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**Title:** Targeting the PI3-kinase pathway in triple negative breast cancer.

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**Abstract:**

Triple negative breast cancer (TNBC) is characterised by poor outcomes and historical lack of targeted therapies. Dysregulation of signalling through the PI3 kinase and AKT signalling pathway is one of the most frequent oncogenic aberrations of triple negative breast cancer. Although mutations in individual genes occur relatively rarely, combined activating mutations in *PIK3CA* and *AKT1*, with inactivating mutations in *PTEN*, occur in approximately 25-30% of advanced TNBC. Recent randomised trials suggest improved progression-free survival with AKT-inhibitors in combination with first-line chemotherapy for patients with TNBC and pathway genetic aberrations. We review the evidence for PI3 kinase pathway activation in TNBC, and clinical trial data for PI3 kinase, AKT and mTOR inhibitors in TNBC. We discuss uncertainty over defining which cancers have pathway activation and the future overlap between immunotherapy and pathway targeting.

**Keywords:** triple-negative breast cancer, PI3K, AKT, PTEN, targeted therapy, predictive biomarkers.

**Key Message:** The PI3 kinase and AKT signalling pathway is frequently altered in TNBC, with two phase 2 clinical trials showing evidence for improved disease-free survival with AKT inhibitors selectively in pathway altered patients, although at a cost of increased toxicity. The optimal methods of selecting patients for AKT inhibition, and the role of alternative pathway inhibitors, remain unanswered.

## INTRODUCTION

Triple negative breast cancer (TNBC) is the most aggressive histological subtype of breast cancer, characterised in the advanced setting by short responses to chemotherapy, adverse overall survival and until recently a lack of routine targeted therapies with non-selective chemotherapy being the mainstay of treatment [1]. Approximately 10% of TNBC harbour inactivating mutations in *BRCA1* or *BRCA2*, with substantial evidence that platinum chemotherapy offers improved outcome for this subpopulation, both in early and metastatic scenarios [2-4]. Recently phase III studies of PARP inhibitors have also shown substantial activity in *BRCA1 or BRCA2* mutant breast cancer [5-7], demonstrating the potential of mutation directed therapies and crucial importance of biomarkers in TNBC therapy selection [8]. Recently the further relevance of biomarkers, and segmentation of TNBC to guide therapy, has been demonstrated for immunotherapy, with substantial activity of atezolizumab in TNBC with PDL1+ve infiltrating immune cells. The era of treating TNBC as a single cancer type with unselective chemotherapy has closed.

Mutations in the PI3 kinase pathway characterise a major additional subgroup of TNBC, with substantial evidence from phase II trials to suggest this subgroup may be targeted therapeutically. The phosphoinositide 3-kinase (PI3K) signalling pathway has an important role linking receptor tyrosine kinase signalling in breast cancer to the regulation of cell growth and survival, and its molecular mechanisms, although continuously refined, have long been well described [9]. Downstream of PI3K the most relevant nodes are protein kinase B (also known as AKT) and mammalian target of rapamycin (mTOR). The pathway is regulated by multiple phosphatases, including phosphatase and tensin homolog (PTEN) and inositol

polyphosphate-4-phosphatase type II B (INPP4B) (figure 1). In cancer this pathway plays a fundamental oncogenic role, extensively interacting with other canonical signalling pathways, to drive tumour evolution and resistance to therapies [10]. Multiple genomic alterations lead to a hyper-activated PI3K pathway including activating events in oncogenes *PIK3CA*, *AKT* and *MTOR* or inactivating events in tumour suppressor genes such as *PIK3R1*, *INPP4B, PTEN, TSC1, TSC2, and LKB1.* Mutations in *PIK3CA* are the single most common event in breast cancer, mutated at substantially higher rates in hormone receptor positive breast cancer compared to TNBC as a whole [11]. Nevertheless, *PIK3CA* is the second most frequently mutated gene after *TP53* in TNBC, with additional inactivating alterations in *PTEN* [12], and additional activating mutations in *AKT1*. Summed together pathway mutations/alterations occur in approximately 25% of primary TNBC, and possibly at a modestly higher frequency in metastatic TNBC.

Here we review the mechanisms through which PI3 kinase pathway is activated in TNBC, and recent clinical trials suggesting this pathway can be targeted for therapy.

## ACTIVATION OF THE PI3K PATHWAY IN TNBC

### *PIK3CA* activating mutations

The phosphoinositide-3 kinases (PIK3s) are intracellular signalling enzymes that phosphorylate the free 3- hydroxyl of the phosphoinositides in the cell membrane. The different PI3Ks are commonly grouped in different classes, with class I PI3 kinase the most commonly altered in cancer, formed of a heterodimer composed by a regulatory (p85) and a catalytic (p110) subunit. There are multiple paralogs of the regulatory subunits (p85α and p85β), and the catalytic subunits (p110α, β, γ and δ)

[13]. In triple negative breast cancer, the vast majority of activating mutations occur in the p110α (alpha subunit encoded by *PIK3CA*) overall mutated in 9% of primary TNBC. The rate of mutations in *PIK3CA* in advanced TNBC is likely enhanced reflecting a subset of originally ER positive breast cancer that relapse losing ER expression, becoming “secondary” TNBC, yet retain the high rate of *PIK3CA* mutations overserved in ER positive breast cancer [14]. *PIK3CA* mutations result in activated alpha PI3 kinase, leading to an enhanced phosphorylation and accumulation of PtdIns-3,4,5-P3 and/or PtdIns-3,4-P2 in the membrane, thus activating downstream pathway.

