER2− breast cancer in combination with fulvestrant after progression following endocrine therapy from the phase III PALOMA-3 trial (Cristofanilli *et al.* 2016) (Table 2).

Also approved for clinical use are ribociclib and abemaciclib. The phase III MONALEESA-2 trial with ribociclib and letrozole as first-line treatment for advanced ER+ HER2− breast cancer showed improved PFS, leading to FDA approval in March 2017 (Hortobagyi *et al.* 2016, 2018). More recently, the phase III MONALEESA-3 trial with ribociclib and fulvestrant as first- and second-line treatment for advanced ER+ HER2− breast cancer demonstrated an improved PFS (Slamon *et al.* 2018). Abemaciclib was FDA approved in September 2017 in combination with fulvestrant as a second-line treatment after the phase II MONARCH-2 trial (Sledge *et al.* 2017) and as monotherapy after progression on endocrine therapy and chemotherapy from the phase II MONARCH-1 trial (Dickler *et al.* 2017). It was later approved in February 2018 in combination with letrozole as first-line treatment based on results from the phase III MONARCH-3 trial (Goetz *et al.* 2017).

CDK4/6 inhibitors are given as oral tablets and are generally well tolerated. Common toxicities include nausea, diarrhoea, fatigue, neutropenia (however, febrile neutropenia is uncommon), leukopenia, anaemia and thrombocytopenia (Shah *et al.* 2018). Patients require regular full blood counts, liver function tests and ECGs.

Current clinical questions include which CDK4/6 inhibitor to choose, how to best sequence therapy and whether to add to the same endocrine therapy regime. The recent TREND study (Malorni *et al.* 2018) shows that adding palbociclib to the previously administered endocrine therapy led to a PFS advantage in patients who received prior endocrine therapy for >6 months (HR 0.53; 95% CI 0.3–0.9, exploratory P-value=0.02), but not in those who had received less than 6 months of endocrine therapy.

Resistance to CDK4/6 inhibitors is now the major emerging consideration in pre-clinical and clinical drug development. Clinical areas of interest to address resistance include identifying predictive biomarkers for CDK4/6 inhibitors and novel treatment combinations.

Does endocrine therapy resistance affect CDK4/6 inhibitor sensitivity?

Endocrine resistance occurs when tumours bypass the cell cycle inhibition of endocrine therapy and return to a proliferative phenotype. Many mechanisms of acquired endocrine resistance have been described, including the upregulation of ER coactivators (e.g. FOXA1), cyclins (particularly D and E class), CDK proteins (CDK2 and CDK6) and mitogen signalling pathways (PI3K and RAS pathways) and/or downregulation of CDK inhibitor proteins ($p16^{INK4A}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$) (Musgrove & Sutherland 2009). Genomic and epigenetic mechanisms of endocrine resistance have also been identified, including activating *ESR1* mutations, which can occur in up to 40% of patients with metastatic disease (Jeselsohn *et al.* 2014, Schiavon *et al.* 2015) and hypermethylation of estrogen-responsive enhancers, which is associated with reduced ER binding and decreased gene expression of key regulators of ER activity (Stone *et al.* 2015). Importantly, in most cases functional Rb protein is retained during the development of endocrine resistance (Musgrove *et al.* 2011), rendering these tumours amenable to CDK4/6 inhibition (Fig. 1).

CDK4/6 inhibition acts directly downstream of endocrine therapy, and it is therefore inevitable that some mechanisms of resistance will be common to both types of treatments. Endocrine resistance associated with dysregulation of the Rb axis could, in theory, reduce sensitivity to CDK4/6 inhibition as many of these mechanisms could also impinge on the effectiveness of CDK4/6 inhibitors. Despite this, the success of CDK4/6

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Table 2 Seminal phase II/III trials of CDK4/6 inhibitors that led to FDA approval for the treatment of advanced ER+ breast cancer.

