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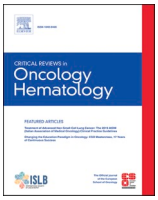

Navigating the complexity of PI3K/AKT pathway in HER-2 negative breast cancer: biomarkers and beyond

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ABSTRACT

The results of the SOLAR-1 and CAPItello-291, highlight the benefit of the α-selective phosphoinositide 3-Kinase Pathway inhibitor (PI3Ki) alpelisib and the AKT inhibitor (AKTi) capivasertib in patients with hormone receptor-positive (HR+)/Human Epidermal Growth Factor Receptor 2 (HER2)- negative metastatic breast cancer (mBC) that have *PIK3CA*/AKT1/PTEN tumour alterations. Although effective, these drugs are associated with significant toxicities, which often limit their use, particularly in frail patients. Following the recent incorporation of these agents into clinical practice, and with many others currently in development, significant challenges have emerged, particularly those regarding biomarkers for patient selection. This review will discuss biomarkers of response and their resistance to PI3K/AKT inhibitors (PI3K/AKTis) in HR+/HER- BC in early and advanced settings to ascertain which populations will most benefit from these drugs. Of the biomarkers that were analysed, such as *PIK3CA*, AKT, PTEN mutations, insulin levels, ^{18F}-FDG-PET/TC, only the *PIK3CA*-mutations (*PIK3CA*-mut) and the AKT pathway alterations seem to have a predictive value for treatments with alpelisib and capivasertib. However, due to the retrospective and exploratory nature of the study, the data did not provide conclusive results. In addition, the different methods used to detect *PIK3CA*/AKT1/PTEN alterations underline the fact that the optimal diagnostic companion has yet to be established. We have summarised the clinical data on the approved and discontinued agents targeting this pathway and have assessed the drugs development, successes, and failures. Finally, because of tumour heterogeneity, we emphasise the importance of reassessing the mutational status of *PI3KCA* in both metastatic tissue and blood at the time of disease progression to better tailor treatment for patients.

1. Introduction

The *PIK3CA*/AKT1/PTEN signalling pathway has a crucial role in cellular proliferation, metabolism, survival, and apoptosis (Erickson and Toker, 2021; Mortazavi et al., 2022). In patients with a hormone receptor-positive (HR+)/ human epidermal growth factor receptor 2-negative (HER2-) breast cancer (BC), the *PIK3CA*/AKT1/PTEN pathway is the most commonly deregulated signalling pathway and has been associated with endocrine therapy (ET) resistance (Bosch et al., 2015; Khan et al., 2024). Approximately 20–40% of early BC (Mosele

Abbreviations: RAS, Rat sarcoma; HER, Human epidermal growth factor receptor; IGF1R, Insulin growth factor 1 receptor; IRS1, insulin receptor substrate-1 gene; MAPKs, Mitogen activated protein kinases; GFR, Growth factor receptor; PTEN, Phosphatase and TENsin homolog deleted on chromosome 10; PIP3, Phosphatidylinositol-3,4,5-triphosphate; PDK1, Pyruvate Dehydrogenase Kinase 1; AKT, protein kinase B; TSC2, Tuberous sclerosis complex 2; MTOR, mammalian target of rapamycin; Bcl2, B-cell leukemia/lymphoma 2 protein; BAD, Bcl-XL/Bcl-2-associated death promoter; FoxO, Forkhead box, class O; GSK-3 beta, Glycogen synthase kinase-3 beta.

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et al., 2020) (eBC) patients and 40% of metastatic BC (mBC) patients exhibit phosphoinositide 3-kinases mutations (*PIK3CA*-mut*)* (Koboldt et al., 2012). It can be abnormally activated by somatic and germline mutations in *PIK3CA*, PIK3R1, AKT, PTEN, and mTOR genes (Thorpe et al., 2015; Cerma et al., 2023).

They present a non-random distribution, as over 90% of them are concentrated in three hotspots: E542K, E545K, and H1047R (Tikoo et al., 2012). In detail, both single and multiple concomitant mutations have been identified in BC. A co-existing *PIK3CA* mutation can occur in 8–13% of BC patients, and the majority of them are double mutations (Vasan et al., 2019).

Even though their precise downstream effects have not been completely elucidated, they can be targeted by pharmacological agents. However, widespread use of these drugs has yet to be achieved due to their intrinsic, high toxicity profile, included nausea, diarrhea and hyperglycemia as well as the crosstalk with bypass alteration, such as the activation of the MAPK pathway, which makes targeting this pathway particularly challenging (Engelman, 2009; Hanker et al., 2019; Loibl et al., 2017; Yang et al., 2019) (Fig. 1).

Therefore, to improve their clinical application, it is necessary to determine whether alterations in key pathways can predict the clinical benefits of PI3K inhibitors (PI3K-i) and if they have clinical utility in patient selection. Hence, identifying biomarkers of response and resistance is paramount.

In this article, we provide an overview of the preclinical and clinical evidence related to biomarkers of the PI3K pathway activation in BC. Additionally, we discuss some emerging clinical issues such as the ideal time to evaluate the mutational status of *PI3KCA*, and the most effective diagnostic companion to detect tumour alterations of *PIK3CA*, AKT, and PTEN.

2. Potential biomarkers of response and resistance to PI3K inhibitors

2.1. Preclinical data

Given the great clinical interest in developing potential biomarkers of sensitivity or resistance to PI3Ki, several pre-clinical studies have been conducted in *vitro* and *in vivo* to test sensitivity to PI3Ki in human cancer cells (Gustin et al., 2009; Edgar et al., 2010; O'Brien et al., 2010; Serra et al., 2011; Fritsch et al., 2014). For example, O'Brien *et al.* found that BC models harbouring mutations in *PIK3CA* were sensitive to the antitumour effects of the pan-class I PI3Ki, pictilisib (O'Brien et al., 2010). Similarly, through panels of non-isogenic cancer cell lines, Beaver *et al.* reported that *PIK3CA* helical domain mutant MCF-7 cells had a 5-fold lower half maximal inhibitory concentration (IC50) for pictilisib than *PIK3CA* wild-type cells (Beaver et al., 2013). Consistent with these findings, Serra *et al.* showed that a dual inhibitor of the PI3K and the downstream mammalian target of rapamycin (mTOR), had potent antitumour activity in mutated p110-alpha BC cell lines (O'Brien et al., 2010; Serra et al., 2008).The predictive value of these mutations remains controversial despite the amount of preclinical data demonstrating the sensitivity of *PIK3CA*-mut BC cells to PI3Ki (Serra et al., 2011; O'Brien et al., 2010; Beaver et al., 2013) (Table 1).