### ***AKT1*** **activating mutations**

*AKT1* mutations are found in multiple tumour types including breast cancer, with ~2.5% breast cancer having a mutation resulting in a single amino acid substitution E17K first described by Carpten et al. [15]. The *AKT* family of three different isoforms (*AKT1*, *AKT2* and *AKT3)* forms a node downstream of PI3 kinase [16]. AKT signalling has many functions in the cell such as survival, cell growth, cell cycle regulation and metabolism. An anti-apoptotic function has been previously reported through phosphorylation and inhibition of anti-apoptotic proteins such as BAD and BAX (both implicated in caspase pathway) and also NF-κB (serving as a transcriptional factor for expression of anti-apoptotic genes) which are activated in response to stress leading to cell death. Therefore the combination of AKT inhibition and chemotherapy (acting as stress inductor) has been studied and proved to be synergistic pre-clinically [17]. Recent studies have attributed isotype specific roles to each isoform. AKT1 upregulation has been shown to modulate cell proliferation through S6 and cyclin D1, whilst AKT2 regulates cytoskeleton components [18]. Interestingly, AKT3 isoform has been identified as overrepresented specifically in

TNBC both at DNA and mRNA levels, inducing cell proliferation and tumour growth but not promoting invasiveness [19].

As well as being mutated in TNBC, AKT phosphorylation is strongly enhanced in TNBC compared to luminal breast cancer, a marker of AKT activation in TNBC [14]. Outside *AKT1* E17K activating mutation, there is uncertainty over alternative AKT activation genetic events. *AKT1* L52R, Q79K and D323H mutations have also been found to promote *AKT1* activity compared to wild-type, but are rare [20]. Also, very rare E17K mutation have also been identified in *AKT2* [21] and *AKT3* [22], both resulting in activation of final AKT protein and both being targetable. Beyond these hotspot mutations, some potentially targetable recurrent indels in the pleckstrin homology domain of both *AKT1* and *AKT2* have been identified, with downstream activation and sensitivity to AKT inhibitors confirmed [23]. All AKT activating genetic alterations, other than AKT1 E17K, are rare in TNBC.

### *mTOR* activating mutations

mTOR is a serine-threonine kinase that can form two complexes mTORC1 and mTORC2, defined by binding to RAPTOR and RICTOR respectively, which are activated both downstream of AKT (mTORC1) and phosphorylate AKT (mTORC2). Mutations in *mTOR* are found in just 1.8% cases in TCGA primary breast cancer, with only a small minority recognised as putative drivers. Other rare mutations that activate mTOR have been found such as mutations in the tumour suppressor *LKB1* which upregulates mTOR and activation of downstream pathway [24] and also mutations arising in the TSC1-TSC2 complex, which is a negative regulator of mTORC1 and promote activation of mTORC2 [25]. In contrast to genetic activation, mTOR is activated both by PI3 kinase and MAPK signalling, and allosteric inhibitors

of mTOR have substantial activity in ER positive breast cancer where mTOR inhibitor everolimus has approval for the treatment of previously treated advanced HR+/HER2- breast cancer [26]. The possible role of everolimus in TNBC is discussed later.

### *PTEN* inactivating mutations/loss

*PTEN* gene on the chromosome 10 was first identified as a tumour suppressor in 1997 by a number of different groups [27-29] and shortly after associated with Cowden syndrome, an autosomal dominant condition in part caused by *PTEN* germline mutation, which is associated to an increased risk of malignancies including breast cancer [30]. The PTEN protein serves as a tumour suppressor through its 3'-phosphatase action dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate ($PIP_3$) resulting in an inhibition of AKT and hence the rest of the signalling cascade. A phosphatase-independent function in the nucleus serving as a chromosomal stability controller has been described [31] and more recent evidence also suggest that PTEN acts as an scaffold protein in both the nucleus and cytoplasm [32]. PTEN contains two key domains for its tumour suppression function: the phosphatase domain and the C2 domain. Loss of PTEN causes activation of PI3 kinase, and in particular the PI3 kinase beta (PIK3CB) isoform through its lipid kinase domain [33, 34], and consequently tailored inhibition of this isoform in the context of PTEN-deficiency has been proposed [35].

Loss of PTEN either at genomic or proteomic level is associated with an increased risk of breast cancer and associated with worse prognosis, decreased expression of the oestrogen receptor and overall adverse phenotypes [36-38]. *In vitro* continued exposure to trastuzumab treatment of HER2+ cells harbouring PTEN

loss caused transformation to TNBC via epithelial-mesenchymal transition (EMT) regulation [39]. In the TNBC subset of an african-american and hispanic/latina women study involving 318 patients it was noted that TNBC associated with loss of PTEN and a CD44+/CD24- phenotype (a marker of stem-cellness). Upon the african-american cohort PTEN loss resulted in poorer disease-free survival [40]. Also, in a tissue microarray of 1000 primary breast cancers from Middle Eastern ethnicity, *PTEN* loss using immunohistochemistry and fluorescence in situ hybridization (FISH) was significantly associated with large tumour size, high grade, recurrence and triple negative phenotype [41].