Study	NCT	Phase	No.	Menopausal status	Description	Median PFS; Hazard ratio (95% CI)	ORR (%)	FDA approval
1st Line								
PALOMA-1 TRIO-18 (Finn *et al*. 2015)	00721409	II	165	PMP	Palbociclib + letrozole vs letrozole	20.2 vs 10.2 months; 0.49 (0.32–0.75); $P = 0.0004$	55.0 vs 39.0	Feb 2015
PALOMA-2 (Finn *et al*. 2016)	01740427	III	666	PMP	Palbociclib + letrozole vs letrozole	24.8 vs 14.5 months; 0.58 (0.46–0.72); $P < 0.001$	55.3 vs 44.4	Feb 2016
MONALEESA-2 (Hortobagyi *et al*. 2018)	01958021	III	668	PMP	Ribociclib + letrozole vs letrozole	25.3 vs 16.0 months; 0.57 (0.46–0.70) $P < 0.0001$	52.7 vs 37.1	Mar 2017
MONALEESA-7 (Tripathy *et al*. 2018)	02278120	III	660	Pre-MP	Ribociclib + OFS + tamoxifen/AI vs OFS + tamoxifen/AI	23.8 vs 13.0 months; 0.55 (0.44–0.69); $P < 0.0001$	51.0 vs 44.0	
MONARCH-3 (Goetz *et al*. 2017)	02246621	III	493	PMP	Abemaciclib + AI vs AI	NR vs 14.7 months; 0.54 (0.41–0.72); $P < 0.0001$	59.0 vs 44.0	Feb 2018
2nd Line								
PALOMA-3 (Cristofanilli *et al*. 2016)	01942135	III	521	PMP + Pre-MP	Palbociclib + fulvestrant ± OFS vs fulvestrant ± OFS	9.5 vs 4.6 months; 0.46 (0.36–0.59); $P < 0.001$	24.6 vs 15.0	Feb 2016
MONARCH-2 (Sledge *et al*. 2017)	02107703	III	669	PMP	Abemaciclib + fulvestrant vs fulvestrant	16.4 vs 9.3 months; 0.55 (0.45–0.68); $P < 0.001$	48.1 vs 21.3	Sep 2017
nth line								
MONARCH-1 (Dickler *et al*. 2017)	02102490	II	132	PMP	Abemaciclib single agent	6.0 months	19.7	Sep 2017

AI, aromatase inhibitor; OFS, ovarian function suppression; ORR, objective response rate; PFS, progression free survival; PMP, post-menopausal; Pre-MP, pre-menopausal.

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inhibitors clinically (Table 2) suggests that in general, endocrine-resistant tumours maintain sensitivity to CDK4/6 inhibition, particularly when used in combination with endocrine therapy. This has been retrospectively demonstrated for endocrine-resistant tumours with activating *ESR1* mutations. In the PALOMA-3 trial, fulvestrant plus palbociclib improved PFS in patients with *ESR1* mutant and *ESR1* WT circulating tumour DNA (ctDNA), indicating that CDK4/6 inhibitors are effective irrespective of ESR1 mutation status (Fribbens *et al*. 2016).

In fact, certain manifestations of endocrine therapy resistance may sensitise breast cancer to CDK4/6 inhibitors. Deficiency of mismatch repair caused by MutL mutation in ER+ breast cancer abrogates CHK2-mediated inhibition of CDK4, leading to endocrine resistance. Consequently, CDK4/6 inhibitors are highly effective in MutL-defective ER+ breast cancer cells, and MutL could prove useful as a biomarker to identify patients suitable for CDK4/6 inhibitors (Haricharan *et al*. 2017). In a window of opportunity trial in patients with early stage breast cancer, palbociclib has been shown to downregulate an E2F signature associated with letrozole resistance, suggesting that this signature could be used to identify high-risk patients who should receive adjuvant CDK4/6 inhibitors in combination with endocrine therapy (Guerrero-Zotano *et al*. 2018). Finally, a non-canonical function of palbociclib is proteosomal inhibition via the ECM29 protein. High ECM29 is predictive of poorer relapse-free survival in patients receiving endocrine therapy, and thus, CDK4/6 inhibitor therapy may also prove particularly effective for these patients (Miettinen *et al*. 2018).

Biomarkers of response to CDK4/6 inhibitors for ER+ breast cancer

Patients with advanced ER+ breast cancer are pre-selected for CDK4/6 inhibitor therapy on the basis that these cancers generally have an intact Rb axis, and indeed the incidence of Rb gene deletion/mutation is very rare (3.9%) in ER+ breast cancer (Ciriello *et al*. 2015). Studies in pre-clinical models demonstrate a requirement of intact Rb for effective CDK4/6 inhibition, supporting its utility as a biomarker (Konecny *et al*. 2011, Thangavel *et al*. 2011). This finding has also been validated clinically (Karakas *et al*. 2016, Hunt *et al*. 2017). In an examination of the effects of palbociclib on unselected *ex vivo* tumour explant breast cancer models (Dean *et al*. 2012), the 2 of 13 models that were insensitive to palbociclib lacked Rb expression. Studies in glioblastoma cell lines (Michaud *et al*. 2010) and pancreatic cancer patient-derived xenograft (PDX) models have reached similar conclusions (Chou *et al*. 2017), with even the low expression of Rb sufficient to confer insensitivity to CDK4/6 inhibitors (Chou *et al*. 2017). The use of Rb as a biomarker of CDK4/6 inhibitor response in ER+ breast cancer could be further refined in combination with low molecular weight cyclin E1 (LMWE). LMWE is a promiscuous cytoplasmic cyclin E1 fragment (Karakas *et al*. 2016, Hunt *et al*. 2017) that when complexed with CDK2 can phosphorylate Rb in the presence of CDK4/6 inhibition (Doostan *et al*. 2017). Patients with Rb-/LMWE+ cancers had the shortest PFS in a cohort of 109 patients treated with palbociclib and endocrine therapy (Vijayaraghavan *et al*. 2017).