2.2. Early hormone receptor-positive breast cancer

Regarding a preoperative window-of-opportunity study to assess whether pictilisib plus anastrozole was effective in decreasing Ki-67 in the luminal B subtype, the *PIK3CA*-mut were not predictive of response to pictilisib (Schmid et al., 2016). Nevertheless, the mutations in exon nine compared to mutations in exon 20 conferred a higher sensitivity to pictilisib (Schmid et al., 2016). Both the NEO-ORB and LORELEI trials showed that adding the a-selective PI3Ki alpelisib and the b-sparing

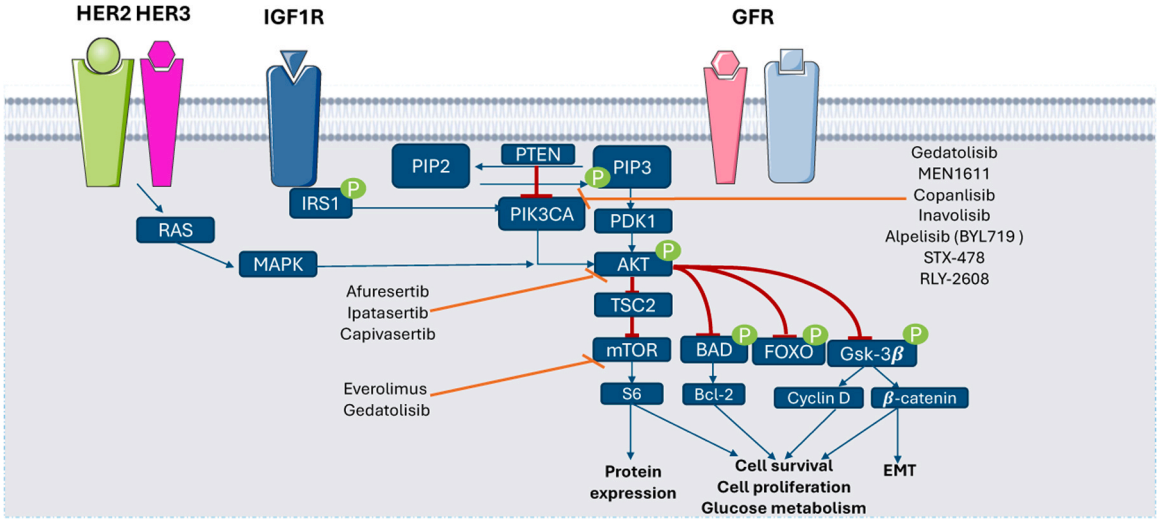

Fig. 1. A simplified illustration of the PIK3CA/AKT/mTOR pathway activation and crosstalk with GFR pathways through RAS/MAPK. The balance between PIP2 and PIP3, which are located close to the cell membrane, is maintained by the activation of PIK3CA (transforming PIP2 to PIP3), and the suppressive action of PTEN (transforming PIP3 to PIP2). The AKT is activated through phosphorylation by PDK1. AKT has a phosphorylation function, inhibiting TSC2, BAD, FOXO, and Gsk-3*β*. The AKT can also be activated by the RAS/MAPK pathway. The members of the conventional PIK3 pathway are regulated at multiple levels. On the cell surface, the tyrosine kinase receptor members, such as HER2 and EGFR, activate their intracellular co-factors to regulate the PIK3 pathways. This activation can be triggered by the Ras pathway or hindered by PTEN. However, these regulations work in loops, resulting in diverse interactions at different levels of the pathways. Additionally, the PI3K/AKT/mTOR and RAS/RAF/MEK/ERK pathways have been found to interact at several nodes, potentially leading to pathway convergence. Like the PIK3CA pathway, the Ras-MAPK cascade is involved in cell growth, proliferation, and survival. Receptor Tyrosine Kinases, including EGFR and HER2, activate PIK3CA by phosphorylating intracellular co-factors. An activated Ras initiates a positive intracellular activation cascade with Raf kinase activation, followed by MEK and MAPKs. MAPKs are crucial in influencing gene expression by interacting with the transcription factors in the nucleus. Both pathways can activate each other, with Akt phosphorylating Ras to activate it. In turn, Ras indirectly stimulates PIK3K by inhibiting negative regulators, such as the TSC2 (tuberous sclerosis complex 2). The two pathways converge to regulate cell proliferation.

Table 1
Overview of clinical trials evaluating biomarkers of response to PI3K/AKTis in breast cancer.

Name	Phase	Experimental arm	Standard arm	Breast cancer subtype	Setting	N° patients	PIK3CA status determination	PIK3CA status value
OPPORTUNE	II	pictilisib plus anastrozole	anastrozole	HR+/HER2-	neoadjuvant	75	tissue sample	not predictive
NEO-ORB	II	alpelisib plus letrozole	placebo plus letrozole	HR+/HER2-	neoadjuvant	257	tissue sample	not predictive
LORELEI	II	taselisib plus letrozole	placebo plus letrozole	HR+/HER2-	neoadjuvant	334	tissue sample	not predictive
BELLE-3	III	buparlisib plus fulvestrant	placebo plus fulvestrant	HR+/HER2-	advanced	432	tissue sample and ctDNA	predictive
BELLE-2	III	buparlisib plus fulvestrant	placebo plus fulvestrant	HR+/HER2-	advanced	1147	tissue sample and ctDNA	predictive
FERGI	II	pictilisib plus fulvestrant	placebo plus fulvestrant	HR+/HER2-	advanced	168	tissue sample	not predictive
BELLE-4	II/III	buparlisib plus paclitaxel	placebo plus paclitaxel	HER2-	advanced	416	tissue sample	not predictive
PEGGY	II	pictilisib plus paclitaxel	placebo plus paclitaxel	HR+/HER2-	advanced	183	tissue sample	not predictive
NCT01296555	II	taselisib plus fulvestrant	N.A.	HR+/HER2-	advanced	60	tissue sample	predictive
NCT01791478	Ib	alpelisib plus letrozole	N.A.	HR+/HER2-	advanced	26	tissue sample	predictive
SOLAR-1	III	alpelisib plus fulvestrant	placebo plus fulvestrant	HR+/HER2-	advanced	572	tissue sample	predictive

PI3Ki taselisib to letrozole in the neoadjuvant HR+/HER2- BC did not improve the rate of pathological complete response (pCR) or the overall response rate (ORR) in either the *PIK3CA*-mutant or the wild-type cohorts (Mayer et al., 2019; Saura et al., 2019). It should be noted that patient recruitment to the NEO-ORB study had to be suspended mainly due to liver toxicity. Only 50 patients were enrolled out of 256 that had been planned (Mayer et al., 2019). Regarding the impact of *PIK3CA*-mut on chemotherapy efficacy, Mosele et al. showed that *PIK3CA*-mutated HR+/HER2- mBC patients present resistance to chemotherapy (Mosele et al., 2020).

Similarly, Wang et al., in a retrospective analysis of HR+/HER2- mBC treated with the combination of everolimus and chemotherapy, observed numerical improvements in PFS, OS and ORR in those harbouring a single PI3K pathway alteration compared to those with more than one alteration (Wang et al., 2024).

Recently, Hayama *et al.* evaluated the impact of *PIK3CA*-mut on neoadjuvant chemotherapy (NAC) and neoadjuvant endocrine therapy (NET), showing that *PIK3CA*-mut are a biomarker of resistance to NAC. However, it does not influence the response to NET (Hayama et al., 2023).

In adjuvant treatment care, Jönsson *et al.* explored the predictive role of *PIK3CA* hotspot mutations in low-risk postmenopausal BC patients randomised to receive tamoxifen vs. no systemic treatment, using formalin-fixed paraffin-embedded tumour samples with digital droplet PCR (ddPCR). The study found no evidence that *PIK3CA*-mut were predictive of tamoxifen efficacy (Jönsson et al., 2023).