PTEN alterations are frequent in TNBC, with genetically mediated loss of function occurring in approximately 15% [14]. AKT inhibition pre-clinically has higher activity in cancers with in PTEN deficient models [42], yet there is a major challenge in determining which PTEN alterations confer sufficient loss of function to be targetable, and the optimal method for identifying this loss of function. Inactivating mutations include truncating and frameshift mutations, and homozygous deletion are considered to be targetable inactivating mutations. In contrast, single nucleotide variants (SNV's) are generally of uncertain pathogenicity, many likely passenger mutations, although some hotspots such as R130X, R233X and R335X are also likely pathogenic [43, 44].

Loss of heterozygosity (LOH) resulting from allelic loss in loci of the 10q23 region, and heterozygous deletion, are generally of uncertain pathogenicity [45]. However, there is uncertainty on whether the classical two-hit model for loss of function for a tumor suppressor gene applies to PTEN. Silencing mutations arising in a single wild-type allele might lead to haploinsufficiency as PTEN mutant heterodimerize with wild-type resulting in hypo-functional PTEN protein [46]. Epigenetic regulation

through silencing of the promoter via methylation is also observed [47], that may inactivate the wild-type allele in heterozygous events to fully inactive PTEN function.

The comprehensive molecular landscape of breast cancer carried out by The Cancer Genome Atlas Network showed PTEN mutation/loss of expression occurred in up to 35% in basal-like TNBC, substantially higher than the rate of genetic alterations. Loss of PTEN expression has been most commonly assessed via immunohistochemistry (IHC), although it is unclear if PTEN loss of expression solely by IHC, without a genomic basis, is a strong enough surrogate for selecting patients for pathway inhibition, as we discuss below. Moreover, different PTEN antibodies have different rates of PTEN loss of expression.

**INPP4B loss**

Inositol polyphosphate 4-phosphatase type II (INPP4B) is a second phosphatase in the PI3 kinase pathway, with knockdown resulting in an enhanced AKT pathway. Decreased INPP4B expression, only uncommonly resulting from genomic events, is very frequent in basal-like breast cancer [48, 49]. INPP4B dephosphorylates phosphatidylinositol (3,4)-bisphosphate ($PIP_2$), instead of the phosphatidylinositol (3,4,5) triphosphate ($PIP_3$) targeted by PTEN. However, in situations of PTEN deficiency it also seems to be involved in $PIP_3$ dephosphorilation [50].

**Cross-talk and functional markers of PI3K activation**

Non-canonical PI3K regulation trough convergence with other pathways has been long recognised, in particular cross-talk with RAS-MAPK being able of both activating or inhibiting PI3K pathway [51], although mutations in MAPK pathway are

not frequent in TNBC. Biomarkers indicating PI3K pathway hyperactivation can also be found downstream at the mRNA and protein level. On a wide multiplatform study including DNA aberrations, mRNA and proteomics interrogation across all major types of cancer, it was found that patients sharing a PI3K/AKT signature correlated at the protein expression level with increased phospho-AKT, GSK3, PRAS40 and TSC [52]. It is unknown if measuring markers of pathway activation could present an alternative approach to predicting sensitivity to pathway inhibitors.

## TNBC SUBTYPES ASSOCIATION WITH ALTERATIONS IN PI3K PATHWAY

Although commonly grouped under the same entity in terms of clinical simplification and prognosis, TNBC can be subdivided into different molecular subtypes that exhibit different behaviours and underlying activation of PI3 kinase pathway. Lehmann et al. using gene expression classifiers identified distinctive TNBC subtypes. In summary basal-like 1 and 2 subtypes were identified (BL1 and BL2), a mesenchymal subtype (M), a mesenchymal stem-like (MSL) subtype that has overlap with claudin-low subtypes and a luminal TNBC subtype luminal androgen receptor (LAR). A further subtype of immunomodulatory (IM) reflects tumors with high lymphocytic infiltration [53].

Large comprehensive studies have shown mutual exclusivity between *PIK3CA* and *AKT1* mutations, although *PIK3CA* and *PTEN* may be found co-mutated [54]. Substantial evidence now shows distinct differences in pathway activation between TNBC subtypes [55]. *PIK3CA* and *AKT1* mutations are relatively uncommon in basal-like TNBC whereas INPP4B is frequently inactivated in basal-like TNBC [56], likely enhancing AKT activation in these tumors [49], and potentially contributes to

sensitivity to AKT inhibitors in this subtype. Lower expression of PTEN protein has been found in basal-like subtype compared with other subtypes with heterozygous loss of PTEN copy number identified in 46.1% [55, 57]. In contrast, *PIK3CA* and AKT1 mutations are more common in luminal TNBC with *PIK3CA* found up to 40% of androgen receptor positive TNBC [55, 58]. Mutations of *PTEN* are possibly more common in LAR TNBC [55]. MSL subtype also display high frequency of *PIK3CA* mutations (23%) [55].