Complicating the use of Rb as a biomarker is the fact that studies of other cancer types suggest that functional Rb is not an absolute requirement for CDK4/6 inhibitors to demonstrate an effect. In bladder cancer models, palbociclib was found to be as effective in Rb-mutant models as it was in Rb wild-type models, and transcriptome analysis identified FOXM1 as the likely target in the context of Rb mutation (Castellano *et al*. 2016). Conversely, the presence of Rb does not guarantee a response to CDK4/6 inhibition, as demonstrated by the poor outcomes of a recent phase II study of Rb+ patients with advanced oesophageal or gastric cancer (Karasic *et al*. 2018).

Whether or not Rb is absolutely required for CDK4/6 inhibitor functionality, it is regarded as the canonical target, and other indicators of intrinsic sensitivity are based on their relationship with Rb. The loss of $p16^{INK4A}$ is postulated to be a marker of sensitivity as this protein inhibits cyclin D1 (Witkiewicz *et al*. 2011). This is supported by two reported cases with homozygous deletion of the $p16^{INK4A}$ gene *CDKN2A*, a collecting duct carcinoma and a uterine leiomyosarcoma, where both were exceptional responders to palbociclib treatment (Elvin *et al*. 2017, Pal *et al*. 2017). This observation has not been borne out in other studies. Low $p16^{INK4A}$ did not predict response in a phase II study of palbociclib monotherapy in Rb+ breast tumours (DeMichele *et al*. 2015) or in the PALOMA-1 study of palbociclib with letrozole for ER+ breast cancer. In PALOMA-1, the treatment of patients with tumours harbouring *CDKN2A* loss had similar PFS compared to unselected patients when assessing combination palbociclib and letrozole treatment versus letrozole monotherapy (Finn *et al*. 2015). A potential explanation of the difference between the exceptional responders and the patients of the PALOMA-1 trial may be that the PALOMA-1 patients were selected for loss of heterozygosity rather than homozygous deletion.

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Conversely, elevated $p16^{INK4A}$ could be associated with reduced CDK4/6 inhibitor sensitivity as $p16^{INK4A}$ is often elevated in the absence of functional Rb (Witkiewicz *et al.* 2011). For example, Rb-/$p16^{INK4A}$ elevated tumours had reduced sensitivity in the study of explant breast cancers (Dean *et al.* 2012). The NeoPalAna trial (neoadjuvant anastrozole with or without palbociclib) identified another INK4 protein, $p19^{INK4D}$, which was elevated in gene-expression analysis across patients with tumours intrinsically resistant to palbociclib (Hunt *et al.* 2017). One possible explanation may be that $p19^{INK4D}$ is transcribed downstream of E2F activity due to non-functional Rb, and further E2F targets, cyclin D3 and *CDKN2D* were also identified in the patients with refractory tumours. The same group subsequently identified that another E2F target, thymidine kinase 1, may be useful as a pharmacodynamic biomarker to monitor the initial patient response to CDK4/6 inhibitors (Bagegni *et al.* 2017).

The PALOMA-1 study assessed *CCND1* amplification as a biomarker for use of CDK4/6 inhibitors, but patients receiving palbociclib plus letrozole showed the same improvement regardless of *CCND1* amplification status (Finn *et al.* 2015). However, a systematic screen of 560 cancer cells identified cell lines that were highly sensitive to abemaciclib had high cyclin D-CDK4/6 function or 'D-cyclin-activating features' (Gong *et al.* 2017). Notably absent from these was *CCND1* amplification, but instead it included *CCND2*/*CCND3* amplification, *CCND1* translocation or 3′UTR loss, and loss of *FBX031*, which drives the turnover of cyclin D1. While this study confirmed the important role of cyclin D1 in CDK4/6 inhibitor function, it did not identify a suitable single biomarker for CDK4/6 inhibitor sensitivity, including cyclin D1 itself.