2.3. Metastatic hormone receptor-positive breast cancer

The predictive value of *PIK3CA*-mut remains controversial in the advanced setting, as some studies demonstrated that the *PIK3CA*-mut have a predictive role when observed in both blood and tissue (Leo et al., 2018; Baselga et al., 2017), whereas other findings did not confirm this (Krop et al., 2016; Martín et al., 2017; Vuylsteke et al., 2016).The BELLE-2 and BELLE-3 trials demonstrated that PI3K signalling could be targeted in a clinical setting (Leo et al., 2018; Baselga et al., 2017). These studies assessed the efficacy of the pan-PI3K inhibitor buparlisib combined with fulvestrant in patients with mBC whose disease progressed after the first line of ET. Although both studies demonstrated a modest improvement in progression-free survival (PFS) in the overall population, an exploratory analysis showed a longer PFS in patients harbouring *PIK3CA*-mut in the circulating tumour DNA (ctDNA), suggesting their potential predictive role (Leo et al., 2018). In addition, Mayer *et al.*, evaluating the alpelisib plus letrozole safety, tolerability, and preliminary activity in patients with HR+/HER2- mBC, observed clinical activity independent of *PIK3CA* mutational status, even if a clinical benefit was seen in a higher proportion of *PIK3CA*-mut patients (Mayer et al., 2017). Three years later, in the SANDPIPER trial, the β-sparing PI3Ki taselisib showed a numerically higher overall response rate (ORR) and clinical benefit rate (CBR) among patients with *PIK3CA*-mut tumours compared to *PIK3CA*-wild type (WT) tumours (Dickler et al., 2018). In line with these results, alpelisib significantly prolonged PFS in patients with mBC whose tumours had *PIK3CA*-mut, in the large SOLAR-1 trial (André et al., 2019). This PFS benefit was not observed in patients with *PIK3CA*-WT tumours. Importantly, no OS advantage was demonstrated in either cohort. One of the greatest limitations of the SOLAR-1 study was the small proportion (6%) of patients previously treated with cyclin-dependent kinase 4 and 6 inhibitors (CDK4/6i), which currently represent the standard first-line of treatment for HR+/HER2 mBC (André et al., 2019).

To overcome this issue, alpelisib plus fulvestrant was tested on HR+/HER2-, *PIK3CA*-mut mBC patients after progression on CDK4/6i plus an aromatase inhibitor (AI) within the BYLieve trial (Rugo et al., 2021). This study is consistent with the SOLAR-1 trial results, which showed the activity of this combination in this population, establishing a benchmark in the therapy sequences following a CDK 4/6i-based regimen (Rugo et al., 2021). Conversely, in the BELLE-4 trial, the combination of buparlisib and paclitaxel failed to improve PFS compared to buparlisib plus placebo in the overall or PI3K pathway-activated population. Nevertheless, it is worth noting that the BELLE-4 trial, also enrolled patients with triple-negative BC (TNBC) and that a PI3K mutational status was mainly determined in archival tissue specimens, which may not reflect the current PI3K mutational status. Additionally, the ctDNA was not evaluated (Cidado and Park, 2012). During the phase 2 FERGY trial, the pan-PI3Ki pictilisib did not significantly improve PFS, regardless of the presence or absence of *PIK3CA*-mut. The high-grade toxicity induced by this pan-PI3Ki could have potentially limited its efficacy (Krop et al., 2016).

Interestingly, the data showed that patients with HR+ mBC harbouring *PIK3CA*-mut could benefit from taselisib and alpelisib and, to a lesser degree, from buparlisib, as a result of the reduced selective nature of pan-PI3Ki (Chopra and Turner, 2017). Triplet combination targeting ER, CDK4/6 and PI3K/AKT/PTEN pathways are ongoing. To this regard, at SABCS23, it has been presented the results of the INAVO 120 trial,

exploring the efficacy of an experimental drug combination of the next generation orthosteric PI3Kα-inhibitor inavolisib, palbociclib, and fulvestrant in patients with *PIK3CA*-mutated, HR+/HER2- endocrine-resistant, locally advanced or mBC. Surprisingly, the median PFS more than doubled from 7.3 months to 15.0 month, with a stratified hazard ratio (HR) of 0.43. (95% CI 0.32, 0.59; $p<0.0001$). Moreover, this combination had a manageable safety profile, consistent with the known safety profiles of each individual drugs. Although overall survival (OS) data are immature, these results should be taken into account and potentially inavolisib in combination with palbociclib and fulvestrant may represent a new standard of care for patient with *PIK3CA*--mutated, HR+/HER2- endocrine-resistant, locally advanced or mBC (Inavolisib Combo Meets Primary End Point in PIK3CA, nd).

The inconsistent predictive role of *PI3KCA*-mut in early and advanced settings may be due to several factors. Firstly, in the early clinical setting, the estrogen receptor (ER) signalling is dominant, so the oncogenic potential of *PI3KCA*-mut may be less prevalent (Mayer and Arteaga, 2014). On the other hand, the acquisition of *PI3KCA*-mut represents a mechanism of endocrine resistance in mBC after progression on CDK4/6i. Thus, in this specific setting, the presence of *PI3KCA*-mut may predict the benefit of PI3Ki. Furthermore, to prevent the development of resistant clonal driven by *PI3KCA*-mut, potential triple combination therapy (ER, CD4/6i, PI3K) should be further evaluated (O'Leary et al., 2018; Fribbens et al., 2016; Presti and Quaquarini, 2019). Secondly, due to tumour heterogeneity, *PI3KCA*-mut may not be an early clonal event but an acquired subcloned driver found only in metastatic lesions. Hence, the use of PI3Ki should be discouraged in treatment-naïve patients. Due to the counterintuitive findings concerning the predictive role of *PI3K3CA*-mut, clinical trials, with and without upfront stratification, are underway (Table 2).

3. The controversial predictive role of PIK3CA mutational status

The *PIK3CA* mutational status seems to have a variable oncogenic potential, with different degrees of tumour cell addiction to the PI3K/AKT/PTEN pathway (Zardavas et al., 2014). Interestingly, Loi *et al.* revealed a gene-expression signature driven by *PIK3CA* (*PIK3CA*-GS) that could discriminate the *PIK3CA* mutational status (Loi et al., 2013). They showed that a high value of the *PIK3CA*-GS was associated with low mTORC1 input and low levels of pathway activation. On the contrary, a low value of *PIK3CA*-GS scores (i.e., with a greater pathway activation) seems to be associated with m TORC1 output and benefits from letrozole/everolimus (Loi et al., 2013; Loi et al., 2010). Furthermore, in a proteogenomic analysis of TCGA BC samples, Mertins *et al.* demonstrated that some *PI3KCA*-mutant BC were not associated with downstream pathway activation, confirming the variable oncogenic potential of the *PIK3CA*-mut (Mertins et al., 2016).

Another possible explanation is that full pathway activation might require a second hit. In this regard, it must be noted that ~ 4% of all BC, or ~ 12% of all patients with *PIK3CA*-mut BC, have double *PIK3CA*-mut (Migliaccio et al., 2022; Martínez-Sáez et al., 2020). Vasan *et al.* showed that the occurrence of double *PIK3CA* mutations in cis elicits hyperactivation of the PI3K pathway and downstream signalling in BC compared to tumour cells harbouring only a single *PIK3CA*-mut or multiple *PIK3CA*-mut occurring in trans (Vasan et al., 2019).