## CLINICAL DATA OF STUDIES TARGETING PI3K PATHWAY IN TNBC

There has been substantial progress in targeting the PI3 kinase pathway in breast cancer. The mTOR inhibitor everolimus in HR+ metastatic breast cancer [26] has substantial activity, although everolimus is similarly active in *PIK3CA* wildtype and mutant cancers, likely suggesting that mTOR is sufficiently down stream of PI3 kinase, with multiple inputs from MAPK signalling and LKB1, to be a therapy largely independent of PI3 kinase activation in the clinic. More recently multiple phase III studies have shown that PI3 kinase inhibitors are active in *PIK3CA* mutant HR positive breast cancer, two with pan-class I PI3 kinase inhibitor buparslisib in BELLE-2 and -3 [59, 60], one with the β-sparing inhibitor taselisib in SANDPIPER [61]and one with alpelisib alpha selective inhibitor in SOLAR-1 [62]. However, the clinical development of pathway inhibitors in TNBC has only recently started to show some evidence of activity for targeted therapies.

### PI3 kinase inhibitors

Following the efficacy of buparlisib in BELLE-2 and -3, buparlisib was also tested in combination with paclitaxel or placebo in the BELLE-4 trial, a study conducted in HER2 negative patients who had not received previous chemotherapy for advanced disease and where TNBC patients represented around 25% [63]. After an adaptive interim analysis 338 patients enrolled it was concluded that the addition of buparlisib to paclitaxel did not improve progression-free survival (PFS) in the overall study, with median PFS 9.2 in the placebo group versus 8.0 months with buparlisib (HR 1.18, 95% CI 0.82-1.68). The TNBC patients showed a worse prognosis with the addition of buparlisib with 5.5 versus 9.3 months in the placebo group (HR 1.86, 95% CI 0.91-3.79). The results were communicated including 125 patients displaying a *PIK3CA* mutation or a PTEN loss of expression by IHC for which also no benefit was found for the addition of the targeted agent (HR 1.17, 95% CI 0.63–2.17). The adverse events (AEs) more frequently appearing in the combination group were diarrhoea, alopecia, rash, nausea and hyperglycaemia, being this toxicity profile quite characteristic of PI3 kinase inhibition. Duration of paclitaxel was lower in the buparlisib group, and the dose intensity not reported, suggesting that the toxicity of buparlisib may have compromised the delivery of the backbone chemotherapy. Following the results from this trial it appeared that TNBC do not benefit of the addition of a PI3 kinase inhibitor, although no selection in TNBC was made for the minority of patients with *PIK3CA* mutations, and caution must be applied for generalising the results. Two open-label phase II clinical trials recruited TNBC patients to be treated with buparlisib as a single agent (NCT01790932, NCT01629615) although no results have been published so far.

The dominance of *PIK3CA* mutations in specific subset of TNBC suggests the potential for combination targeted therapy, with *PIK3CA*-mutant TNBC both showing sensitivity to CDK4/6 inhibition [64] and AR targeting [58] suggesting combination approaches. A phase Ib clinical trial assessing the combination of the β-sparing *PIK3CA* inhibitor taselisib in combination with palbociclib in metastatic breast cancer includes a cohort of TNBC and has already finished its recruitment (NCT02389842). Another phase I clinical trial is combining the α-specific *PIK3CA* inhibitor alpelisib with enzalutamide in AR+ and PTEN+ breast cancer including a cohort of TNBC (NCT03207529). The results from these trials may give us a better perspective on how PI3 kinase inhibition affects triple-negative patients.

In TNBC models it was shown that pan-PI3K inhibitor buparlisib downregulated BRCA1/2 expression leading to homologous recombination deficiency, with combination efficacy of buparlasib and the PARP inhibitor olaparib in *BRCA1* WT TNBC [65]. In parallel, a second study also showed combined enhanced activity in *BRCA1*-mutant models [66]. A subsequent phase I trial of the buparlisib and olaparib combination reported activity in both germline *BRCA*-mutant and wild type recurrent breast (some of which where TNBC) and ovarian cancer, albeit requiring a reduction in buparlisib dose [67].

**AKT inhibitors**

Early AKT inhibitors, such as perifosine and MK2206, failed to show clinical impact in phase II trials ([68], NCT01277757), but more modern AKT inhibitors showed promising responses in phase I trials [69, 70]. Recently there is preliminary evidence of substantial activity of AKT inhibtors of two different phase II clinical trials in first-line treatment: LOTUS and PAKT (table 1). The randomized, placebo-

controlled, phase-II LOTUS trial enrolled 124 patients with locally advanced or metastatic triple negative breast cancer and randomized them to first-line weekly paclitaxel +/- ipatasertib, an ATP-competitive pan-AKT inhibitor. This trial showed an increase in PFS from 4.9 to 6.2 months (HR 0.60, 95% CI 0.37–0.98; *p*=0.037) with the addition of the AKT-inhibitor ipatasertib to paclitaxel in the intention-to-treat population [71]. A co-endpoint analysis using PTEN IHC as a biomarker demonstrated no enrichment for benefit in PTEN IHC low cancers(defined as IHC 0 in at least 50% of tumour cells, HR 0.59, 95% CI 0.26–1.32: *p*=0.18). In contrast, a non-stratified secondary endpoint analysis involving 42 patients with *PIK3CA/AKT1/PTEN*-mutated tumors assessed by tumor sequencing showed median PFS improvement from 4.9 months in the placebo group versus 9.0 months with ipatasertib (non-stratified HR 0.44, 95% CI 0.20–0.99, *p*=0.041). Interestingly, out of the 15 patients with a *PTEN* inactivating alteration with PTEN IHC assessment, 15 (93%) showed a PTEN protein expression loss. However, only 28.57% with PTEN loss by IHC had a *PTEN* genetic alteration. This difference in efficacy suggested that PTEN IHC was unhelpful, and that genomic mutations were likely more useful in selecting patients likely to benefit from AKT inhibition. The results for overall survival (OS) at 50% OS events in both arms suggested OS advantage in the ITT population from 18.4 to 23.1 months (HR 0.62, 95% CI 0.37-1.05), with greater benefit in the pathway altered tumors. In terms of safety, this trial showed that the most common grade 3/4 toxicity were diarrhoea (arising in 23% of ipatasertib-treated versus 0% in placebo-treated), decreased neutrophil count (8% vs 6%) and neutropenia (10% vs 2%). Overall, serious adverse events were reported in 28% of the experimental group versus 15% of the placebo. A phase 3 trial of