With the dearth of suitable single biomarkers for CDK4/6 inhibitor sensitivity, researchers are now assembling signatures of sensitivity. The 'D-cyclin-activating features' signature described above is one such example, and a similar approach is the RBsig signature, a gene expression signature of Rb loss of function derived from E2F1 and E2F2 expression in breast cancers that can predict cell lines with sensitivity to palbociclib (Malorni *et al.* 2016). An alternative approach is a gene signature for inactive CDK4, which predicted insensitivity to palbociclib across different cell lines (Raspe *et al.* 2017). This 11 gene expression signature is currently being validated in the NeoRHEA phase II trial (NCT03065621), in biopsies before treatment and following four cycles of neo-adjuvant, pre-operative treatment with palbociclib and endocrine therapy.

The emerging tide of CDK4/6 inhibitor resistance in ER+ breast cancer

The clinical trials on CDK4/6 inhibitors, while highly successful at increasing PFS, have universally yet to demonstrate significant improvement in overall survival. This has slowed the uptake of CDK4/6 inhibitors worldwide, as health economic analyses conclude that the current high costs lead to poor cost-effectiveness ratios for CDK4/6 inhibitor use (Mamiya *et al.* 2017). Despite this, governments are still being lobbied to support their cost, as their advantages of being orally available with a relatively low toxicity profile means that they provide a tangible improvement in quality of life for patients with advanced ER+ breast cancer. The development of resistance to CDK4/6 inhibitors is inevitable (Xu *et al.* 2017, Condorelli *et al.* 2018). In the reported trials that have led to FDA and EMA approval, at least 1/3 of patients recurred on CDK4/6 inhibitors within 2 years, and in the PALOMA-2 trial >70% of patients treated with the palbociclib plus letrozole had progressive disease by 40 months (Finn *et al.* 2016).

Current knowledge of the molecular mechanisms of CDK4/6 inhibitor resistance is far from complete and is largely based in single agent studies using cell line models. However, several studies have pointed to the acquisition of resistance being a multi-step process: first cells can undergo adaptive changes that may affect durability of response, and this is later followed by long-term acquisition of hard-wired resistance mechanisms (Herrera-Abreu *et al.* 2016, Martin *et al.* 2017). An important factor in considering these pre-clinical findings is that CDK4/6 inhibition in ER+ breast cancer will predominantly occur in the context of endocrine therapy treatment and/or resistance (Fig. 2).

Short-term adaptation to CDK4/6 inhibitors

CDK4/6 inhibition results in an immediate and profound G_0/G_1 cell cycle arrest in Rb+ cells (Fry *et al.* 2004), but in some cancer models, this wanes within several days. This 'adaptive response' is postulated to play a role in the acquisition of resistance or at least in the durability of therapeutic response. In ER+ breast cancer cell lines acutely exposed to palbociclib, cell cycle inhibition was temporary; Rb phosphorylation and markers of S phase entry returned within 72 h of first exposure, including increased expression of cyclin D1 (Herrera-Abreu *et al.* 2016). Non-canonical complexes of cyclin D1 and CDK2 were observed and proposed to be the cause of continued Rb phosphorylation. Increased CDK2 activity was also

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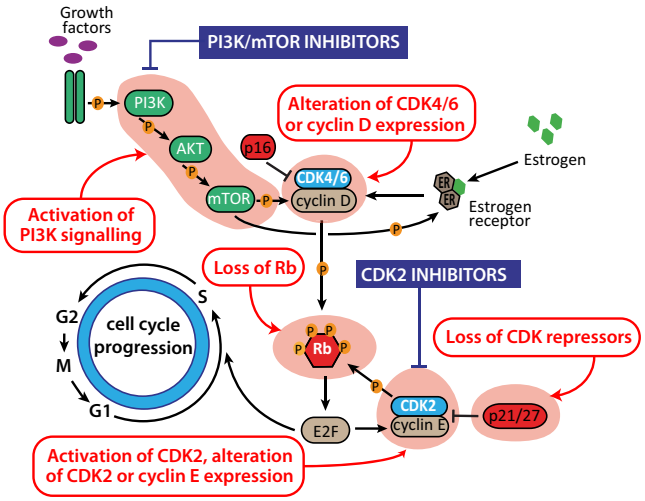

Figure 2
Mechanisms of CDK4/6 inhibitor resistance. The key mechanisms that have so far been implicated in the development of resistance to CDK4/6 inhibitors are highlighted. Possible targets for intervention with currently available drugs or drugs in development are shown.

observed in acute myeloid leukaemia cells after 96 h of palbociclib treatment, and in this model, the increase in CDK2 activity correlated with a decrease in the $p27^{Kip1}$ inhibitor protein (Wang *et al.* 2007).