Indeed, the authors demonstrated that the presence of a double *PIK3CA*-mut in cis, through enhanced membrane binding, increases the sensitivity to selective PI3Kα inhibitors, such as alpelisib and inavolisib (Vasan et al., 2019). This report may suggest that multiple hits on the *PIK3CA* gene might have a synergistic effect. In addition, Sivakumar *et al.* have recently shown that multi-*PIK3CA*-mut are usually clonal and in cis, while subclonal *PIK3CA*-mut frequently occur in trans (Sivakumar et al., 2023). Recently, Hutchinson et al. confirmed preclinical data in a phase III clinical-genomics dataset from the SANDPIPER trial; patients with clonal multiple PIK3CA-mut exhibited prolonged PFS and ORR compared to those with subclonal mutations (Hutchinson et al., 2023). This trend was consistent with the results from a preliminary analysis of *PIK3CA*-mutational status from an ongoing I/Ib study of inavolisib alone or when combined with ET, with or without palbociclib (NCT03006172). Specifically, the authors observed that patients with multiple *PIK3CA*-mut at baseline ctDNA were more likely to achieve partial response or stable disease compared to those with only a single *PIK3CA*-mut (Jhaveri et al). Instead, Dakota *et al.*'s comprehensive genetic evaluation of 161 primary BC, found that a subset of tumours with activating mutations in the *PIK3CA* gene also had the DNA copy number increased in the *PIK3CA* locus (Kadota et al., 2009). Similarly, Migliaccio *et al.,* analysing a dataset of over 2000 samples, observed that the *PIK3CA* copy number (CN) gain occurred preferentially in *PIK3CA* mutant tumours (Migliaccio et al., 2022). Additionally, they found better responses to alpelisib in pan-cancer than in BC cell lines and patient-derived xenografts (PDX) with *PIK3CA* mutated/CN gain compared to *PIK3CA* mutated/CN neutral, suggesting that response to alpelisib depends primarily on the *PIK3CA* mutational signature rather than *PIK3CA* CN status (Migliaccio et al., 2022).

However, due to the limited sample size and the small number of alpelisib-treated patients in this study, the potential predictive role of *PIK3CA*-mutated/CN gain has yet to be established. Interestingly, in an exploratory analysis of the SANDIPER trial, patients with ≥2 *PIK3CA*-mut, detected through ctDNA, showed a more favourable PFS and a higher ORR compared to those with a single mutation, suggesting that in patients with more than one *PIK3CA*-mut, PI3Ki may be more effective (Dent et al., 2021). Conversely, Varkaris et al., has recently showed secondary *PIK3CA*-mut, within the catalytic pocket as a novel mechanism of resistance to orthosteric PI3Kα-inhibitor (Varkaris et al., 2023). Indeed, through serial liquid biopsies and rapid autopsies in 39 patients with mBC developing acquired resistance to PI3Kis, they described the emergence of secondary resistance *PIK3CA*-mut, such as Trp780 residue, driving resistance to both PI3Kα and pan-PI3Ki. They also identified that a novel allosteric PI3Kα-inhibitor, RLY-2608, which may overcome these resistance mechanisms (Varkaris et al., 2023).

Overall, these data suggest a larger clinical benefit of PI3Ki in patients with compound mutations or gains in copy number, encouraging further study of *PIK3CA* gain and mutations as a marker of PI3Ki response. Nevertheless, it should be taken into considerations that some mutations may drive resistance by alterating affinity of alpelisib and inavolisib, however novel allosteric PI3Ki are underway.

4. Potential biomarkers of resistance to PI3K inhibitors

4.1. PTEN loss

PTEN is a tumour suppressor belonging to the PI3K signalling pathway (Shi et al., 2003). In many human cancers, the gene is frequently inactivated through point mutations, as well as through other genetic and biochemical mechanisms, leading to high AKT activity and abnormal growth regulation (Steelman et al., 2011; Chalhoub and Baker, 2009).

Preclinical data indicate that cells with loss of PTEN expression are more sensitive to PI3Ki (O'Brien et al., 2010). Juric *et al.* reported a case in which *de novo* loss of PTEN expression was observed in all progressing lesions in a patient under treatment with alpelisib (Juric et al., 2015). Moreover, PTEN-null xenografts derived from this patient were found to be also resistant to alpelisib, suggesting that parallel genetic evolution of separate metastatic sites with different PTEN alterations, may induce a PTEN-null profile resistant to PI3Ki (Juric et al., 2015). On the other hand, Wee *et al.* found that PTEN-deficient tumours are dependent on the p110β signalling (Wee et al., 2008) explaining why in the OPPORTUNE trial, patients with PTEN-positive and PTEN-negative BC, derived benefit from the pan-PI3Ki pictilisib (Schmid et al., 2016). Despite preclinical data, in the BOLERO-2 and the TAMRAD trial, PTEN mutations or low protein expression by immunohistochemistry (IHC), failed to predict everolimus activity in patients with HR+/HER2− mBC

Table 2
Ongoing clinical trials with PI3K inhibitors in early and metastatic breast cancer settings.

Trial name	Phase	Study Design	Patient population	Stratification for Pi3K/PTEN	Method	Intervention	Primary Endpoint	Status
Early breast cancer								
NCT02626507	I	18 Participants Single Group AssignmentOpen Label	Untreated HR+/ HER2- eBC	no		Neoadjuvant Gedatolisib + Fulvestrant and Palbociclib	AEs	Active, not recruiting
NCT05306041 (GeparPiPPa)	II	170 ParticipantsRandomisedParallell AssignmentOpen Label	HR+/HER2+ eBC	PI3KCA mutated	Determined by NGS	neoadjuvant PHESGO and ET +/- Inavolisib	pCR	Recruiting
NCT04216472	II	8 ParticipantsSingle Group AssignmentOpen Label	Anthracycline refractory eTNBC	PIK3CA mutated/ PTEN loss	Determined by NGS	Neoadjuvant Alpelisib and Nab-paclitaxel	pCR	Recruiting
Advanced breast cancer								
NCT05143229 (ASSET)	I	18 Participants InterventionalOpen Label	Stage III/IV HR+/ HER2- BC	no		Alpelisib + Sacituzumab Govitecan	RP2D	Recruiting
NCT05508906	I	60 ParticipantsNon-RandomisedParallel AssignmentOpen Label	HR+/HER2- MBC	no		OP-1250 + Ribociclib or Alpelisib	DLTsMTD	Recruiting
NCT01791478	I	46 ParticipantsSingle Group AssignmentOpen Label	Post-Menopausal HR+/HER2- MBC	no		BYL719 and Letrozole	DLTsMTD	Active, not recruiting
NCT03207529	I	18 ParticipantsSingle Group AssignmentOpen Label	AR+ MBC	PTEN loss	Determined by using CLIA	Alpelisib + Enzalutamide	MTD	Active, not recruiting
NCT05230810	I/II	40 ParticipantsSingle Group AssignmentOpen Label	HER2+ MBC	PIK3CA mutated	Determined with a solid or liquid biopsy by an assay approved for clinical decision making by an FDA-approved test	Tucatinib + Alpelisib and Fulvestrant	AEs	Recruiting
NCT04108858	I/II	12 ParticipantsRandomisedParallel AssignmentOpen Label	HER2+/HR- MBC	PIK3CA mutated/ PTEN loss	Presence of actionable mutation on molecular testing	Copanlisib + Trastuzumab and Pertuzumab	RP2D	Recruiting
NCT01872260	I/II	255 Participants RandomisedParallel AssignmentOpen Label	HR+/HER2- MBC	no		Alpelisib and LEE011 + Letrozole	DLTsSafety	Active, not recruiting
NCT03284957 (AMEERA-1)	I/II	136 ParticipantsRandomisedParallel AssignmentOpen Label	Post-Menopausal HR+/HER2- MBC	no		Amnecestrant Monotherapy and in Combination with other treatments (Palbociclib, Alpelisib, Everolimus, Abemaciclib)	DLTs	Active, not recruiting
NCT05768139	I/II	160 ParticipantsNon-RandomisedOpen Label	Advanced Solid Tumour HR+/HER2- MBC (cohort A)	no		STX-478 Monotherapy and in Combination with other treatments	AEs	Recruiting
NCT04851613	I/II	20 Participants Non-RandomisedSingle Group AssignmentOpen Label	Locally advanced or HR+/HER2- MBC	no		Afuresertib + Fulvestrant	ORR	Active, not recruiting
NCT04345913	I/II	106 ParticipantsRandomised Sequential AssignmentOpen Label	mTNBC	no		Eribulin + Copanlisib	MTDPFS	Recruiting
NCT05025735	II	25 Participants Single Group AssignmentOpen Label	HR+/HER2- MBC	PI3KCA mutated	Identified by a either in a CLIA certified tumour genomic assay or ctDNA assay	Dapagliflozin + Alpelisib and Fulvestrant	Incidence of all grade hyperglycemia	Recruiting
NCT05810870 (SABINA)	II	28 ParticipantsNon-RandomisedParallel AssignmentOpen Label	Metaplastic MBC	PIK3CA/PTEN-altered	Identified by IHC	MEN1611 +/- Eribulin	CBRORR	Recruiting
NCT04762979	II	44 ParticipantsSingle Group AssignmentOpen Label	HR+/HER2- MBC	PIK3CA mutated	Identified via local testing from tumour tissue or blood	Alpelisib + AI/Fulvestrant	PFS	Recruiting
NCT05501886 (VIKTORIA-1)	III	701 Participants RandomisedOpen Label	HR+/HER2- MBC after CDK4/6i and AI	no		Alpelisib + FulvestrantGedatolisib + Fulvestrant +/- Palbociclib	PFS	Recruiting
NCT05646862 (INAVO121)	III	400 ParticipantsRandomisedParallel AssignmentOpen Label	HR+/HER2- MBC after CDK4/6 and ET	PIK3CA mutated	Detection of specified mutations via specified test	Fulvestrant + Inavolisib/Alpelisib	PFS	Recruiting
NCT04251533 (EPIK-B3)	III	137 ParticipantRandomisedParallel AssignmentDouble blind	mTNBC	PIK3CA mutated/ PTEN loss	Identified from tumour tissue	Nab paclitaxel + Alpelisib/placebo	PFSORR	Active, not recruiting
NCT05038735 (EPIK-B5)	III	234 ParticipantsRandomisedCrossover AssignmentDouble Blind	HR+/HER2- MCB after CDK4/6i and AI	PIK3CA mutated	Determined in tumour tissue	Alpelisib + Fulvestrant	PFS	Recruiting

