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Cotargeting of CYP-19 (aromatase) and emerging, pivotal signalling pathways in metastatic breast cancer

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Aromatase inhibition is one of the cornerstones of modern endocrine therapy of oestrogen receptor-positive (ER+) metastatic breast cancer (MBC). The nonsteroidal aromatase inhibitors anastrozole and letrozole, as well as the steroidal aromatase inactivator exemestane, are the preferred drugs and established worldwide in all clinical phases of the disease. However, although many patients suffering from MBC experience an initial stabilisation of their metastatic burden, drug resistance and disease progression occur frequently, following in general only a few months on treatment. Extensive translational research during the past two decades has elucidated the major pathways contributing to endocrine resistance and paved the way for clinical studies investigating the efficacy of novel drug combinations involving aromatase inhibitors and emerging drugable targets like mTOR, PI3K and CDK4/6. The present review summarises the basic research that provided the rationale for new drug combinations involving aromatase inhibitors and the main findings of pivotal clinical trials that have already started to change our way to treat hormone-sensitive MBC. The challenging situation of oestrogen receptor-positive and human epidermal growth factor receptor 2-positive (HER2+) MBC is also shortly reviewed to underline the complexity of the clinical scenario in the heterogeneous subgroups of hormone receptor-positive breast cancer patients and the increasing need for personalised medicine. Finally, we summarise some of the promising findings made with the combination of aromatase inhibitors with other potent endocrine treatment options like fulvestrant, a selective oestrogen receptor downregulator.

Following several decades with antioestrogen dominance concerning first line-therapy in oestrogen receptor-positive (ER+) metastatic breast cancer (MBC), aromatase inhibitors (AIs) of the third generation (letrozole, anastrozole, exemestane) became the preferred drugs in this setting approximately two decades ago (Geisler and Lønning, 2005). The pivotal trials comparing tamoxifen with letrozole, anastrozole or exemestane as first-line therapy in the metastatic setting are summarised elsewhere and are not given in details here (Gibson *et al*, 2009). Meanwhile, AIs of the third generation have been established as one of the preferred choices in all clinical phases of ER+ MBC as well as in the neoadjuvant and adjuvant setting for selected groups of patients.

Many patients with ER+ MBC experience an initial stabilisation or even regression of their metastatic burden during first-line

therapy with an AI. However, in the majority of patients, endocrine resistance and disease progression occurs frequently, following only a few months on therapy. Thus, intensive research during the past two decades has elucidated several mechanisms contributing to tumour adaptation during aromatase inhibition and oestrogen suppression (Dowsett *et al*, 2005; Ma *et al*, 2015). Some of the signalling pathways involved in the development of endocrine resistance include targetable molecules like the kinase mammalian target of rapamycin (mTOR), the phosphatidylinositol-3-kinase (PI3K) and the cyclin-dependent kinases 4 and 6 (CDK4/6). Development of novel drugs targeting key players in the signalling intracellular cascades paved the way for new studies testing these novel compounds given in combination with AIs compared with more traditional AI monotherapy.

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The aim of the present paper is to summarise the experience made hitherto with the most promising new drugs that have proven clinical efficacy in combinations with AIs, as well as to review novel agents that are currently tested in ongoing early-phase clinical trials. The challenging situation of ER+ and human epidermal growth factor receptor 2-positive (HER2+) MBC is also shortly reviewed to underline the complexity of the clinical scenario in the heterogeneous subgroups of ER+ breast cancer patients and the increasing need for personalised medicine.

AROMATASE INHIBITORS VS AROMATASE INACTIVATORS

Nonsteroidal AIs act as competitive, reversible CYP-19/aromatase inhibitors, whereas steroidal AIs act as irreversible ('suicide') inactivators of aromatase, causing decomposing of the aromatase molecule after binding to the inactivator (Hong and Chen, 2006). Many compounds with aromatase inhibitory effects have been tested in MBC patients during the past three decades (Geisler, 2003). Meanwhile, the orally administered compounds belonging to the third generation are currently used worldwide in the majority of countries. The drugs are classified as either nonsteroidal AIs (triazoles like anastrozole and letrozole) or steroidal aromatase inactivators (exemestane) because of their molecular structures and mode of action (Figure 1). Importantly, a lack of cross-resistance has been documented between these two major groups of aromatase inhibitory compounds, allowing sequential use in the metastatic setting (Bertelli *et al*, 2005; Lønning, 2009; Beresford *et al*, 2011; Van Asten *et al*, 2014). However, the precise mechanisms explaining the clinically observed lack of cross-resistance have not been fully clarified yet. Some of the possible explanations for this important clinical phenomenon have been discussed elsewhere (Lønning, 2009).

MECHANISMS OF RESISTANCE TO AROMATASE INHIBITORS

Breast cancer tumours are highly heterogeneous showing early subclonal development because of a variety of mutations and

activated pathways that are only incompletely understood (Yates *et al*, 2015). So far, we know that most of the tumour cells become resistant to AIs after exposure for a given time (secondary or acquired resistance). However, primary or 'de novo' resistance towards AIs has been reported to occur in up to 30% of ER+ MBC patients (Ma *et al*, 2015). An important goal of ongoing research is therefore to identify these nonresponding patients before therapy or early during therapy in order to either avoid nonfunctional therapies or to adapt treatment to the underlying tumour biology from the start. To predict the latter and at the same time find out how we may treat women who experience disease progression on AI treatment, we need to investigate the individual mechanisms that are responsible for endocrine resistance in a particular patient. In the following chapters we will briefly summarise some of the best documented mechanisms that are likely to cause resistance to AIs in breast cancer patients, as these mechanisms guided the way for the development of potentially meaningful new drug combinations.

Activation of growth factor receptors (GFRs) and bidirectional crosstalk between the epidermal growth factor receptor family (EGFR/HER1, HER2, HER3, HER4) and ER α (Figure 2) has been associated with both *de novo* and acquired resistance to AIs (Gee *et al*, 2005; Lupien *et al*, 2010). Other growth factors besides the EGFR family are the insulin-like growth factor receptor-1 (IGFR-1), fibroblast growth factor receptor 1 (FGFR1) and their downstream signalling pathways, including the PI3K/Akt and the mitogen-activated protein kinase (MAPK) pathway (Liu *et al*, 2014; Ma *et al*, 2015). Activation of these tyrosine kinases may induce downstream effects like phosphorylation of ER α and alterations in the recruitment of its co-regulators. Overexpression of GFR signalling through EGFR or HER2 may also lead to activation of MAPK in ER+ breast cancer, causing loss of ER α expression (Ma *et al*, 2015). Primary significant overexpression of HER2 has been observed in as many as 10–15% of MBC and is associated with an increased metastatic potential, decreased disease-free and overall survival (OS) rates as well as early onset of endocrine resistance (Fan *et al*, 2015). Increased HER2 expression has been seen in sequential tumour biopsies from breast cancer patients after initiation of neoadjuvant AI therapy (Flågeng *et al*, 2009). This may indicate that AI therapy somehow triggers the expression of HER2, representing one of the many mechanisms causing acquired resistance. Additional inhibition of the HER2 pathway may reactivate ER α and sensitise the tumour to antihormonal therapies (Ma *et al*, 2015).