Interestingly, combination therapies have been shown to inhibit this adaptive response. In breast cancer cells, the addition of a PI3K inhibitor to palbociclib delayed the resumption of S phase entry and abrogated the accumulation of cyclin D1, consistent with the role of the PI3K pathway in promoting cyclin D1 expression (Herrera-Abreu *et al.* 2016). In an independent study, mTOR pathway inhibition synergised with CDK4/6 inhibition to prevent resumption of proliferation of breast cancer cells, and the combination therapy induced a significant downregulation of E2F target genes (Michaloglou *et al.* 2018). Finally, endocrine therapy co-treatment with CDK4/6 inhibitors is able to suppress the activation of cell metabolism and cell growth in breast cancer cells (Knudsen & Witkiewicz 2016).

CDK4/6 inhibition mediates cell senescence, as defined by a prolonged proliferation arrest in combination with molecular markers such as β-galactosidase (Bollard *et al.* 2017). A major CDK4/6 target is FOXM1 (Anders *et al.* 2011), and when CDK4/6 is inhibited, the hypo-phosphorylated forms of FOXM1 promote a program of senescence (Sharpless & Sherr 2015). Consequently, the durability of response to CDK4/6 inhibition may affect whether or not cells become senescent with treatment. The program of senescence induced by CDK4/6 inhibitors can be augmented through co-treatment to inhibit other pathways. For example, reduced mTOR signalling can augment entry into senescence induced by CDK4/6 inhibition (Yoshida *et al.* 2016) and autophagy inhibitors in combination with CDK4/6 inhibitors can augment senescence (Karakas *et al.* 2016, Hunt *et al.* 2017, Valenzuela *et al.* 2017).

Effective induction of senescence by CDK4/6 inhibitors could potentially avert long-term resistance, but equally, it appears that the induction of a G_1 arrest without senescence allows for better synergy with other classes of therapies. For example, palbociclib treatment of melanoma cells for more than 3 days led to the induction of senescence in association with a decreased sensitivity to vemurafenib (Yoshida *et al.* 2016). Likewise, Rb-expressing sarcoma cell lines with reversible palbociclib-induced cell cycle arrest were sensitive to co-treatment with the WEE1 kinase inhibitor AZD1775 (Francis *et al.* 2017). Unfortunately, CDK4/6 inhibitor-induced senescence may also result in an undesirable outcome in the stroma through the promotion of a proinflammatory, senescence-associated secretory phenotype (SASP). This SASP phenotype could augment insensitivity to CDK4/6 inhibitors: when palbociclib-induced senescent fibroblasts were co-injected with melanoma cells into an immune-proficient, syngeneic mouse model, it resulted in accelerated tumour growth (Guan *et al.* 2017).

Long-term acquisition of CDK4/6 inhibitor resistance

Prolonged exposure to CDK4/6 inhibitors eventually gives rise to resistant cell populations that undergo hard-wired changes that are distinct to 'the adaptive response'. A handful of mechanisms have been described in this context, including the loss or mutation of Rb, changes to CDK4/6 and CDK2 signalling and activation of growth signalling pathways. So far, no reports of mutations in either *CDK4* or *CDK6* that reduce the affinity of CDK4/6 inhibitors have emerged. However, this remains a plausible route for the development of resistance and with the advent of CDK4/6i now being standard of care in the first-line setting, it is likely that previously undescribed mechanisms of CDK4/6i resistance will begin to emerge in the clinic.

Loss or mutation of Rb

By far the most frequently observed change in cells resistant to CDK4/6 inhibitors is loss or mutation of the Rb protein. This is observed in multiple cell line models from different tumour types (Taylor-Harding *et al.* 2015, Herrera-Abreu *et al.* 2016, Bollard *et al.* 2017).

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Of particular interest is a PDX model exposed to chronic ribociclib treatment that developed partial treatment resistance concurrent with the clonal expansion of a pre-existing Rb-null population (Herrera-Abreu *et al*. 2016). Interestingly, the parental PDX model was derived from a patient who had been previously treated with endocrine therapy and harboured an activating ESR1 mutation (Y537S), a mutation in *TP53* and loss of genes encoding $p16^{INK4A}$, $p15^{INK4B}$ and $p14^{ARF}$. Despite these changes, presumably acquired in response to endocrine therapy, the model responded initially to palbociclib treatment. Following on from these pre-clinical results, the first examples of putative attenuation of Rb function through the emergence of potentially deleterious Rb mutations in ctDNA, acquired during the development of resistance to CDK4/6 inhibitors in patients, have begun to emerge (Xu *et al*. 2017, Condorelli *et al*. 2018).