Abbreviations: AEs: adverse event; AI: aromatase inhibitors; AR: Androgen Receptor; BC: breast cancer; CBR: clinical benefit rate; CDK4/6i: cyclin-dependent kinase 4/6 inhibitors; CLIA: Clinical Laboratory Improvement Amendments; eBC: early breast cancer; ctDNA: circulating tumour DNA; ET: endocrine therapy; HER2: human epidermial growth factor receptor 2; HR: hormone receptor; IHC: immunohistochemistry; MBC: metastatic breast cancer; NGS: next-generation sequencing; ORR: objective response rate; pCR: pathologic complete response; PHESGO: Pertuzumab/trastuzumab/hyaluronidase; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PFS: progression-free survival; PTEN: Phosphatase and TENsin homolog; RP2D: recommended phase 2 dose; TNBC: triple-negative breast cancer.

(Baselga et al., 2012; Treilleux et al., 2015; André et al., 2016). Nevertheless, it is noteworthy that HER2-positive (HER2+) mBC harbouring PTEN loss or *PI3KCA*-mut, derive PFS benefit from everolimus, suggesting that the predictive value of these alterations may vary based on the BC subtype (André et al., 2016).

Significantly, it has been recently established that, in post-menopausal luminal BC patients, PTEN loss is associated with resistance to CDK4/6i with potential clinical implications, as these two drugs represent the standard first- and second-line treatment options for mBC (Costa et al., 2020; Gennari et al., 2021). Moreover, the authors reported that low PTEN expression does not influence response to either NAC or NET, but it is associated with a shorter recurrence-free survival (RFS) in patients receiving NAC (Hayama et al., 2023).

Due to varying mechanisms of inactivation, such as promoter hypermethylation, gene deletion, expression of various interacting proteins, miRNAs, and others, the evaluation of PTEN loss may be challenging (Schmid et al., 2016; Saura et al., 2019; Baselga et al., 2017; André et al., 2016; Nuciforo et al., 2019). In addition, these studies have adopted different methods to define PTEN status, making comparisons between them difficult. So far, despite pre-clinical data, there is no evidence to present PTEN mutations as a clear and clinically useful biomarker of resistance to PI3Ki.

4.2. *High insulin levels and ^{18}FFDG-PET/CT imaging*

Metabolic alterations, including hyperglycaemia/diabetes and hyperinsulinemia, are well-known AE from PI3K/AKTis, which could theoretically reactivate the insulin receptor (IR)/PI3K/AKT/mTORC1 pathway, thereby compromising treatment effectiveness (Fruman et al., 2017). In this regard, Hopkins *et al.* showed that preventing insulin feedback, using dietary or pharmaceutical approaches, enhances PI3Ki efficacy (Hopkins et al., 2018). In further detail, in PDX models of advanced endometrial adenocarcinoma with PTEN deletion and *PIK3CA*-mut and FGFR amplified bladder cancer, the addition of ketogenic diet in combination with PI3Ki was shown to reduce the levels of phosphorylated IR, phosphorylated AKT, and phosphorylated S6, leading to decreased proliferative activity indicated by Ki67 staining (Hopkins et al., 2018).

On the other hand, the use of metformin for ten days before PI3Ki had only a minimal effect on the PI3Ki-induced elevation of glucose and insulin levels (Hopkins et al., 2018). Caffa *et al.* confirmed these results, demonstrating that in mouse models of HR+ BC, a fasting-mimicking diet (FMD), increased the activity of tamoxifen and fulvestrant by reducing circulating IGF1, insulin, and leptin and by inhibiting PI3K/AKT/mTOR signalling via upregulation of EGR1 and PTEN (Caffa et al., 2020).

Interestingly, FMD was able to inhibit PI3K/AKT/mTOR pathway, without any rebound of hyperglycaemia and hyperinsulinemia correlated with the use of PI3Ki and with PI3Ki resistance (André et al., 2019; Hopkins et al., 2018; Di Biase et al., 2017; Pizzuti et al., 2017). Taken together, the data supports the hypothesis that a ketogenic diet, FMD, or pharmacological measures, enhances PI3Ki activity by reducing blood insulin and the subsequent ability of insulin to activate IR in tumours. Consistently, in the SANDIPER trial, taselisib is more effective in Asia compared to Europe and Latin America (Dent et al., 2021). Probably, the diversity in diet or degree of insulin resistance, as well as the management of hyperglycaemia, could partially explain this discrepancy.

Apart from PI3Ki, the EverExt and subsequently the EVERMET study provided evidence that both baseline and on-treatment glycemia are associated with the efficacy of the mTOR inhibitor everolimus combined with exemestane. More specifically, in the EVERMET trial, patients who were normoglycemic at baseline and experienced on-treatment diabetes had lower PFS than patients who had higher glycemic at baseline and suffered from diabetes during everolimus-exemestane treatment (median PFS, 6.34 vs. 10.32 months; HR, 1.76; 95% confidence interval, 1.15–2.69; P = 0.008)(Vernieri et al., 2021).