The PI3K/Akt/mTOR pathway (Figure 2) is another pivotal and frequently altered signalling pathway in human breast cancer. Studies have shown that at least 20–50% of all breast cancer tumours harbour mutations in PI3K (Sanchez *et al*, 2011; Lopez-Knowles *et al*, 2014), with mutations of the catalytic subunit of PI3K (PIK3CA) being the most common (Miller *et al*, 2011; Geuna *et al*, 2015). There is a growing body of evidence suggesting that alterations of PI3K play a crucial part in the development of acquired resistance towards AIs by regulating the ER α expression (Ma *et al*, 2015). This has caused considerable efforts to synthesise selective PI3K inhibitors for clinical use (Bendell *et al*, 2012; Liu *et al*, 2013; Ndubaku *et al*, 2013; Ando *et al*, 2014; Mayer *et al*, 2014; Rodon *et al*, 2014; James *et al*, 2015; Sarker *et al*, 2015; Wang *et al*, 2015; Massacesi *et al*, 2016). Further downstream along this pathway, we find an additional drug target, the kinase mTOR, that is essential to several intracellular signalling pathways promoting cell proliferation, differentiation, metabolism, migration and survival (Alberts *et al*, 2015; Geuna *et al*, 2015). The mTOR may be activated from aberrant upstream signalling or may be altered itself. Mutation of a subunit in the mTOR1 complex may phosphorylate and thereby activate the function domain 1 of the ER (Geuna *et al*, 2015). Beyond mutations that involve activation of PI3K and mTOR specifically, the entire PI3K/Akt/mTOR

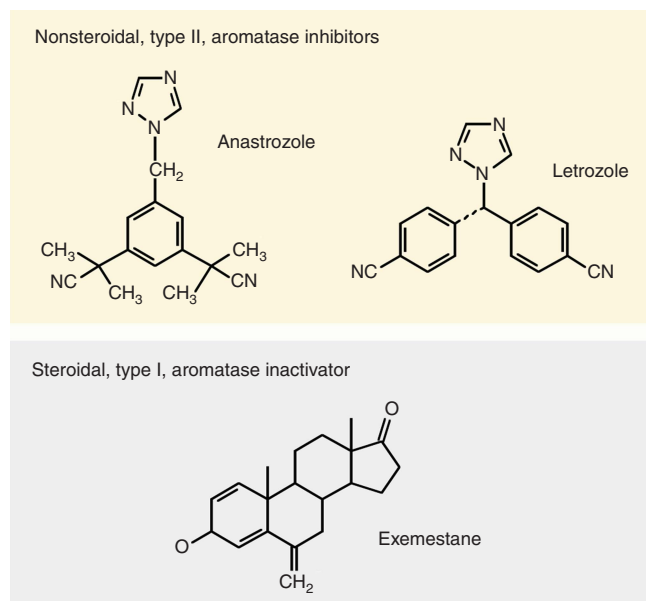


Figure 1. Chemical structures of anastrozole, letrozole and exemestane.

