PIK3CA in breast cancer: a Belgian practical testing guideline

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SUMMARY

The PI3K/AKT pathway plays an important role in the oncogenesis of breast cancer. Activating mutations in PI3K, more specifically in the p110α catalytic unit of the class IA PI3K isoform (encoded by the *PIK3CA* gene), lead to an increased conversion of phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) inducing a cell signalling cascade for cell proliferation and cell survival. *PIK3CA* mutations are found in 20-32% of all breast cancers (BC), particularly in hormone sensitive (HR+) BC. In breast cancer, activation of the PI3K pathway coexists with the activation of the oestrogen receptor pathway. Inhibition of one of these pathways may lead to compensatory activation of the other pathway. Therefore, monotherapy with PI3K inhibitors has limited activity in HR+ BC. On the other hand, this explains the efficacy of a PI3K/ER dual blockade. This dual blockade is researched in the phase III SOLAR-1 trial. In the *PIK3CA*-mutated cohort of this study, there is an improved outcome for patients with advanced or metastatic HR+ HER2- BC, harbouring activating hotspot mutations in *PIK3CA* and previously treated **with an aromatase inhibitor and no more than one line of endocrine therapy for MBC**, who received fulvestrant (a selective oestrogen receptor degrader) and alpelisib (a p110α-isoform specific inhibitor) in comparison to the patients that received fulvestrant and placebo. Based on these results, a medical need program for alpelisib in **a heavily pre-treated setting** and an amendment were approved by the EMA and the Belgian FAMHP. Supporting this data, we propose the mutational analysis of *PIK3CA*, preferably by next generation sequencing on FFPE tumour material, in advanced or metastatic HR+ HER2- BC, previously treated with three lines of systemic therapy.

(BELG J MED ONCOL 2021;15(6):304-14)

In the ever-evolving field of oncology, targeted therapy has gained great importance. Targeted therapeutic modality is not new anymore and has been, and still is, thoroughly researched in many types of cancers, such as lung cancers, melanomas, haematologic malignancies, colorectal cancers, *etc*. On the other hand, available targeted therapy is yet somehow limited in other types of cancers. Breast cancer is one of them with only two types of targeted therapy available: hormone therapy for breast cancers with hormone receptor expression and anti-HER2 therapy for *HER2*-amplified breast cancers. Besides these two targets, a third one exists: PARP inhibitors for germline *BRCA*1/2 mutated breast

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Conflict of interest: The authors have nothing to disclose and indicate no potential conflict of interest.

Keywords: breast cancer, guideline, molecular biology, PIK3CA.

cancer. Although not yet approved in Belgium, it is available in a medical need program. Yet, there is still a significant proportion of patients with breast cancer, who still have progressive disease in advanced stages. As such, there is a need for searching and finding new therapeutic targets. An important improvement in breast cancer is the finding of the importance of the PI3K pathway and mutations in *PIK3CA*. This paper aims to provide insights in the PI3K/AKT pathway to understand its role as an oncogenic driver, the underlying mechanisms of overcoming resistance to endocrine therapy and the rationale as a therapeutic driver. The latter will be endorsed with results of the SOLAR1 clinical trial. Based on these insights we provide a practical testing guideline in analysing *PIK3CA* mutations in breast cancer.

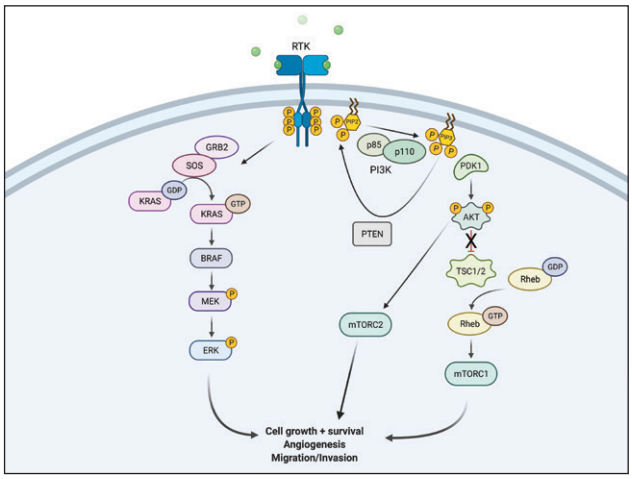

FIGURE 1. Schematic overview of the PI3K/AKT pathway next to the RAS/MAPK pathway (created with BioRender.com).