ipatasertib for PI3K-pathway alterations including first-line treatment for TNBC patients is already ongoing (NCT03337724).

A similar phase II double-blind, placebo-controlled study enrolling 140 patients with metastatic TNBC with no prior treatment for metastatic disease or taxane treatment during the previous 12 months has recently showed an advantage in median PFS from 4.2 months on paclitaxel plus placebo versus 5.9 months with capivasertib plus paclitaxel (HR 0.75, 95% CI 0.52-1.08; one-sided *p*=0.06). In addition, an OS benefit was also observed for patients in the capivasertib group with median OS increasing from 12.6 to 19.1 months compared with placebo (HR 0.64, 95% CI 0.40-1.01; one-sided *p*=0.02). Similar to LOTUS a pre-planned analysis of the *PIK3CA*/*AKT1*/*PTEN*-altered tumors by central assessment suggested a median PFS of 3.7 months with placebo versus 9.3 months with capivasertib group (HR 0.30, 95% CI 0.11- 0.79; two-sided *p*=0.01), and similarly improvements in overall survival (HR 0.37, 95% CI 0.12-1.12; two-sided *p*=0.07). No significant differences were observed for the *PIK3CA*/*AKT1*/*PTEN*-non-altered tumors in PFS or OS, highlighting the importance of biomarker assessment for better selection of these inhibitors. Similar to ipatasertib, the most frequent serious adverse event in the trial was diarrhoea (13.2% vs 1.4%) and fatigue (4.4% vs 0%), potentially with a higher incidence of rash (4.4% vs 0%) and infections (4.4% vs 1.4%). In this study, the rates of neutropenia were similar between both treatment groups [72].

The biomarker selection results of LOTUS and PAKT do contrast with results from prostate cancer. In a phase Ib/II study conducted by de Bono et al. 253 metastatic castration-resistant prostate cancer patients previously treated with docetaxel were randomized to one of three treatment arms to be treated with ipatasertib 400 mg,

ipatasertib 200 mg or placebo in combination with abiraterone acetate 1000 mg and prednisone 10 mg daily. Median radiographic PFS (rPFS) for the ITT population was 8.18 months with ipatasertib 400 mg schedule versus 6.37 months with placebo (HR 0.75, 90% CI 0.54-1.05; *p*=0.17). PTEN loss was observed in 43% (71/165) tested tumors associated with an improved rPFS (HR 0.39, 90% CI 0.22-0.70) [73]. It must be noted that this study used the CST138G6 PTEN antibody, whereas LOTUS trial used clone SP218, a difference that could conceivably account for differences observed between studies. In this particular study no associations could be found for efficacy based on stratification at the genomic level. Although this suggests that PTEN IHC is useful in prostate, but not in breast cancer, this raises some doubts over the conclusion that PTEN IHC is unhelpful in breast cancer patient selection.

Capivasertib has also been tested in ER+/HER2- metastatic breast cancer in BEECH study, a phase II clinical trial assessing weekly paclitaxel plus capivasertib 400mg or placebo for patients not previously treated with chemotherapy showing negative results. Median PFS was 10.9 months for capivasertib plus paclitaxel versus 8.4 months on placebo plus paclitaxel (HR 0.80, 80% CI 0.6–1.06, *p*=0.31). A pre-specified analysis in *PIK3CA* mutated subgroup revealed a median PFS of 10.9 versus 10.8 months for capivasertib versus placebo added to paclitaxel (HR 1.11, 80% CI 0.73–1.68, *p*=0.76), showing no benefit increased based on this selection [74].

Ipatasertib has been explored in neoadjuvant TNBC in a phase II study randomizing patients to receive weekly paclitaxel plus ipatasertib or placebo before surgery. Endpoints were pCR rates in ITT, PTEN-low population assessed via IHC and PIK3CA/AKT1/PTEN-altered tumors using NGS. Addition of ipatasertib showed a

numerically but non-significant increase in pCR rates in those 3 groups, with differences more pronounced in patients with PTEN-low tumors (32% vs 6%) and PIK3CA/AKT1/PTEN-altered tumors (39% vs 9%) [75].

In a similar way PI3K and PARP inhibitors combined have demonstrated some efficacy in early-phase trials, AKT inhibitors could potentially be also more effective when combined with PARP inhibitors. A phase II trial looking at different combinations for olaparib included an arm where patients receive both olaparib and the AKT inhibitor capivasertib (NCT02576444).