Activation of CDK4/6 signalling

Another mechanism of resistance to CDK4/6 inhibition is the upregulation of CDK4 or CDK6 and their cognate cyclins. While CDK6 amplification has been reported (Yang *et al*. 2017), CDK4 amplification has not been detected in resistance models. Both high and low expression of CDK4 has been noted in resistance models (Bollard *et al*. 2017, Martin *et al*. 2017). This may be because while some expression of CDK4 or CDK6 is required for sensitivity to CDK4/6 inhibition, high level amplification of CDK4 on the other hand, as seen in rhabdomyosarcoma, can lead to reduced sensitivity to palbociclib (Olanich *et al*. 2015). Cyclins D1 and D2 are also upregulated in models of CDK4/6 inhibitor resistance (Taylor-Harding *et al*. 2015, Jansen *et al*. 2017, Martin *et al*. 2017) and high cyclin D3 was observed in patients with ER+ tumours that did not respond to palbociclib (Hunt *et al*. 2017). Resistance could occur by either non-canonical activation of CDK2 (Herrera-Abreu *et al*. 2016) or through formation of cyclin D3-CDK4/6 complexes, which appear to phosphorylate Rb even in the presence of synthetic CDK4/6 inhibitors (Paternot *et al*. 2014).

Activation of CDK2 signalling

In normally cycling cells, cyclin E-CDK2 (cyclin E1-CDK2 or cyclin E2-CDK2) complexes phosphorylate Rb subsequent to phosphorylation by cyclin D-CDK4/6, as part of a second wave of signalling. CDK4/6 inhibition has multiple inhibitory effects on CDK2 action. Without the priming of Rb by cyclin D1-CDK4/6 phosphorylation, endogenous levels of cyclin E-CDK2 complexes cannot efficiently phosphorylate Rb to release E2F transcription factors. Cyclin E2 is a transcriptional target of E2F, and hence cyclin E2-CDK2 complexes are also reduced after CDK4/6 inhibition (Caldon *et al*. 2009). Finally, cyclin E1-CDK2 complexes probably have suppressed activity from the redistribution of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ inhibitors after depletion of cyclin E2-CDK2 complexes (Caldon *et al*. 2009). In this context, the upregulation of cyclins E1 or E2, or downregulation of their inhibitors can subvert CDK4/6 inhibition.

Cyclin E1, cyclin E2 and CDK2 are upregulated in CDK4/6 inhibitor resistance models (Taylor-Harding *et al*. 2015, Herrera-Abreu *et al*. 2016, Bollard *et al*. 2017, Martin *et al*. 2017, Yang *et al*. 2017). Mechanistically this can occur through *CCNE1* gene (which encodes cyclin E1) amplification in a single agent CDK4/6 resistant model (Herrera-Abreu *et al*. 2016), and *CCNE2* gene (which encodes cyclin E2) amplification in a combination endocrine therapy/palbociclib resistance model (Martin *et al*. 2017). Ablation of either cyclin E1 or CDK2 resensitised resistant cells to palbociclib-induced cell cycle arrest (Herrera-Abreu *et al*. 2016). There are currently no specific CDK2 inhibitors available for clinical use, but newer CDK2 inhibitors currently in development could potentially have a role in CDK4/6 inhibitor-resistant tumours (Caldon *et al*. 2012).

Growth factor signalling pathways

Rb, CDK4/6 and CDK2 are all conduits for growth regulatory signalling pathways to upregulate cell growth and cell cycle progression, and it is not surprising that several signalling pathways can be deregulated to overcome CDK4/6 inhibitors. A kinome-wide siRNA screen identified the PI3K pathway kinase, PDK1, was highly expressed in ribociclib-resistant cells, and sensitised cells to ribociclib (Jansen *et al*. 2017). While no changes in mTOR signalling components have been documented in long-term resistance models, CDK4/6 inhibitor-resistant cell lines have demonstrated sensitivity to mTORC1/2 inhibition (Michaloglou *et al*. 2018). Other signalling changes include *NRAS* amplification or mutation in an NRAS model of melanoma co-treated with MEK1 inhibition and CDK4/6 inhibition (Teh *et al*. 2018), and alterations in FGF/FGFR signalling in resistance to CDK4/6 inhibitors both alone and in combination with endocrine therapy (Cruz *et al*. 2018, Formisano *et al*. 2018, Mao *et al*. 2018, Shee *et al*. 2018). Finally, an activating mutation in $PIK3CA^{E545K}$, was shown to display a resistance phenotype to combination

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Table 3 Clinical trials with CDK4/6 inhibitors in combination with inhibitors of PI3 kinase pathway and endocrine therapy in advanced HR+HER2- breast cancer.