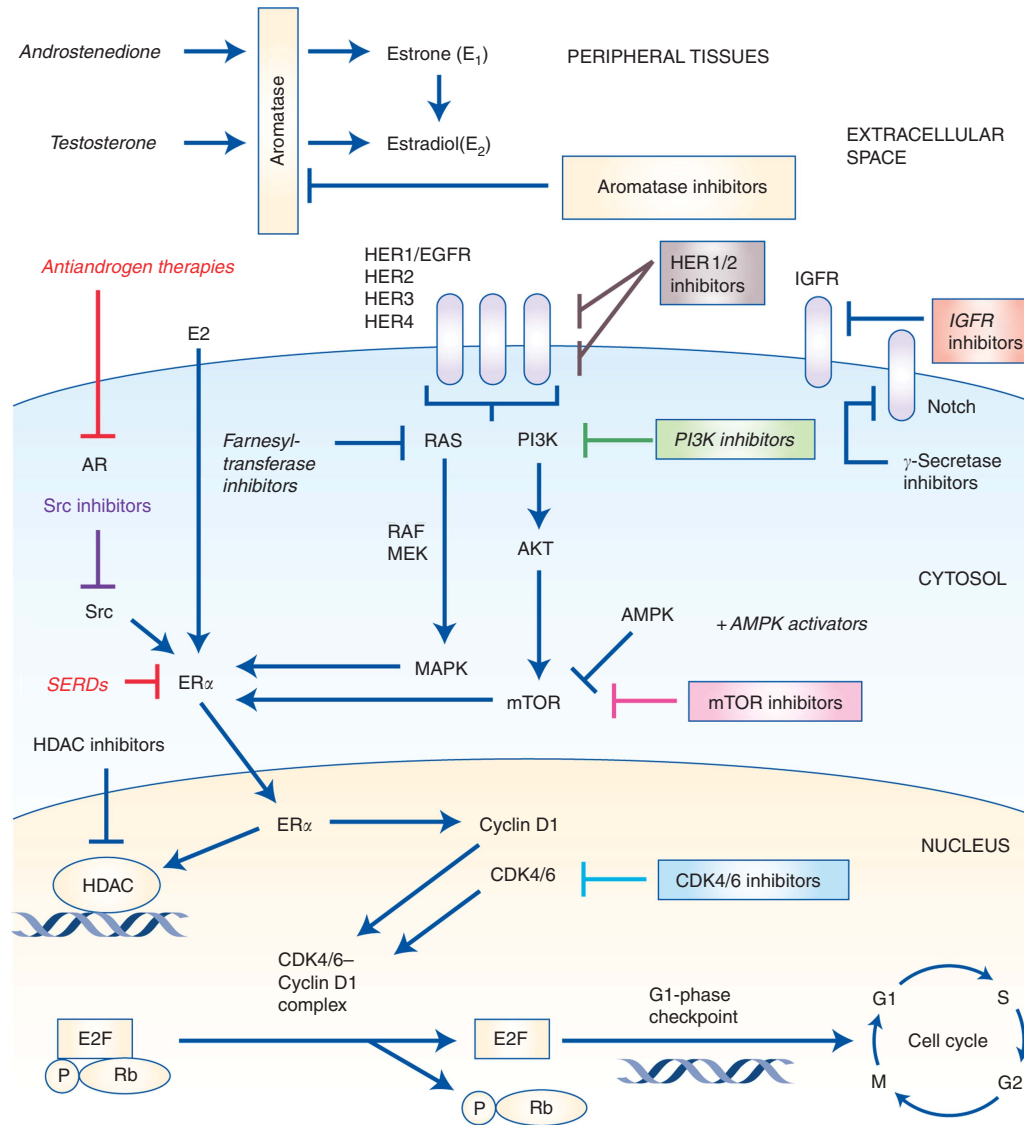


Figure 2. Pivotal intracellular signalling cascades involved in endocrine resistance and site of action for selected targeted anticancer drugs. AMPK = adenosine monophosphate-activated protein kinase; AR = androgen receptor; CDK4/6 = cyclin-dependent kinase 4/6; E2 = oestradiol; E2F = E2F transcription factors; ER α = oestrogen receptor- α ; HDAC = histone deacetylase; HER2 = human epidermal growth factor receptor-2; IGFR = insulin like growth factor receptor; P = phosphate; Rb = retinoblastoma protein; MEK = mitogen-activated ERK-activating kinase; mTOR = mammalian target of rapamycin; SERD = selective oestrogen receptor downregulator.

pathway may become abnormally activated following, for example, loss of PTEN (the pivotal negative regulator of the PI3K pathway), loss of inositol polyphosphate-4-phosphatase or mutations of Akt1 (Ma *et al*, 2015).

Altered ER signalling may also cause ligand-independent gene transcriptions and cell proliferation (Thomas and Gustafsson, 2015). Thus, it is thought to be one of the crucial mechanisms involved in endocrine resistance in general and in resistance during AI therapy in particular. The ER mutations most commonly cluster in the carboxy-terminal ligand-binding domain of oestrogen receptor 1 (ESR1), with Y537S, C or N and D538G being the most common. These mutations are rare in treatment-naïve breast cancer, but more common in MBC, ranging from 11% to 55% (Ma *et al*, 2015). The incidence of ESR1 mutations seems to increase in patients who have a progressive disease following several endocrine treatment lines including AIs. Mechanisms such as translocations, amplifications, point mutations or aberrant expression or mutations of ER co-regulators have been described (Clarke *et al*, 2015; Ma *et al*, 2015).

In addition, active androgen receptor (AR) signalling has also been suggested to contribute to endocrine resistance during therapy with nonsteroidal AIs (Rechoum *et al*, 2014). Although the AR is expressed in 50–70% of all breast cancers, it is reported to be positive in 80–90% of all ER+ BC (Park *et al*, 2010). The AR positivity and overexpression have been linked to HER2 stimulation and activation of other signalling pathways by transcriptional upregulation of AR-dependent genes (Fujii *et al*, 2014; O'Shaughnessy *et al*, 2016). The AR negativity has also been shown to predict early treatment failure during adjuvant AI therapy (Elebro *et al*, 2015). As a consequence, subgroups of breast cancer patients may benefit from treatment with antiandrogens or selective AR modulators.

Aberrant expressions of molecules involved in the cell cycle machinery have recently been associated with endocrine resistance as well (Rocca *et al*, 2014). The CDKs play an important role in the control of check points during the cell cycle (Figure 2). Their levels rise and fall in a cyclic manner throughout the cell cycle, leading to cyclical phosphorylation and dephosphorylation of intracellular

proteins that regulate important steps in the cell cycle. Of the 13 CDKs described in human cells, cyclin D is particularly important in the late G1 phase by binding CDK4/6 and thereby inducing the synthesis of cyclin E. This leads to the formation of the CDK2–cyclin E complex that in turn contributes to cell cycle progression through the restriction point and into the S phase of the cell cycle (Vidula and Rugo, 2016). Several signalling cascades induce the expression of cyclin D: Ras/Raf/MAPK, NF- κ B, Jak/STAT and steroid hormones (Alberts *et al*, 2015). The mechanism of action of the CDK4/6–cyclin D complex is phosphorylating and thus inactivating the tumour suppressor gene retinoblastoma (Rb) and its related proteins. When phosphorylated, the Rb protein relieves the inhibition of the transcription factor E2F (Figure 2), a promoter of genetic sequences encoding products that mediate S-phase entry and mitosis (Dickson, 2014; Rocca *et al*, 2014). Typical mechanisms involved in endocrine resistance at this stage are loss or phosphorylation of the Rb protein, deletion of the CDK4/6-inhibitor p16 or amplification of CDKs (Carey and Perou, 2015). Downregulation of the Rb pathway in ER+ breast cancer seems also to be associated with a more aggressive tumour growth and rapid recurrence on endocrine therapy (Cadoo *et al*, 2014; Witkiewicz and Knudsen, 2014). Loss of CDK4/6 cell cycle regulation occurs in 20–35% of breast cancer and promotes CDK2 activity, driving the cell cycle progression without the need of CDK4/6 (Vidula and Rugo, 2016). As a consequence, specific CDK4/6 inhibitors like palbociclib have no activity in Rb-deficient cells (Cadoo *et al*, 2014). The majority of ER+ breast cancer cells, however, do have intact Rb and it is therefore urgent to identify other clinically useful predictive biomarkers before treatment with CDK4/6 inhibitors (Carey and Perou, 2015).

Finally, numerous noncoding RNAs (ncRNA) have recently been shown to play a part in breast cancer progression and acquired endocrine resistance. MicroRNA (miRNA) and long non-coding RNA (lncRNA) are small molecules involved in the regulation of gene expression via translational transcription or degradation of mRNA transcripts. Overexpression of miRNAs and lncRNAs are associated with aberrant signalling and unregulated survival of cancer cells. More detailed information covering the contribution of miRNAs and so on during the development of endocrine resistance have been summarised in recent overviews and are not given here in detail (Hayes and Lewis-Wambi, 2015).