Considering the impact of PI3Ki on glycolytic metabolism, it was hypothesized that the assessment of the tumour uptake of radiolabelled glucose could be a reliable predictive biomarker of response. In a phase II trial, evaluating the combination of buparlisib and letrozole in mBC, the absence of a metabolic response by fluorodeoxyglucose positron emission tomography scan (^{18}F-FDG-PET/CT), at two weeks from the beginning of therapy, was associated with rapid disease progression (Mayer et al., 2014). In the aforementioned study, the authors observed that the PI3Ki-induced spikes in insulin could cause transient increases in glucose uptake in these tumours (Hopkins et al., 2018). Although interesting, these data are still preliminary, and the role of ^{18}F-FDG-PET/CT as a biomarker of response to PI3Ki has yet to be clarified and requires further validation in larger studies.

5. AKT mutations and the role of AKT inhibitors in breast cancer

The activation of the PI3K/AKT/PTEN pathway may also be related to AKT-activating mutations which are detected in 7% of mBC (Bertucci et al., 2019). In a large-scale genomic study of human cancer AKT1 pleckstrin homology domain mutations occur in around 3% of BC (Koboldt et al., 2012). A dominant hotspot mutation has been found in glutamate 17 to lysine (E17K) substitution, and it is associated with improved efficacy of AKT-inhibitors (AKTis) in solid tumours (Nuciforo et al., 2019; Fruman et al., 2017; Hopkins et al., 2018). The inhibition of AKT is responsible for the blockade of mTORC1, thus controlling the downstream effects of the PI3K/AKT/mTOR pathway (Engelman, 2009; Smyth et al., 2020). Therefore, due to its role as a key molecular regulator of this pathway, AKT could be an interesting target. So far, the majority of AKTis investigated in clinical trials inhibits all three AKT subunits, hence they are defined as pan-AKTis.

Among these drugs, ipatasertib and capivasertib target AKT signalling *in vitro* and *in vivo* (Blagden et al., 2019; Isakoff et al., 2020; Smyth et al., 2020). Ipatasertib is a highly selective oral ATP-competitive pan-AKTis, showing encouraging activity, especially in cancers with markers of AKT activation, namely high-basal phospho-AKT levels, PTEN loss, and *PIK3CA* kinase domain mutations (Isakoff et al., 2020; Saura et al., 2017). In the phase II randomised LOTUS trial patients with metastatic TNBC had longer PFS when treated with paclitaxel plus ipatasertib with an enhanced effect in those with *PIK3CA*/AKT1/PTEN-altered tumours and PTEN loss (Kim et al., 2017). However, due to the small sample sizes and TNBC heterogeneity, interpretation within biomarker-defined subgroups was not feasible.

Conversely, in the phase III IPATunity130 trial, the addition of ipatasertib to paclitaxel did not give any benefit in *PIK3CA*/AKT/PTEN-altered HR+/HER2− mBC (Turner et al., 2022). Interestingly, in the neoadjuvant setting, the ongoing phase II trial, BARBICAN trial (NCT05498896), is evaluating the effects of adding ipatasertib to chemotherapy and atezolizumab in patients with and without *PIK3CA*/AKT1/PTEN genetic alterations. The IPATHER trial (NCT04253561) is evaluating if the combination of ipatasertib, trastuzumab, and pertuzumab is safe and effective in patients with HER2+ mBC harbouring *PIK3CA*-mut.

Capivasertib (AZD5363) is a selective ATP-competitive pan-AKT kinase inhibitor of the three AKT isoforms (AKT1, AKT2, and AKT3) (Landel et al., 2020). It has been evaluated both *in vitro e in vivo* showed to inhibit tumor growth of mouse xenograft models with HR/HER-2 BC harbouring PI3KCA, AKT1 and PTEN mutations (Luboff and DeRemer, 2024).

In 2020, the FAKTION trial showed that capivasertib plus fulvestrant improved PFS in patients with AI-resistant mBC (Jones et al., 2020). Moreover, although there was not enough statistical power to confirm this finding, the effect size of capivasertib was independent of PI3K pathway alteration status. However, the expanded biomarker analysis using the FoundationOne CDx (F1CDx) Next Generation Sequencing (NGS) Clinical Trial Assay (Foundation Medicine, Cambridge, MA, USA), suggested that capivasertib predominantly benefits patients with

PIK3CA/AKT/PTEN-altered BC (Howell et al., 2022). Similarly, Smyth et al., demonstrated that capivasertib alone or in combination with fulvestrant had a favourable safety profile and had promising anticancer activity in patients with AKT1E17K-mutant HR+/ mBC (Smyth et al., 2020).

Further compelling results were derived from the phase III study CAPItello-291 phase III trial (Turner et al., 2023). This trial randomised patients with HR+/HER2- mBC, who were experiencing disease progression during or after treatment with an AI, with or without CDK4/6i, to receive capivasertib plus fulvestrant or placebo plus fulvestrant (Turner et al., 2023). The dual primary endpoint was investigator-assessed PFS evaluated both in the overall population and among patients with AKT pathway–altered (*PIK3CA*, AKT1, or PTEN) tumours. Interestingly, the capivasertib and fulvestrant combination resulted in a significantly longer PFS compared to fulvestrant alone (Turner et al., 2023). Notably, the PFS benefit was consistent in the overall population and among those with AKT-altered tumours identified with the F1CDx assay (AstraZeneca A Phase III, nd). Among 289 patients with *PIK3CA*/AKT1/PTEN-altered tumours, those treated with capivasertib-fulvestrant had a median progression-free survival (PFS) of 7.3 months (95% CI: 5.5, 9.0), whereas those treated with placebo-fulvestrant had a median PFS of 3.1 months (95% CI: 2.0, 3.7). The HR was 0.50 (95% CI: 0.38, 0.65) with a p-value of less than 0.0001. However, an exploratory analysis of PFS in the 313 (44%) patients whose tumours did not have a *PIK3CA*/AKT1/PTEN-alteration showed an HR of 0.79 (95% CI: 0.61, 1.02), showing that the difference in the overall population was mainly attributed to the results seen in the population of patients whose tumours have *PIK3CA*/AKT1/PTEN-alteration.

Moreover, it is worth mentioning that, compared to the ~40% of patients with any AKT alteration, around 50% of patients with a not-altered pathway in both arms did not have any detectable mutations in the PI3K pathway, due to the lack of available samples or pre- or post-analytical errors.

Therefore, with the information gathered so far, it is not possible to conclude the predictive potential of AKT mutations. Hopefully, future analysis of ctDNA as well as ongoing phase 3 studies will contribute to further elucidation of the predictive value of AKT pathway alterations. Following these results, in November 2023, the Food and Drug Administration (FDA) approved capivasertib with fulvestrant in patients with one or more *PIK3CA*/AKT1/PTEN-alterations detected by F1CDx assay as a companion diagnostic device (Research, C. for D.E. and FDA, nd). Table 3 summarizes the main ongoing trials evaluating AKTis in BC.

On the contrary, in the BEECH trial, the association of paclitaxel and

Table 3
Ongoing clinical trials with AKT inhibitors in early and metastatic breast cancer settings.