NCT number	Phase	Estimated/actual participants	PI3K pathway target	PI3K pathway inhibitor	Stage	Endocrine therapy/CDK4/6 inhibitor backbone
03006172	I	156	PI3 Kinase	GDC-0077	Advanced	AI, fulvestrant/palbociclib
02154776	I	13	PI3 Kinase	Buparlisib	Advanced	AI/ribociclib
01872260	Ib	253	PI3 Kinase	Alpelisib	Advanced	AI/ribociclib
02684032	I	120	PI3 Kinase/mTOR	Gedatolisib	Metastatic	AI, fulvestrant/palbociclib
01655225*	I	130	PI3 Kinase/mTOR/DNA-PK	LY3023414	Advanced	AI, fulvestrant/abemaciclib
02057133*	Ib	198	PI3 Kinase mTOR/DNA-PK	LY3023414 Everolimus	Metastatic	AI, fulvestrant/abemaciclib
01857193	Ib	132	mTOR	Everolimus	Advanced	AI/ribociclib
03128619	I, II	102	PI3 Kinase	Copanlisib	Stage I–IV	AI/palbociclib
02088684	Ib, II	70	PI3 Kinase	Alpelisib and buparlisib	Advanced	Fulvestrant/ribociclib
02732119	I, II	51	mTOR	Everolimus	Advanced	AI/ribociclib
02599714	I, II	54	mTORC1/2	Vistusertib	Metastatic	Fulvestrant/palbociclib
02871791	Ib, II	32	mTOR	Everolimus	Metastatic	AI/palbociclib

*HR+/HER2– breast cancer arms as part of a larger study.
AI, aromatase inhibitor.

MEK and CDK4/6 inhibitors, and the outgrowth of a clone expressing this mutation caused recurrence in a melanoma patient treated with these inhibitors (Romano *et al.* 2018).

Strategies to avert and overcome CDK4/6 inhibitor resistance

The most frequently detected mechanism of resistance, loss or mutation of Rb, is unfortunately not amenable to targeted therapy. In this situation chemotherapy may be a renewed option, as Rb disruption sensitises tumours to chemotherapy (Zagorski *et al.* 2007, Witkiewicz *et al.* 2012), although the efficacy of this is very dependent upon the combination of tumour type and drug mode of action. Also, CDK4/6 inhibitors can be antagonistic when combined with chemotherapy, especially those with an anti-mitotic effect (Franco *et al.* 2014, Yoshida *et al.* 2016), and therefore a cautious approach should be taken with such combinations. As more patients present clinically, these therapies can be systematically assessed to determine if they prolong survival effectively.

The clinical efficacy of endocrine therapy doublet combination therapy with mTOR, PI3K, and CDK4/6 inhibitors (Baselga *et al.* 2012, Finn *et al.* 2015, Cristofanilli *et al.* 2016, Finn *et al.* 2016, Baselga *et al.* 2017, Goetz *et al.* 2017, Sledge *et al.* 2017, Slamon *et al.* 2018), and the cross talk between these pathways (Fig. 2), have led to the logical development of clinical trials of triplet therapy combinations of CDK4/6 and PI3K pathway inhibitors with an endocrine therapy backbone (Table 3). Further supporting this strategy is data that has demonstrated that CDK4/6 inhibitors can sensitize PIK3CA mutant tumours to PI3K inhibitors (Vora *et al.* 2014), and the converse, that CDK4/6 resistant cells have been shown to be sensitive to mTORC1/2 inhibition (Michaloglou *et al.* 2018). Finally, combined targeting of CDK4/6 and PI3K pathways resulted in greater tumour regression compared with PI3K or CDK4/6 inhibition alone; and triplet therapy with CDK4/6 and PI3K inhibitors was more effective than dual therapy with respect to tumour regression (O'Brien *et al.* 2014, Herrera-Abreu *et al.* 2016). In two recent studies, cohorts of heavily pre-treated patients who had received everolimus obtained limited benefit from the addition of palbociclib, suggesting that CDK4/6 inhibitors should be used prior to or concurrently with drugs targeting the PI3K pathway (Dhakal *et al.* 2018, du Rusquec *et al.* 2018). The multiple types of PI3K inhibitors in clinical development, combined with the three lead CDK4/6 inhibitors, multiple classes of ER-directed therapies, and next generation selective ER degraders, in different lines of therapy, have resulted in a large number of combinations and permutations, creating a challenge when determining the optimal therapeutic strategy for a given patient. Further complicating matters are the potential for overlapping toxicity and financial implications.