NOVEL STRATEGIES TO DELAY DRUG RESISTANCE DURING AROMATASE INHIBITOR THERAPY IN MBC

Aromatase inhibitors given in concert with drugs targeting HER1 (EGFR) and/or HER2 in MBC. One of the first strategies widely tested in clinical trials in this respect was the combination of epidermal growth factor receptor-1 (EGFR/HER1) targeting compounds and AIs (Leary and Dowsett, 2006). The European Organisation for Research and Treatment of Cancer (EORTC) recently published a randomised, multicentre phase II study comparing anastrozole monotherapy with the combination including the EGFR-inhibitor gefitinib (Tryfonidis *et al*, 2016). Disappointingly, gefitinib did not improve progression-free survival (PFS) when added to anastrozole. To the contrary, a phase II study published in 2010 compared the same combination and could show an improvement of ~6-month PFS in the combination arm (Cristofanilli *et al*, 2010). However, both studies included ER+ MBC patients without defining the EGFR (mutational) status as an inclusion criterion, resulting in minor subgroups of enrolled patients actually being EGFR mutation positive. Interestingly, EGFR T790M mutations have recently been observed in biopsies of completely untreated primary breast cancer, indicating immediate drug resistance against most of the

available EGFR targeting drugs in subgroups of MBC patients (Bemanian *et al*, 2015).

In contrast, simultaneous use of HER2 targeting drugs and AIs has been challenged in a few pivotal trials that have already changed the standard care for ER+/HER2+ stage IV breast cancer patients (Riemsma *et al*, 2012). We mention here only two pivotal phase III trials in detail. The randomised, placebo-controlled TANDEM trial (Figure 3) showed that adding trastuzumab, a recombinant IgG1 monoclonal HER2 antibody against HER2, to anastrozole significantly improved the outcome in ER+/HER2+ MBC (Kaufman *et al*, 2009). Briefly, 207 postmenopausal ER+/HER2+ patients with MBC were included and randomly assigned to receive either anastrozole with trastuzumab or single-agent anastrozole. Patients in the combination arm experienced significantly improved mean PFS of 4.8 months (compared with 2.4 months in the monotherapy group, $P=0.0016$). There were more grade 3 and 4 adverse events in the combination arm, but all side effects were reversible and none were life threatening. The second major phase III trial was published by Johnston *et al* (2009; EORTC 30008; Figure 3), where letrozole was combined with lapatinib, an orally active dual HER1/HER2 inhibitor, that works by inhibiting the domains of the intracellular tyrosine kinases of both EGFR/HER1 and HER2 receptors (Paul *et al*, 2008). The results revealed that a combined strategy of lapatinib and letrozole significantly increased PFS in patients with ER+/HER2+ MBC (Schwartzberg *et al*, 2010). A total of 1286 ER+ patients were enrolled in the study and randomly assigned to receive either letrozole and lapatinib or letrozole and placebo. Approximately 17% of patients in each arm were HER2+. In the HER2+ subgroup ($n=219$), PFS significantly increased from a mean of 3.0 to 8.2 months ($P=0.019$). Importantly, there was no improvement in PFS in the ER+/HER2- subgroup when lapatinib was added to letrozole. Finally, in preclinical models, Curley *et al* (2015) showed that HER3 signalling mediates resistance to letrozole, suggesting that MBC patients expressing HER3 may benefit from adding a specific ERBB3 (HER3) inhibitor such as the anti-ERBB3 antibody seribantumab to antihormonal therapy.

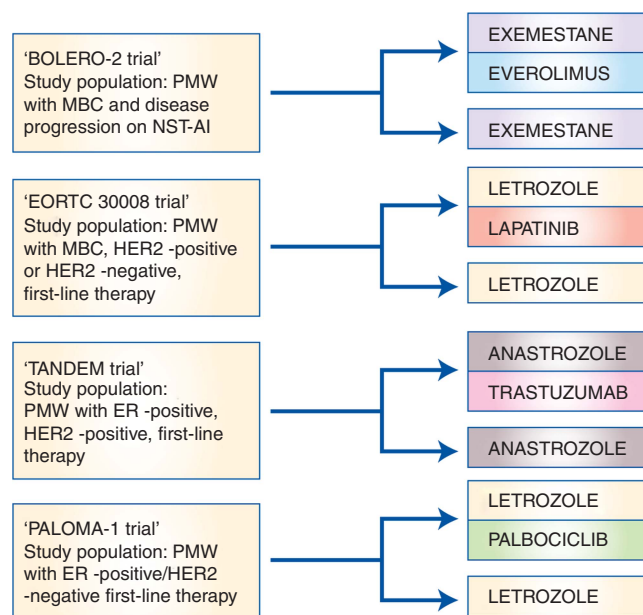


Figure 3. Design of important clinical trials comparing aromatase inhibitors alone vs aromatase inhibitors given in combinations with novel targeted therapies. MBC = metastatic breast cancer; NST-AI = nonsteroidal aromatase inhibitor; PMW = postmenopausal women.

In summary, both trastuzumab and lapatinib have been established as targeting drugs that should be combined with traditional AIs in selected, ER+/HER2+ patients. Meanwhile, ongoing trials are testing a variety of novel combinations of aromatase inhibitors and anti-HER2 targeting drugs given in concert with mTOR inhibitors or CDK4/6 inhibitors.

Aromatase inhibitors given in combination with mTOR inhibitors. Two mTOR inhibitors have been tested so far in combination with AIs: temsirolimus and everolimus. Both compounds unfold their action by binding to FKBP12, a protein receptor on the mTOR complex 1 (mTORC1) (Klumpen *et al*, 2010), a complex that has the ability to phosphorylate and thereby activate the function domain 1 of the ER. By inactivating mTORC1, mTOR inhibitors block the activation of the ER. Combining mTOR inhibitors with AIs has therefore been suggested to be a promising strategy to delay the onset of AI resistance (LoRusso, 2013). The pivotal BOLERO-2 trial (Table 1 and Figure 3), a randomised multicentre, phase III trial, included 724 postmenopausal women with ER+ MBC who had recurrence or progression on nonsteroidal AI treatment in the adjuvant or metastatic setting (Baselga *et al*, 2012). Patients were randomly assigned in a 2:1 ratio, the largest group receiving the mTOR inhibitor everolimus in combination with the steroidal AI exemestane. The BOLERO-2 trial showed significantly longer PFS in the combination group compared with the exemestane monotherapy group (10.6 vs 4.1 months). However, a follow-up publication 2 years later showed no statistically significant improvement of OS (Piccart *et al*, 2014). Following publication of the BOLERO-2 results, the combination of exemestane and everolimus was established as a standard combination in patients progressing on monotherapy with nonsteroidal AIs in many countries. The HORIZON study, a randomised, phase III, placebo-controlled study, tested the efficacy and safety of letrozole in combination with the mTOR inhibitor temsirolimus vs letrozole in monotherapy (Wolff *et al*, 2013). A total of 1112 patients were enrolled. Surprisingly, there was no overall improvement in PFS in the combination arm. However, an exploratory analysis showed significantly longer PFS in patients below the age of 65 years (9.0 vs 5.6 months; Wolff *et al*, 2013).