**mTOR inhibitors**

The mTOR inhibitor everolimus in combination with exemestane has activity in HR+/HER2- metastatic breast cancer [26], although there is suggestions of activity of everolimus in other subtypes. The combination of everolimus with first-line trastuzumab plus paclitaxel in HER2 positive metastatic breast cancer patients was assessed in a large phase 3 trial involving 719 patients including both HR-positive and negative conducted by Hurvitz et al [76]. In the ITT population no differences between treatment arms were observed with median PFS 14.95 months with everolimus versus 14.49 months with placebo (HR 0.89, 95% CI 0.73–1.08; *p*=0.12). However, when selecting the 311 HR-negative patients, differences in efficacy appeared. Median PFS was 20.27 versus 16.56 with the addition of everolimus (HR 0.66, 95% CI 0.48–0.91; *p*=0.0049) although not reaching the pre-specified significant level (*p*=0·0044). Even though not strictly statistically significant, this numerical difference strongly suggested that this combination might be more beneficial in HR negative HER2 positive breast cancer. The biomarker stratification taken out from this study in addition to the results from BOLERO-3 study which

stratified trastuzumab-resistant HER2 positive patients to weekly trastuzumab and vinorelbine plus everolimus or placebo showed in combination that activation of the PI3K-pathway in HER2 positive patients could potentially identify patients more likely to benefit from everolimus [77].

A phase I study exploring the combination of the chemotherapeutic agent liposomal doxorubicin, the antiangiogenic agent bevacizumab and the mTOR inhibitors temsirolimus or everolimus included 52 patients with metaplastic TNBC [78]. The selection for metaplastic TNBC was used as a surrogate of MSL subtypes, known to have relatively frequent PIK3-pathway alterations as well as high expression of vascular endothelial growth factor (VEGF). The objective response rate (ORR) was 21% including 4 complete responses and 7 partial responses. 32 (74%) patients were found to display a PIK3-pathway alteration defined by a mutation in the pathway detected by sequencing or a PTEN loss of expression by IHC. Interestingly, PIK3-athway activation was found to correlate with improved response rates ($p$=0.04). A phase I assessing the combination of everolimus and eribulin in previously treated metastatic TNBC patients is also ongoing (NCT02616848), but no results have been published yet.

**Dual inhibitors**

Inhibition of mTOR, AKT and PI3 kinase initiates feedback loops that potentially limit the effectiveness of these agents. mTOR inhibition was found to upregulate upstream receptor tyrosine kinases (RTKs), resulting in rebound activation of AKT [79]. AKT inhibition initiates FOXO-dependent transcription and activation of RTKs [80]. PI3 kinase inhibition, although preventing AKT activation, also results in enhanced MAPK signalling [81]. Taken together, this evidence stands as a good

rationale for investigating dual inhibitors of the PI3 kinase-AKT-mTOR pathway that may control both target pathway activation and feedback loop in response to it, thus preventing or delaying resistance. For example, the combination of mTOR and AKT inhibitors on basal-like patient-derived xenograph models showed synergistic efficacy [82].

However, the class of PI3K/AKT/mTOR dual-blockade agents has been severely limited in clinical activity due to adverse effects. For example the catalytic mTOR inhibitor AZD2014, which inhibits both mTORC1 and mTORC2, was less effective in the clinic than everolimus in HR positive breast cancer, despite preclinical evidence of higher activity of AZD2014 [83]. A number of PI3K/mTOR inhibitors continue in clinical development including gedatolisib (PF-05212384), with activity in breast cancer and acceptable safety profile and tumoural activity [84]. Bimiralisib (PQR-309) [85] and apitolisib [86] represent other inhibitors in early development.

**ONGOING TRIALS**

In particular with evidence of AKT inhibitor activity in pathway mutant TNBC, there are several ongoing clinical trials exploring PI3-kinase pathway inhibition (table, S1). Ipatasertib is now being tested in combination with paclitaxel compared to paclitaxel and placebo as upfront treatment for TNBC with *PIK3CA*/*AKT1*/*PTEN*-altered tumors in the phase III IPATunity130 trial [87], and phase III trials are planned with capivasertib.

Current evidence suggests that AKT targeting for TNBC has most efficacy in pathway-aberrant tumors. It will be important to establish in future phase III trials if all

genetic events share a similar degree of sensitivity to AKT inhibition, if they all benefit equally from AKT inhibition or if individual genetic events (e.g. *AKT1* mutations) derive more benefit than others.

## IMMUNOTHERAPY VERSUS TARGETED THERAPY

Immunotherapy for the treatment of cancer has been a revolution in last years but clinically meaningful efficacy for breast cancer patients has remained elusive until recently. Previous work had established that immune biomarkers as programmed cell death ligand 1 (PD-L1) are overexpressed in TNBC [88] and early clinical evidence of activity of immune agents targeting both PD-1 and PD-L1 on breast cancer patients showed that TNBC was the subtype where most durable clinical activity is found [89, 90]. More recently, a phase 3 trial with the PD-L1 inhibitor atezolizumab in addition to nab-paclitaxel demonstrated benefit in PFS for TNBC patients when used as upfront treatment [91].