Trial name	Phase	Study Design	Patient population	Stratification for Pi3K/PTEN	Intervention	Primary Endpoint	Status
Early breast cancer							
NCT05498896 (BARBICAN)	II	146 ParticipantsRandomisedParallel AssignmentOpen Label	eTNBC	no	Neoadjuvant Ipatasertib + Atezolizumab and CT (Paclitaxel, Doxorubicin, Cyclophosphamide)	pCR	Active, not recruiting
Advanced breast cancer							
NCT04253561 (IPATHER)	I	25 Participants Single Group AssignmentOpen Label	HER2+ MBC	PI3KCA mutated	Ipatasertib + Trastuzumab and Pertuzumab	RP2D	Recruiting
NCT03959891 (TAKTIC)	I	77 ParticipantsNon-RandomisedParallel AssignmentOpen Label	HR+/HER2- MBC	no	Ipatasertib + Fulvestrant and AI +/- Palbociclib	TEAE	Active, not recruiting
NCT05390710	I/II	101 Participants Randomised Sequential AssignmentOpen Label	mTNBC	no	LAE005/Afuresertib/both + Nab-Paclitaxel	AEs DLT	Recruiting
NCT03853707	I/II	28 ParticipantsRandomised Parallel AssignmentOpen Label	mTNBC	no	Ipatasertib + Carboplatin/ Carboplatin and Paclitaxel/ Capecitabine and Atezolizumab	RP2DPFS	Active, not recruiting
NCT04920708 (FAIM)	II	324 Participants Randomised Parallel AssignmentOpen Label	HR+/HER2- MBC Without ctDNA Suppression	no	Fulvestrant and Palbociclib +/- Ipatasertib	PFS	Active, not recruiting
NCT04464174 (PATHFINDER)	II	54 Participants Non-randomisedParallel AssignmentOpen Label	Taxane-Pretreated mTNBC	no	Ipatasertib + Non-Taxane CT	Safety	Active, not recruiting
NCT05720260	II	56 Participants RandomisedParallel AssignmentOpen Label	Premenopausal MBC	no	Goserelin and Fulvestrant +/- Capivasertib/Capivasertib and Durvalumab/ Durvalumab	PFS	Recruiting
NCT03801369	II	320 Participants Non-Randomised Parallel AssignmentOpen Label	mTNBC	no	Olaparib + Durvalumab/ Selumetinib/Capivasertib/ Ceralasertib	ORR	Recruiting
NCT04060862 (IPATunity150)	I/III	20 Participants RandomisedParallel AssignmentDouble blind	HR+/HER2- MBC	no	Palbociclib and Fulvestrant + Ipatasertib/Placebo	PFS	Active, not recruiting
NCT04862663 (CAPItello-292)	I/III	700 Participants RandomisedParallel AssignmentDouble blind	HR+/HER2- MBC	no	CDK4/6i and Fulvestrant + Capivasertib/Placebo	safetyPFS	Recruiting
NCT04650581 (FINER)	III	250 Participants RandomisedParallel AssignmentDouble blind	HR+/HER2- MBC after CDK4/6i and AI	no	Ipatasertib and Fulvestrant	PFS	Recruiting
NCT04305496 (CAPItallo-291)	III	818 Participants RandomisedParallel AssignmentDouble blind	HR+/HER2- MBC after AI	no	Fulvestrant + Capivasertib/ Placebo	PFS	Active, not recruiting

Abbreviations: AE: adverse event; AI: aromatase inhibitors; AR: Androgen Receptor; BC: breast cancer; CBR: clinical benefit rate; CDK4/6i: cyclin-dependent kinase 4/6 inhibitors; eBC: early breast cancer; ET: endocrine therapy; HER2: human epidermial growth factor receptor 2; HR: hormone receptor; MBC: metastatic breast cancer; ORR: objective response rate; pCR: pathologic complete response; PHESGO: Pertuzumab/trastuzumab/hyaluronidase; PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PFS: progression-free survival; PTEN: Phosphatase and TENsin homolog; RP2D: recommended phase 2 dose; TNBC: triple-negative breast cancer.

capivasertib did not provide benefit in terms of PFS either in the overall population or in the *PIK3CA*-altered population determined using ddPCR (Turner et al., 2019). A possible explanation could be the small number of patients in each subgroup. In addition, due to the crosstalk between ER and PI3K signalling (Bosch et al., 2015), the lack of ER blockade may have contributed to these disappointing results. Lastly, the PAKT trial, which tested the efficacy of capivasertib in TNBC, reported a significant improvement in PFS in the overall population, however, the benefit emerged predominantly in tumours with activating mutations in *PIK3CA* or AKT1 or inactivating alterations in PTEN (Kim et al., 2017; Schmid et al., 2020).

Overall, differences in methods, as well as the retrospective and exploratory nature of these analyses, based on a small number of patients, could partially explain these results. Therefore, identifying a tumour molecular profile that can predict the response to AKTis remains challenging (Goodman).

6. Discordance in PIK3CA status between the primary tumour and metastatic tissue or ctDNA

Due to the incorporation of PI3Kis in the management of patients with HR+ BC, there are many concerns about the ideal timing to assess the presence of the predictive biomarker. Indeed, a series of methodological and analytical variables may influence the results of the search for *PIK3CA*-mut in ctDNA or tissue, both in fresh and archival tumour samples (Fusco et al., 2021). Other tumour-related factors may affect the validity of this biomarker research, namely spatial tumour heterogeneity and its clonal evolution throughout the disease.

In the pivotal SOLAR-1 study, the benefit of alpelisib was demonstrated in the *PIK3CA*-mut population even though the mutation status was assessed in samples from primary tumours in the majority (77%) of cases, and only 22% of metastatic lesions (André et al., 2019; Rugo et al). In the above-mentioned BELLE-2 study, the addition of buparlisib to fulvestrant did not significantly increase PFS compared to placebo in the PI3K pathway-activated group. However, there was a significant difference in PFS favouring the buparlisib group in a pre-planned analysis including patients with ctDNA *PIK3CA*-mut (Baselga et al., 2017). Interestingly, the primary definition of PI3K pathway status was based almost entirely (97% of cases) on archival biopsies, obtaining a median of 3.8 years before study entry, while only 3% were assessed from fresh biopsies (Baselga et al., 2017). In this study, the concordance of *PIK3CA* mutation status between biopsy and ctDNA was 77%. However, 21% of patients with *PIK3CA* wild-type tumour tissue had *PIK3CA* mutant ctDNA, suggesting tumour evolution over time (Baselga et al., 2017).

A recent meta-analysis, including data from 25 studies, provided data on the discordance rates of *PIK3CA*-mut between primary BC and their matched metastases (Rosin et al., 2023). They observed that the overall discordance rate of *PIK3CA*-mut between primary and matched metastatic tumours was approximately 10% without significant differences within BC subtypes or metastatic sites (Rosin et al., 2023). In light of this discordance and the resulting clinical implications, the authors concluded by suggesting performing biopsies of metastatic tissue for *PIK3CA*-mutation analysis.

On the contrary, data from AURORA indicated a high concordance rate of *PIK3CA*-mutational status between primary tumour and matched metastasis (Aftimos et al., 2021). An explanation for this result may be the heterogeneity of NGS techniques used in the AURORA program (Aftimos et al., 2021). Moreover, the majority of patients were enrolled early in the course of mBC, while acquired additional driver mutations represent a late event in the metastatic process. Overall, these data suggest that due to molecular evolution, selective pressure, and the increased metastatic potential of clones with a pathway alteration, it could be useful to repeat the metastatic tissue biopsy, in case a wild *PIK3CA* mutation status is obtained from the primary/archival tumour sample (Aftimos et al., 2021; Yates et al., 2018).

Given recent advancements in functional imaging and artificial intelligence (AI), virtual biopsies have emerged as advanced non-invasive diagnostic techniques used to obtain detailed information about the structure and characteristics of tissue and organs inside the body (Murray et al., 2022). This could lead to a less invasive and more personalized era in medicine.