Aromatase inhibitors given in combination with PI3K inhibitors. The PI3K mutations occur in up to 50% of ER+ MBC. Some of these mutations leave the enzyme constitutively active (Figure 2) and thereby a drugable target (Sanchez *et al*, 2011; Geuna *et al*, 2015). Preclinical models have showed that inhibition of the PI3K/mTOR pathway decreases tumour activity and induced apoptosis when combined with oestrogen deprivation in ER+ tumour cells (Sanchez *et al*, 2011), providing a rationale for combining the two regimens in a clinical setting. Both pan-PI3K and the PI3K α -selective inhibitors have been developed, but only phase I–II clinical trials have been published so far (Table 1). Mayer *et al* (2014) published a phase Ib trial in 2014, involving 51 postmenopausal women with histologically confirmed ER+/HER2– MBC. All patients received continuously letrozole as the basic endocrine therapy. In addition, all participants were randomised to either continuous or intermittent administration of the pan-PI3K inhibitor buparlisib. Maximum tolerated dose (MTD) in both treatment arms was 100 mg day⁻¹, and the combination of letrozole and buparlisib was all in all well tolerated. Other dose-escalation studies with buparlisib show that 100 mg day⁻¹ was a well-tolerated recommended dose for use in future studies (Bendell *et al*, 2012; Ando *et al*, 2014; Rodon *et al*, 2014). Common drug-related adverse events were hyperglycaemia, nausea, diarrhoea, fatigue, transaminitis, mood disorders and rash (Bendell *et al*, 2012). Several other PI3K inhibitors (such as pictilisib, pilarisib, voxalisib and idelalisib) are currently tested in clinical trials (Liu *et al*, 2013; Ndubaku *et al*, 2013; Blackwell *et al*,

2015; James *et al*, 2015; Sarker *et al*, 2015). Although the latter has just recently been approved by the US Food and Drug Administration (FDA) for various haematological malignancies, we are currently waiting for crucial data from ongoing phase II–III trials to define the role of PI3K inhibitors as additional therapy to AIs.

Combinations of aromatase inhibitors and CDK4/6 inhibitors. The CDKs play an important role in the cell cycle checkpoints by interacting with the E2F/Rb complex (Figure 2). Following phosphorylation by the cyclin D1–CDK4/6 complex, the transcription factor E2F is released from Rb, leaving E2F available for initiating the transcription of genetic products necessary for entering the S phase of the cell cycle. Without the cyclin D1–CDK4/6 complex, E2F remains bound to Rb, and the cell does not proceed to the G1 phase. The recently FDA-approved, potent and highly selective inhibitor of CDK4/6, palbociclib, represents one of the new promising drugs prohibiting cell cycle progression (Rocca *et al*, 2014; Beaver *et al*, 2015).

The PALOMA-1/TRIO-18 trial (Table 1 and Figure 3) was published in 2015 and represents the first and so far the only phase II study comparing letrozole monotherapy with the palbociclib/letrozole combination (Finn *et al*, 2015). The trial was an open-label, randomised study of postmenopausal women with advanced ER+/HER2– MBC. A total of 165 patients were enrolled in two separate cohorts: cohort 1 included 81 patients on the basis of their ER and HER2 status alone, whereas the 84 patients in cohort 2 were characterised by either amplification of cyclin D1 or loss of p16 or both. Patients were randomly assigned in both cohorts to receive continuous single-agent letrozole or the combination of letrozole and palbociclib. Overall, median PFS was 10.2 months in the letrozole monotherapy group that increased to 20.2 months in the combination group. In cohort 1, the PFS was 5.7 months in the letrozole monotherapy group and 26.1 months in the group treated with palbociclib in addition to letrozole. For cohort 2, the PFS was 11.1 months (letrozole monotherapy) and 18.1 months (combination), respectively. All in all, the study showed that patients with ER+/HER2– MBC had significantly longer PFS on combination therapy compared with single-agent letrozole. This finding was later on confirmed to be true for nearly all subgroups of patients (Finn *et al*, 2016). Currently, PALOMA-2, a phase III trial, is ongoing, resembling PALOMA-1 in a larger patient cohort. The final results are expected soon. Another ongoing phase III trial comparing palbociclib with exemestane or chemotherapy (capecitabine) in ER+/HER2– MBC patients with resistance to nonsteroidal AIs is currently recruiting.

In October 2016, Hortobagyi *et al* (2016) presented an early report covering a phase III clinical trial evaluating the efficacy of another CDK4/6 inhibitor, ribociclib, combined with letrozole vs letrozole given alone as systemic therapy in MBC patients. After 18 months, the PFS rate was 63% in the combination arm (ribociclib and letrozole) and 42.2% in the letrozole monotherapy arm. Although the PFS was significantly longer among those receiving ribociclib plus letrozole, this came at the cost of a higher rate of myelosuppression.

Additional trials involving other CDK4/6 inhibiting agents such as abemaciclib (LY2835219) and AIs in the metastatic setting are currently recruiting patients.