Although immuno-oncology and targeted inhibition of the *PI3KCA/AKT/mTOR* pathway can be seen as non-redundant approaches to tackle the disease there is emerging evidence that they can be combined to exploit potential synergy. PTEN loss has been demonstrated to confer resistance to PD-L1 blockade in pre-clinical work based on melanoma models and combined treatment with a PIK3β inhibitor and anti-PD-L1 resulted in enhanced tumor growth inhibition [92]. This immunoresistance based on PTEN loss has also been found in other tumor types [93]. Now that AKT inhibition and immunotherapy have both demonstrated clinical efficacy in large clinical trials designed for TNBC population it seems important to

explore potential triplet combinations, and studies have also been setup to test such combinations (NCT03424005, NCT03395899).

Caution will have to be taken in investigating potential biomarkers to predict increased sensitivity to PI3K pathway inhibitors in combination with immunotherapy, as PI3K pathway signalling plays major roles in immune cell function and immune surveillance. It is therefore possible that the tumor derived biomarkers may predict sensitivity to PI3 kinase pathway inhibitors less effectively in combination, with the potential need to consider markers of PI3K pathway activation in tumor infiltrating lymphocytes and other immune cells.

## CONCLUSIONS

Enormous effort has been put into development of new strategies for TNBC patients and the era of precision medicine for TNBC has finally arrived. Increasing evidence suggests the potential of AKT inhibitors in cancers with genetic alterations in the *PI3KCA/AKT1/PTEN* axis, with phase III trials ongoing. Whether activity is limited to pathway inhibitors has not been fully resolved by the current studies, with the low expression of INPP4B in the substantial majority of basal-like cancers raising the possibility of activity outside pathway mutant cancers, along with the potential for AKT inhibitors to synergise with immune checkpoint inhibitors if sufficiently tolerable. The possible role of everolimus in TNBC has not been extensively explored, as has the potential role of alpha selective PI3 kinase inhibitors in *PIK3CA* mutant TNBC.

Selection of *PI3KCA/AKT1/PTEN* alterations appears to optimize the selection of patients for AKT inhibition, although the potential role of PTEN status assessment by

IHC remains unanswered. The survival advantage in the existing phase II trials has come at a cost of increased toxicity. These data suggest the potential for precision medicine in TNBC, and the further sub-dividing of TNBC into therapeutically defined subtypes. The era where TNBC lagged behind other breast cancer subtypes in innovation appears to be over. Now substantial evidence suggests distinct therapeutic approaches for different TNBC subtypes, with the potential to be translated to substantial improvements in overall survival.