7. Companion diagnostic test for the evaluation of *PI3KCA*-mut

In May 2019, alpelisib was approved by the FDA and by the European Medicines Agency (EMA) for the treatment of patients with advanced *PIK3CA*-mut HR+/HER2− BC previously treated with ET (FDA Approves Alpelisib for Metastatic Breast Cancer. FDA 2019..). Following its approval, *PIK3CA*-mut were included in the tier of evidence IA of genomic alterations in BC of the ESMO Scale for Clinical Actionability of molecular Targets (ESCAT) for predicting benefit from alpelisib, when used in combination with fulvestrant in the metastatic setting (André et al., 2019).

Notably, alpelisib was approved in the USA with the diagnostic test Therascreen® *PIK3CA* RGQ PCR kit, used in SOLAR-1 to select patients who had *PIK3CA*-mut in tumour tissue and/or in circulating ctDNA isolated from plasma specimens. The Therascreen® assay is a real-time qualitative PCR that can identify 11 *PIK3CA* hotspot mutations, mostly found in exons nine and 20, using genomic DNA extracted from formalin-fixed, paraffin-embedded breast tumour tissue or ctDNA from plasma.

In this regard, Martínez-Sáez *et al.* performed a comprehensive analysis of *PIK3CA*-mut in BC to understand to what extent the Therascreen® assay detects most *PIK3CA*-mut (Martínez-Sáez et al., 2020). They found that N345K mutation in exon four, representing 6% of all tumour samples with *PIK3CA*-mut, is not included in the Therascreen® assay. Even though its clinical role is still unknown, it increased sensitivity to PI3K pathway inhibition in a preclinical model (Martínez-Sáez et al., 2020). Regarding the distribution of *PIK3CA*-mut in plasma ctDNA, they analysed 48 tested plasma samples from 48 consecutive patients with HR+/HER2− mBC from the Hospital Clinic of Barcelona, using the G360 NGS platform standardised assay. It turned out that five patients (28%) had *PIK3CA*-mut were not included in the Therascreen® assay. Overall, this study demonstrated that the Therascreen® assay does not detect approximately 20–30% of patients with known *PIK3CA*-mut. Furthermore, they observed that it would not efficiently capture double *PIK3CA*-mut, since only 5% of patients known to harbour two or more *PIK3CA*-mut would have mutations represented in the Therascreen® assay (Martínez-Sáez et al., 2020). Supporting this viewpoint, Rugo et al., using comprehensive genomic profiling, detected approximately 72% *PIK3CA*-mut in tissue biopsies from 33,977 patients with mBC, demonstrating that up to 20% of patients carried *PIK3CA*-*mut,* which had not been detected in the Therascreen® assay (Pizzuti et al., 2017). Of note, this study pointed out that patients with different *PI3KCA*-mut also showed a better PFS when treated with alpelisib plus fulvestrant compared to fulvestrant alone (Pizzuti et al., 2017).

Indeed, Arsenic *et al.* evaluated 186 BC patients to find a concordant between NGS and Sanger sequencing (SGS) for the *PI3KCA* hotspot mutations and investigated additional hotspot mutations using NGS (Arsenic et al., 2015). Overall, they found a high degree of concordance between the two genomic assays as only three mutations found by NGS were not detected with SGS due to low variant frequencies (below 10%). Yet, consistent with previous reports (Rohlin et al., 2009; Walsh et al., 2006), these results showed that NGS is more sensitive compared to SGS, especially for low-frequency mutations. Notably, they discovered multiple mutations, which had not been described previously, in four cases (Arsenic et al., 2015). Although the significance of these mutations is unknown, it has been speculated that these tumours are multiclonal, and a potential second hit was necessary to induce a selective expansion, if the first mutation proved insufficient to activate the pathway (Arsenic et al., 2015). As previously described, the FDA has recently approved the F1CDx assay as a companion diagnostic device to identify mBC patients

Table 4
Multiple targets in the PI3K/AKT/mTOR signalling pathway.

Drug name	p110α	p110β	p110γ	p110δ	mTOR	AKT
Gedatolisib	X	X	X	X	X	-
MEN1611	X	X	X	-	-	-
Copanlisib	X	-	-	X	-	-
Inavolisib	X	-	-	-	-	-
Alpelisib (BYL719)	X	-	-	-	-	-
STX-478	X	-	-	-	-	-
Everolimus	-	-	-	-	X	-
Afuresertib	-	-	-	-	-	X
Ipatasertib	-	-	-	-	-	X
Capivasertib	-	-	-	-	-	X

with *PIK3CA*/AKT1/PTEN-alterations, for treatment with capivasertib with fulvestrant (Research, C. for D.E. and FDA Approves Capivasertib with Fulvestrant for Breast Cancer. FDA 2023). Similarly, in the INAVO120 trial, central testing for of *PIK3CA-mut* was done on ctDNA using F1®Liquid CDx, which was the only FDA-approved companion diagnostic able to analyzes guideline-recommended genes from a simple blood draw. On the other hand, within the same study, but in China, the central ctDNA test was PredicineCARE NGS assay (Inavolisib Combo Meets Primary End Point in PIK3CA, nd).

Given the aforementioned results, the optimal method to detect *PI3KCA*-mut in clinical practice has yet to be established. In this context, physicians may prefer alternative tests rather than the Therascreen® assay, such as NGS, which provides a more comprehensive mutational analysis of *PIK3CA*-mut. Prospective clinical trials are eagerly awaited to leverage the data and use cutting-edge technologies for better functional characterization of the *PIK3CA* mutational landscape. The aim is to predict the benefits of alpelisib or other PI3Kis in patients with *PIK3CA*-mut that have not been detected by the Therascreen® assay.

8. Conclusions

In recent years, the advent of novel PI3K/AKTis has significantly improved the treatment landscape for patients with HR+/HER2- BC. Currently, second line treatment decision rely on PI3K/AKT/PTEN pathway mutations. Therefore, as these new drugs will soon become available, there is great urgency to find predictive biomarkers that can aid clinicians in choosing an optimal, tailored therapy for BC. Although the preclinical and clinical data have provided encouraging evidence, a composite biomarker able to assess precisely PI3K/AKT/PTEN pathway activations is in high demand.

So far there is a lack of consensus regarding a standard biomarker assessment, timing of tissue collection, and the technology used. Furthermore, beyond *PIK3CA-mut*, AKT, and PTEN, mutations could also have a potential predictive role. Additionally, as PI3Ki-induced hyperglycaemia may re-activate the pathway, it will be mandatory to redesign clinical trials, including oral antidiabetic drugs or diet interventions.

In the meantime, detailed translational studies, as well as numerous phase I, II, and even III clinical trials, specifically selecting patients with *PIK3CA*/AKT/PTEN altered BC, are currently underway. The findings of these studies are eagerly awaited as they have the potential to improve our understanding of the complex PI3K/AKT genomic landscape, and consequently, assess biomarkers of sensitivity and resistance.

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M. Sirico, F. Jacobs and C. Molinelli: Conceptualization, Data curation. G. Nader-Marta, V. Debien and E. de Azambuja: Writing-original draft, Writing-review & editing. **C. Gianni, U. De Giorgi, F. Merloni, M. Palleschi, H. Faith Dewhurst**: Conceptualization, Data curation, Formal analysis, Methodology, Writing-original draft, Writing-review & editing.

Declaration of Competing Interest

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