Novel drug combinations involving AIs in MBC. It has been stated for several decades that combinations of traditional endocrine treatment options are not recommended for the treatment of ER+ MBC. Thus, combinations of, for example, antioestrogens and AIs, have not shown superiority compared with monotherapy in sequence. One of the major recently performed trials testing an AI (anastrozole) with an antioestrogen (tamoxifen) was the ATAC-trial in early BC (Cuzick *et al*, 2010). Again, the combination arm was terminated early because of lack of synergy

Table 1. Pivotal clinical trials comparing AI monotherapy with drug combinations including AIs and novel, targeted drugs

Study/phase	Patients	N	AI	Targeted drug	Efficacy/results
I. Studies combining AIs with HER1/2 inhibitors					
EORTC 30008 Phase III, randomised, double-blind, placebo-controlled trial (Johnston <i>et al</i> , 2009)	PMW with MBC HR-pos, HER2-pos or -neg HR-pos/HER2-pos (subgroup) No prior ET for MBC was allowed.	1286 219	Letrozole	Lapatinib	Median PFS in the ER-pos/HER2-pos subgroup: 8.2 months (combination LAP + LET) vs 3.0 months for LET monotherapy, HR 0.71, 95% CI: 0.53–0.96 (P = 0.019).
TAnDEM study Phase III, randomised, open-label clinical trial (Kaufman <i>et al</i> , 2009)	PMW with HR-pos/HER2-pos MBC TAM or ANA as ET for MBC allowed for up to 4 weeks before inclusion.	207	Anastrozole	Trastuzumab	Median PFS: 4.8 months (combination ANA + TZM) vs 2.4 months for ANA monotherapy, HR = 0.63, 95% CI: 0.47–0.84 (P = 0.0016).
NCT00077025 Phase II, randomised placebo-controlled clinical trial (Cristofanilli <i>et al</i> , 2010)	PMW with HR-pos MBC; No prior ET for MBC.	94	Anastrozole	Gefitinib	Median PFS: 14.7 months in combination group (ANA + GEF) vs 8.4 months in the ANA + PLAC subgroup; HR: 0.55, CI: 0.32–0.94; CBR ANA + GEF: 49% CBR ANA + PLAC: 34%.
NCT00066378 Phase II randomised, double-blind, placebo-controlled trial (Tryfonidis <i>et al</i> , 2016)	PMW with HR-pos MBC LABC in subgroup of patients; Prior ET for MBC allowed.	71	Anastrozole	Gefitinib	PFS rate at 1 year: 35% for ANA + GEF vs 32% for ANA + PLAC; ORR: 22% ANA + GEF vs 28% ANA + PLAC; median duration of response: 13.8 months in the ANA + GEF group vs 18.6 months in the ANA + PLAC.
II. Studies combining AIs with mTOR inhibitors					
HORIZON Phase III, randomised, double-blind, placebo-controlled trial (Wolff <i>et al</i> , 2013)	PMW with HR-pos LABC or MBC; at least one measurable lesions by RECIST; no prior AI therapy for either LABC or MBC; no adjuvant AI therapy during the last 12 months before inclusion.	1112	Letrozole	Temsirolimus	Overall PFS was similar in both arms: 8.9 vs 9 months; HR: 0.90, 95% CI: 0.76–1.07 (P = 0.25); PFS in patients ≤ age 65 years (exploratory analysis): 9.0 months in LET + TEM group vs 5.6 months in LET monotherapy arm; HR 0.75, 95% CI: 0.60–0.93 (P = 0.009);
BOLERO-2 Phase III, randomised, double-blind, placebo-controlled trial (Baselga <i>et al</i> , 2012)	PMW with ER-pos/HER2-neg MBC refractory to previous LET or ANA monotherapy (recurrence during or within 12 months after the end of adjuvant therapy or within 1 month after the end of treatment for advanced disease.	724	Exemestane	Everolimus	Median PFS was 6.9 months with EXE + EVE and 2.8 months for EXE monotherapy; HR 0.43, CI 0.35–0.54 (P < 0.001); Median central PFS: 10.6 (EXE + EVE) vs 4.1 months (EXE monotherapy); HR 0.36, 95% CI: 0.27–0.47 (P < 0.001).
III. Studies combining AIs with PI3K inhibitors					
NCT01248494 Phase Ib, open-label clinical trial (Mayer <i>et al</i> , 2014)	PMW with ER-pos/HER2-neg MBC refractory to at least one line of ET in the MBC setting or diagnosed with MBC during or within 1 year of adjuvant ET.	51	Letrozole	Buparlisib	Clinical benefit rate at MTD of buparlisib: 31% 27% grade 3 adverse events. No grade 4 adverse events; Clinical efficacy not dependent on PIK3CA mutation status.
IV. Studies combining AIs with CDK4/6 inhibitors					
PALOMA-1/TRIO-18 Phase II, open-label, randomised trial (Finn <i>et al</i> , 2015)	PMW with advanced ER-pos/HER2-neg BC without prior treatment for advanced disease; Cohort 1: inclusion based on HR/HER2-status; Cohort 2: as cohort 1 but in addition confirmed amplification of cyclin D1, loss of p16 or both.	165	Letrozole	Palbociclib	Median PFS: 20.2 (LET + PAL) vs 10.2 months (LET monotherapy); HR 0.488, 95% CI: 0.319–0.748 (P = 0.0004); Cohort 1: median PFS: 5.7 months (LET) vs 26.1 months for the LET + PAL combination; HR 0.299, 95% CI: 0.156–0.572 (one-sided P > 0.0001); Cohort 2: median PFS: 11.1 months for LET monotherapy vs 18.1 months for the LET + PAL combination, HR 0.508, 95% CI: 0.303–0.853 (one-sided P = 0.0046)
NCT01958021 Phase III, randomised, double-blind, placebo-controlled clinical trial (Hortobagyi <i>et al</i> , 2016)	PMW with HR-pos/HER2-neg recurrent or MBC without prior therapy for advanced breast cancer.	668	Letrozole	Ribociclib	PFS rate at 18 months: 63% for the LET + RIB combination vs 42.2% for the LET monotherapy arm, Overall response rate (patients with measurable disease at baseline): 52.7% for the LET + RIB combination vs 37.1% for the LET monotherapy group (P < 0.001).
V. Studies combining AIs with fulvestrant					
FACT-trial Phase III, open-label, randomised, clinical trial (Bergh <i>et al</i> , 2012)	PMW or PREMPW receiving a GnRH agonist, with HR-pos MBC and relapse after or during primary treatment.	514	Anastrozole	Fulvestrant	Median TTP was 10.8 months for the ANA + FULV combination vs 10.2 months in the ANA monotherapy arm, HR 0.99, 95% CI: 0.81–1.20 (P = 0.91); median overall survival was OS: 37.8 months and 38.2 months, respectively (P = 1.00);

Table 1. (Continued)