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**FIGURES**

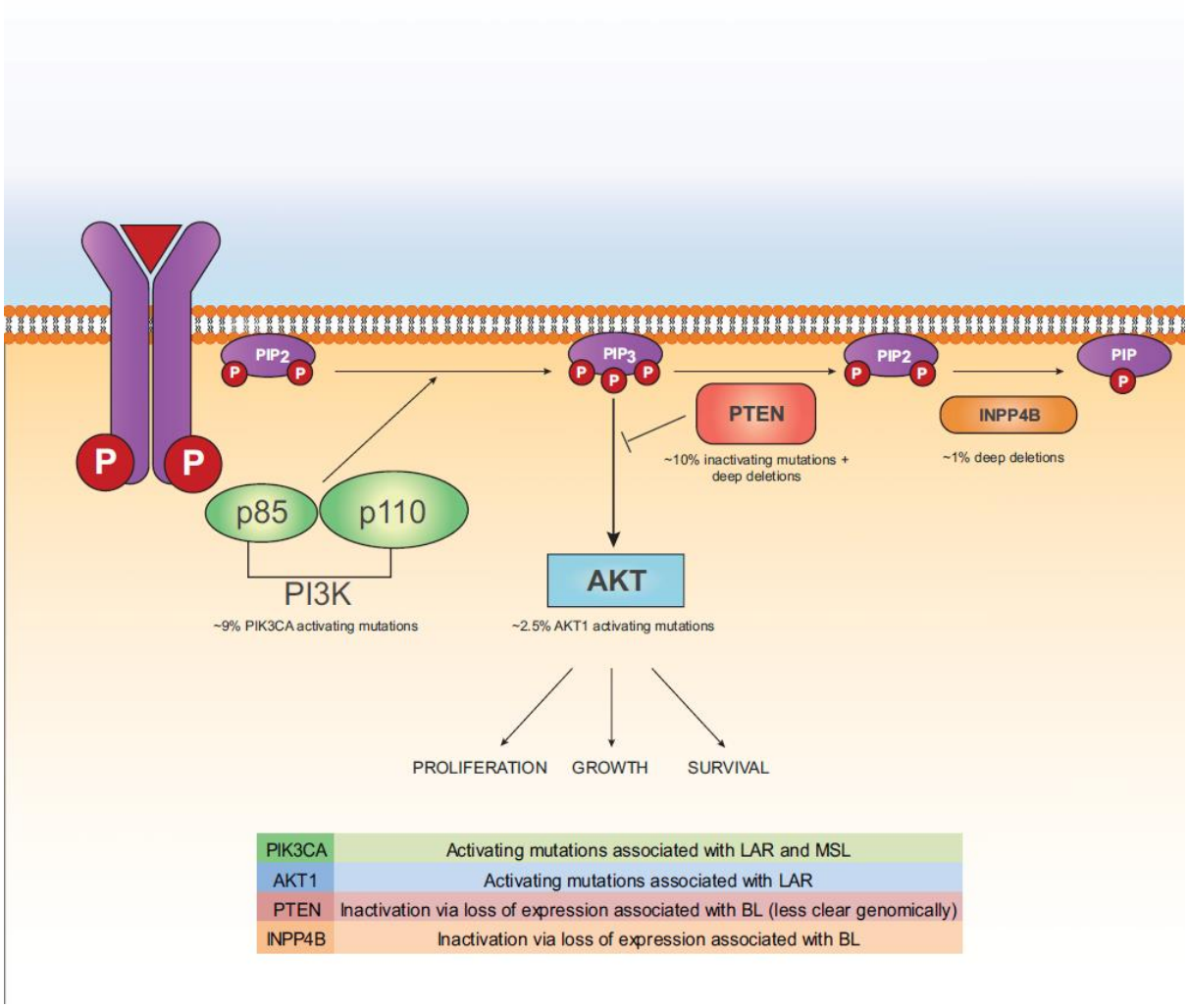


Figure 1. PI3-kinase pathway and simplification of their functions. Main genomic alterations (mutations/copy number variations - CNV) found in each gene in TNBC and association with molecular subtypes.

**TABLES**

| | LOTUS | PAKT |
|---|---|---|
| Design | Randomized, placebo-controlled, phase 2 | Randomized, placebo-controlled, phase 2 |
| Clinical scenario | First-line metastatic TNBC | First-line metastatic TNBC |
| Drugs | Paclitaxel +/- Ipatasertib | Paclitaxel +/- Capivasertib |
| Median PFS ITT | 4.9 vs 6.2 months favouring experimental* | 4.2 vs 5.9 months favouring experimental* |
| Median PFS PTEN low by IHC | 3.7 vs 6.2 months favouring PTEN-low | Not shown |
| Median PFS *PI3KCA/AKT1/PTEN* alteration by NGS | 4.9 vs 9.0 months favouring altered* | 3.7 vs 9.3 months favouring altered* |
| Median OS ITT | 18.4 vs 23.1 months | 12.6 vs 19.1 months* |
| Median OS PTEN low by IHC | 16.1 vs 21.8 months | Not shown |
| Median OS PI3KCA/AKT1/PTEN alteration by NGS | NE vs 19.7 months | 10.4 vs NA |
| Common SAEs | Diarrhoea, decreased neutrophil count, neutropenia | Diarrhoea, fatigue, infection, neuropenia, rash |

Table 1. Clinical data of LOTUS and PAKT trials. "*"= Statistically significant differences.

**SUPPLEMENTARY MATERIAL**

S1. Clinical trials for PIK3-pathway inhibition in TNBC. Data from www.clinicaltrials.gov, accessed on August 16 2018.

**Supplementary Table 1**

| Trial ID | Phase | Drug tested | PI3K-pathway target | Combination |
|---|---|---|---|---|
| NCT03337724 | 3 | Ipatasertib | AKT | Paclitaxel |
| NCT02162719 (LOTUS) | 2 | Ipatasertib | AKT | Paclitaxel |
| NCT02423603 (PAKT) | 2 | AZD5363 | AKT | Paclitaxel |
| NCT02506556 | 2 | Alpelisib | PI3K α | None |
| NCT01623349 | 1 | Buparlisib or Alpelisib | PI3KCA pan-class I or PI3KCA α | Olaparib |
| NCT01920061 | 1 | Gedatolisib | PI3KCA α/γ and mTOR | Docetaxel, Cisplatin or Dacomitinb |
| NCT02307240 | 1 | CUDC-907 | PI3K α/β/δ and HDAC (Histone deacetylase) | None |
| NCT01884285 | 1 | AZD8186 | PI3K β/δ | None or AZD2014 (dual mTORC1/2) |
| NCT02723877 | 1/2b | PQR309 | PI3K pan-class I and mTOR dual mTORC1/2 | Eribulin |

| | | | | |
|---|---|---|---|---|
| NCT02457910 | 1b/2 | Taselisib | PI3K α/δ/γ | Enzalutamide |
| NCT03218826 | 1 | AZD8186 | PI3K β/δ | Docetaxel |
| NCT02637531 | 1 | IPI-549 | PI3K γ | Nivolumab |
| NCT02476955 | 1 | ARQ 092 | PI3K pan-class I and AKT | Carboplatin and Paclitaxel |
| NCT01964924 | 2 | GSK2141795 | AKT | Trametinib |
| NCT02208375 | 1,2 | AZD2014 or AZD5363 | mTOR or AKT | Olaparib |
| NCT03243331 | 1 | Gedatolisib | PI3KCA α/γ and mTOR | PTK7-ADC (anti-PTK7 mAB) |
| NCT03400254 | 1 | Gedatolisib | PI3KCA α/γ and mTOR | Hydroxychloroquine |
| NCT02890069 | 1 | Everolimus | mTOR | PDR001 (anti-PD-1) |
| NCT02583542 | 1,2 | AZD2014 | mTOR | AZD6244 (MEK inhibitor) |
| NCT01964924 | 2 | GSK2141795 (Uprosertib) | AKT | Trametinib |

S1. Clinical trials for PIK3-pathway inhibition in TNBC.