Finally, CDK4/6 inhibitors have also been shown to enhance both tumour antigen presentation, T cell activation and the efficacy of anti-PD-1 immunotherapy,

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Table 4 Ongoing clinical trials of CDK4/6 inhibitors in early stage breast cancer.

Study	NCT no.	Estimated enrolment	Breast cancer subtype	Treatment stage	Treatment	Primary outcome measured
Phase III						
SAFIA	03447132	400	HR+ HER2–	Neo-adjuvant	Palbociclib + fulvestrant vs placebo + fulvestrant	pCR rate
PALLAS	02513394	5600	HR+ HER2–	Following neo-adjuvant chemotherapy	Palbociclib + ET vs ET	IDFS
PENELOPE-B	01864746	1250*	HR+	Following neo-adjuvant chemotherapy	Palbociclib + ET vs placebo	IDFS
EarLEE-1	03078751	52*	HR+ HER2– (high risk)	Adjuvant	Ribociclib + ET vs placebo + ET	IDFS
monarchE	03155997	3580	HR+ HER2– (high risk)	Adjuvant	Abemaciclib + ET vs ET	IDFS
Phase II						
NEOBLC	03283384	100	HR+ HER2–	Neo-adjuvant, stage II or III	AI followed by chemotherapy vs AI + ribociclib	CCCA
neoMONARCH	02441946	224*	HR+ HER2–	Neo-adjuvant	AI vs abemaciclib vs AI + abemaciclib	Change in Ki67 expression
PALLET	02296801	306	HR+ HER2–	Neo-adjuvant	AI vs AI then AI + palbociclib vs palbociclib then AI + palbociclib vs AI + palbociclib	Change in Ki67 expression and cCR

AI, aromatase inhibitor; CCCA, complete cell cycle arrest; cCR, clinical complete response; ET, endocrine therapy; HR, hormone receptor; IDFS, invasive disease-free survival; pCR, pathological complete response. * actual enrolment

representing another potential therapeutic combination for this class of drug (Goel *et al.* 2017, Deng *et al.* 2018).

The future of CDK4/6 inhibitor use and resistance in ER+ breast cancer

The addition of CDK/6 inhibitors into contemporary treatment algorithms for advanced ER+ breast cancer represents a renaissance for the most common subtype of breast cancer and represents the most significant advance in the last decade. While CDK4/6 inhibitors have changed the natural history of ER+ breast cancer by prolonging the PFS, when used in the metastatic context, disease progression and the emergence of resistance is inevitable. As they become standard therapy, combined resistance to endocrine and CDK4/6 inhibitor therapy represents the next wave of clinical challenge to face the breast cancer community. To benefit these patients, we need a detailed mechanistic understanding of CDK4/6 inhibitor resistance in an endocrine sensitive and resistant setting.

The full potential of CDK4/6 inhibitors has yet to be realised, and trials are currently underway to expand its use to other breast cancer subtypes, earlier stages of disease and other cancers. With the success of CDK4/6 inhibitors in advanced ER+ breast cancer, it is now logically being evaluated in early stage ER+ breast cancer. A series of phase II and phase III adjuvant and neoadjuvant trials with CDK4/6 inhibitors are currently underway (NEOLBC (NCT03283384), neoMONARCH (NCT02441946), PALLET (NCT02296801), SAFIA (NCT03447132), monarchE (NCT03155997) and PALLAS (NCT02513394)) (Table 4). The exclusion of HER2+ patients is also being reassessed after the positive results of the abemaciclib monotherapy trial (Patnaik *et al.* 2016), where 36% (4/11) of ER+ HER2+ patients showed a response compared to 28% (7/25) of ER+ HER2- patients, and several trials such as PATINA (NCT02947685), PATRICIA (NCT02448420) and monarcHER (NCT02675231) are evaluating combinations of CDK4/6 inhibitors, anti-HER2 (trastuzumab, pertuzumab) and endocrine therapy. These emerging treatment scenarios will have different genetic backgrounds and selective drug pressures, potentially giving rise to unique resistance mechanisms in each treatment type.

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