Study/phase	Patients	N	AI	Targeted drug	Efficacy/results
SoFEA Phase III, randomised, placebo-controlled clinical trial (Johnston <i>et al</i> , 2013)	PMW with HR-pos relapse or advanced BC (MBC or LABC) during therapy with an NSAI (NSAI given for at least 12 months as adjuvant therapy or at least 6 months for MBC).	723	Anastrozole Exemestane	Fulvestrant	PFS: ANA + FULV: 4.4 months (CI: 3.4–5.4 mo); FULV + PLAC: 4.8 months (CI: 3.6–5.5 mo); EXE mono: 3.4 months (CI: 3.0–4.6 mo); No difference was recorded comparing the ANA + FULV vs the FULV + PLAC groups or between the FULV + PLAC and EXE monotherapy groups.
NCT00075764 Phase III, randomised clinical trial (Mehta <i>et al</i> , 2012)	PMW with HR-pos MBC without prior therapy for MBC; prior adjuvant therapy with AI or TAM was allowed (following an early amendment).	707	Anastrozole	Fulvestrant	The median PFS was 13.5 months (95% CI: 12.1–15.1) for the ANA monotherapy arm vs 15.0 months (95% CI: 13.2–18.4) for the ANA + FULV arm ($P=0.007$); The median overall survival was 41.3 months (95% CI: 37.2–45.0) with ANA alone and 47.7 months (95% CI: 43.4–55.7) with ANA + FULV ($P=0.049$).
VI. Studies combining AIs with alternative compounds					
Exemestane ± Etinostat trial Phase II, randomised, double-blind, placebo-controlled, 'signal-finding' clinical trial (Yardley <i>et al</i> , 2013)	PMW with ER-pos BC experiencing relapse following adjuvant therapy with a NSAI (at least for 12 months) or progression of MBC during a NSAI (given for at least 3 months).	130	Exemestane	Etinostat	Median PFS was 4.3 months in the EXE + ETI group vs 2.3 months in the EXE + PLAC group; HR 0.73, 95% CI: 0.50–1.07 ($P=0.055$); Median overall survival was 28.1 months in the EXE + ETI arm vs 19.8 months in the EXE + PLAC subgroup, HR 0.59, 95% CI: 0.36–0.97 ($P=0.036$).
NCT00405938 Phase II, non-randomised clinical trial (Yardley <i>et al</i> , 2011)	PMW with HR-pos MBC without any previous therapy for MBC were eligible; HER2-pos and HER2neg patients could participate.	79	Anastrozole	Bevacizumab	Median TTP was 21 months for the combination ANA + BEV vs 9 months for the combination FULV + BEV; the overall response rates were 47% and 27%, respectively; both combinations were evaluated as feasible as first-line therapy for MBC and are currently tested in ongoing phase III trials.
LEA-study Phase III, randomised, open-label, clinical trial (Martin <i>et al</i> , 2015)	PMW with HR-pos/HER2-neg MBC; no prior therapy for MBC was allowed.	380	Letrozole or fulvestrant	Bevacizumab	Median PFS was 14.4 months in the ET-group (LET or FULV monotherapy) vs 19.3 months in the ET-B group (LET or FULV combined with BEV); HR 0.83, 95% CI: 0.65–1.06 ($P=0.126$); Overall response rate was 22% with ET vs 41% with ET-B, ($P<0.001$); TTF and OS were comparable in both arms.
NCT00050141 Phase II, randomised, blinded, parallel-group study (Johnston <i>et al</i> , 2008)	PMW with HR-pos BC progressing on tamoxifen either as adjuvant therapy or first line for advanced disease (objective response or stable disease during TAM-therapy was mandatory in MBC patients),	120	Letrozole	Tipifarnib	Median TTP was 10.8 months for the LET monotherapy arm vs 5.6 months for the combination LET + TIP; the ORR was 30% in the LET + TIP arm vs 38% in the LET monotherapy arm. Clinical benefit rate: 49% for the LET + TIP subgroup and 62% for the LET monotherapy cohort.
Abbreviations: AI = aromatase inhibitor; ANA = anastrozole; BC = breast cancer; CBR = clinical benefit rate; CI = confidence interval; ER-pos = oestrogen receptor positive; ET = endocrine treatment; ETI = etinostat; EVE = everolimus; FULV = fulvestrant; GEF = gefitinib; GnRH = gonadotropin-releasing hormone; HER2-pos = human epidermal growth factor receptor-2 positive; HR = hazard ratio; HR-pos = hormone receptor positive; LABC = locally advanced breast cancer; LAP = lapatinib; LET = letrozole; MBC = metastatic breast cancer; MTD = maximum tolerated dose; NSAI = nonsteroidal anti-inflammatory (drugs); OLT = open-label trial; OS = overall survival; ORR = objective response rate; PFS = progression-free survival; PLAC = placebo; PMW = postmenopausal women; PREMPW = premenopausal women; RIB = ribociclib; TAM = tamoxifen; TEM = temsirolimus; TIP = tipifarnib; TTP = time to progression; TZM = trastuzumab.					

and perhaps even detrimental effects. However, although early experience was based on clinical use of tamoxifen in drug combinations, the appearance of fulvestrant challenges this point of view. In contrast to tamoxifen, fulvestrant is a pure steroidal ER antagonist lacking oestrogen agonist effects. When bound to fulvestrant, the ER dimerisation is disrupted, making it unstable and thereby accelerating the degradation of the complex. Fulvestrant has been shown to be as least as efficient compared with AIs in the metastatic setting (Graham *et al*, 2016) when given in the optimal dose of 500 mg per month. Several studies combining fulvestrant with other endocrine agents have shown promising results (Massarweh *et al*, 2014; Cristofanilli *et al*, 2016; Ma *et al*, 2016). A few studies have tested dual endocrine treatment involving fulvestrant and an AI simultaneously. Mehta *et al* (2012) presented results that favoured the combination of fulvestrant and anastrozole vs anastrozole alone, and showed significant increase in both PFS and OS. Unfortunately, neither the FACT study (Bergh

et al, 2012) nor the SoFEA trial (Johnston *et al*, 2013) could confirm that the combination of fulvestrant with either anastrozole or exemestane improves PFS compared with an AI alone. If subgroups of patients benefit from the combination of fulvestrant and an AI, these have to be defined in a better way before this strategy may be established as a standard.

Trials involving other compounds in combination with AIs included the farnesyl transferase inhibitors tipifarnib and lonafarnib (Johnston *et al*, 2008) and the antiangiogenic agent bevacizumab (Yardley *et al*, 2011; Martin *et al*, 2015). One study showed increased PFS compared with previous trials involving hormonal therapy alone when combining bevacizumab with either anastrozole or fulvestrant (Yardley *et al*, 2011). Besides this study, none of the other combinations mentioned have showed a significant improvement in PFS or OS in patients with MBC.

The dual monoclonal antibody against IGF1/II MEDI-573 has showed preliminary activity in a heavily pretreated population of

patients with different solid tumours (Haluska *et al*, 2014), and data from an ongoing study testing MEDI-573 in combination with AIs are expected next year. Another dual IGFI/insulin receptor inhibitor (BMS-754807) has also shown preclinical antitumour effect in combination with letrozole (Hou *et al*, 2011). A third agent targeting the IGF system (IGFR2) is ganitumab, an antibody that binds specifically to the IGF2 receptor. It has shown an acceptable safety profile and preliminary antitumour response (Tolcher *et al*, 2009), but data published by Robertson *et al* (2013) comparing ganitumab with exemestane or fulvestrant showed that adding ganitumab did not improve outcomes.

Simultaneous blockade of both oestrogen and androgen biosynthesis has been suggested as another promising strategy to delay endocrine resistance during AI therapy. O'Shaughnessy *et al* (2016) recently reported a large phase II trial challenging this interesting hypothesis. The 297 ER+ MBC patients who were pretreated with a nonsteroidal AI were randomised (1:1:1) to receive oral once daily either 1000 mg abiraterone acetate (AA) plus 5 mg prednisone (arm A), 1000 mg abiraterone acetate plus 25 mg exemestane (arm B) or 25 mg exemestane monotherapy (arm C; O'Shaughnessy *et al*, 2016). Unfortunately, adding AA to exemestane did not improve PFS in this study. Additional studies testing antiandrogen therapies in combination with AIs are currently ongoing.

Finally, the histone deacetylase inhibitor entinostat showed acceptable safety profile and demonstrated clinical activity in ER+ MBC patients in a phase II study by Yardley *et al* (2013). A phase III trial investigating the clinical benefit of adding entinostat to exemestane in postmenopausal MBC patients is currently recruiting.

DISCUSSION

Following the establishment of AIs belonging to the third generation as first-line monotherapy in ER+ MBC, considerable efforts have been made to increase the duration of response by adding on other targeted therapies simultaneously. Based on crucial findings made in both basic and translational research, several mechanisms of adaptation to oestrogen depletion have been identified and targeted by novel drugs.

One of the first success stories was the co-administration of AIs and HER2 targeted drugs like trastuzumab and lapatinib in dual ER/HER2-positive subgroups of breast cancer patients. The crucial studies published by Johnston *et al* (2009) and Kaufman *et al* (2009) showed that adding either trastuzumab or lapatinib to an AI significantly increases PFS in ER+/HER2+ MBC patients. As a consequence, simultaneous therapy with AIs and anti-HER2 drugs is established as one potential treatment strategy in ER+/HER2+ patients. Following the identification of mTOR as another pivotal player in endocrine resistance, temsirolimus and everolimus were challenged in clinical trials as well. The crucial BOLERO-2 trial caused the worldwide breakthrough of mTOR inhibitors in addition to AIs. BOLERO-2 tested exemestane alone *vs* the combination with everolimus in postmenopausal patients progressing on a nonsteroidal AI (anastrozole or letrozole). The trial was highly positive, favouring the combination of exemestane and everolimus causing an increase in PFS of 6.5 months. Thus, exemestane combined with everolimus is recognised as well as one of the standard combination in clinical use for selected patients according to the design of the BOLERO-2 trial.

Motivated by the success stories following the combination of anti-HER2 and mTOR inhibitors with AIs, PIK3A inhibitors have entered the stage as well because of promising data obtained in early-phase studies (Bendell *et al*, 2012; Ando *et al*, 2014; Mayer *et al*, 2014; Rodon *et al*, 2014). However, the clinical role of PIK3A inhibitors in the future algorithms of MBC treatment remains to be

clarified as we await the data from ongoing phase II and III trials. Finally, CDK4/6 inhibitors, influencing on the cell cycle itself, have shown promising results and are now tested in phase III trials. The phase II PALOMA-1/TRIO-18 presented an increase in PFS of 10 months when combining letrozole with palbociclib compared with letrozole alone (Finn *et al*, 2015). The phase III PALOMA-2 trial is ongoing, resembling PALOMA-1/TRIO-18 in a larger patient cohort. At this stage, palbociclib represents one of the most promising new agents suitable for combinations with AIs.

However, although several new classes of targeted therapies have shown some clinical efficacy when given simultaneously to AIs, endocrine resistance still occurs in all patients, although further delayed for a couple of months compared with AI monotherapy. Thus, additional translational cancer research is urgently needed to find new strategies that may delay tumour growth over longer time periods in ER+ breast cancer. Several new combinations have already been identified like combining AIs with pure antioestrogens or compounds interfering with the IGFR system.

Most importantly, from a clinical point of view, a significant increase in OS could not be demonstrated for the majority of drugs when combined with AIs. This may be because of the early clinical setting of AI drug combinations that are in general followed by a variety of antihormonal and chemotherapy lines. Whether some of the novel drug combinations involving AIs may transfer some time from the chemotherapy period of an individual patient into the on-treatment situation during endocrine therapy is not proven by hard evidence. On the other hand, the lack of influence on OS curves in general may also indicate that the additional blockade of an alternative signalling pathway may not cause a major breakthrough in breast cancer therapy (Tannock and Hickman, 2016) because of the networking of many parallel pathways and the plasticity of malignant cells in general.

The future impact of targeted therapy is further challenged by the emergence of completely novel strategies to treat advanced cancer like immunotherapy. The idea to combine several targeting drugs at the same time in patients is tempting, although increased toxicity has been reported in several trials involving 2–3 new compounds simultaneously. The side effects reported during novel drugs like cell cycle inhibiting drugs resemble partly the toxicity observed during classical chemotherapy (i.e., pancytopenia and so on). Thus, the major advantage of traditional antihormonal therapies like mild side effects and so on may be partly lost when initiating novel drug combinations. However, the introduction of novel screening methods like next-generation sequencing (NGS) will increase the ability to identify important targetable mutations in individual patients and hopefully pave the way for personalised drug combinations causing better clinical responses in selected patients.

In conclusion, we have recently seen significant improvements in PFS in selected breast cancer patients based on novel drug combinations involving AIs, mTOR inhibitors, HER2 directed therapies, PI3K inhibitors and CDK4/6 inhibitors. Unfortunately, no influence on OS in general indicates the urgent need of novel targets, improved patient selection based on novel biomarkers and, if possible, a better understanding of the complex adaptation processes underlying resistance to aromatase inhibition.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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