INTRODUCING THE PI3K/AKT PATHWAY

Unlike the RAS/RAF pathways, the PI3K/AKT pathway uses phosphatidylinositol, a minor membrane phospholipids, in the signal transduction pathway from an activated dimerised receptor tyrosine kinase (RTK) to downstream signalling proteins and complexes AKT, mTORC1 and mTORC2 to promote cell growth, survival, angiogenesis and migration. *Figure 1* shows a schematic overview of all signalling proteins in the PI3K/AKT pathway. In this pathway, the role of phosphatidylinositol-3-kinase (PI3K) is the phosphorylation of the 3-OH group of the inositol ring in phosphatidylinositol-4,5-biphosphate [PtdIns-4,5-P2, PIP2] to form phosphatidylinositol-3,4,5-triphosphate [PtdIns-3,4,5-P3, PIP3].[1,2] PTEN dephosphorylates PIP3 to PIP2. Otherwise, PIP3 can also be hydrolysed by SH2 (Src homology 2) generating PtdIns-3,4-P2, which is not the same as PIP2 and which cannot be used to generate PIP3 anymore.[1]

Three different classes of PI3K exist: Class I, Class II and Class III. The first class is subdivided in Class IA and Class IB. Subdivision depends on the structure and substrate specificities of the different classes.[1,3] All except class II PI3Ks are heterodimers consisting of a catalytic subunit and a regulatory subunit. The catalytic and regulatory subunit make contacts and conformational changes, resulting in maintaining the enzyme in a low state of activity under basal conditions. The components of the PI3K classes are presented in *Table 1*. The PIK3CA gene encodes the p110 catalytic subunit of a Class IA PI3K.[1,3-5]

PIK3CA MUTATIONS IN BREAST CANCER

PREVALENCE

When looking at the p110α protein, five different domains can be described. The first part consists of a p85 binding domain (where the regulatory subunit binds), followed by a RAS binding domain, a C2 domain, a helical domain and finally the kinase domain itself. The C2 domain has been considered as a binding domain to the cellular membranes and also makes contact with the p85 regulatory subunit. Over 80% of the somatic *PIK3CA* missense mutations found in different cancers, are located in the kinase and helical domains.[3,6,7] A lollipop plot of the different domains and the most frequent mutations can be found in *Figure 2*.

Kinase domain mutations are more linked to cell proliferation[9], while helical domain mutations are more linked with features enabling motility and dissemination. The *PIK3CA* mutations are found to be concentrated in several hot spot regions in the coding sequence. One of these regions is located in the p85 binding domain in exon 2, followed by mutations in the C2 domain in exons 5 and 8, another hotspot region is located in the helical domain of p110α in exon 10 and the fifth is located at the end of the catalytic kinase domain in exon 21.[10] In these regions, the most frequent mutations are seen in codon 1,047 (exon 21), codon 545 (exon 10), codon 542 (exon 10), codon 88 (exon 2), codon 345 (exon 5) and codon 420 (exon 8).[1,6,10]

The mutations in the helical domain (*e.g.* mutations in codons 542, 545 and 546) are dependent on RAS, mimics activation of RTK's and abrogate the inhibitory effect of the p85α regulatory unit[1,6,7,10-13], while the mutation at the end of and in the kinase domain (*e.g.* mutations in codon 1,047) affects the conformation of the activation loop of p110α, increasing the interaction of p110α with lipid membrane, resulting in greater access to the substrate PIP2.[1,6,7,12,13] The C2 domain interacts also with the p85 regulatory subunit. Mutations in this domain (*e.g.* mutations in codon 420) hinder this interaction, relieving its inhibitory effect.[7]

While the majority of *PIK3CA* hotspot mutations is well established as oncogenic drivers, several less frequent *PIK3CA*

TABLE 1. Subtypes of epithelial ovarian cancer: histological, molecular and gene mutation characteristics.

Class	Catalytic subunit		Regulatory subunit	
	Gene	Protein	Gene	Protein
IA	*PIK3CA* *PIK3CB* *PIK3CD*	p110α p110β p110δ	*PIK3R1* *PIK3R2* *PIK3R3*	p85α (and splicing variants p55α and p50α) p85β p55y
IB	*PIK3CG*	p110y	*PIK3R5* *PIK3R6*	p101 p87
II	*PIK3C2A* *PIK3C2B* *PIK3C2G*	PIK3C2α PIK3C2β PIK3C2y	/	/ (monomeric)
III	*PIK3C3*	VPS34	VPS15	VPS15

mutations occur in cancer for which pathogenicity remains unclear. In this context, recent data suggest that the rare *PIK3CA* C-terminal mutations resulting in a frame-shifted protein product with an extended C terminus are activating driver mutations in breast cancer.[14]

Sometimes a combination of a kinase domain and a helical domain hotspot mutation can be found in the same molecule. This combination has a great synergistic effect on signalling and oncogenicity.[15] As has been discussed, hot spot mutations account for 78% of *PIK3CA* mutations in cancers.[12] The proteins that show rarer mutations show lower enzymatic activity, mediate lower levels of downstream phosphorylation and induce decreased oncogenic transformation in cell cultures. This might explain the lower frequencies at which these rare mutations are found in cancer.[2,13,15]

PIK3CA mutations are found in 20-32% of all breast cancers. It is one of the most frequently mutated proteins in breast cancer and over 50% of these mutations are located in the kinase domain, yet mutations in the helical domain and C2 domain can also be found in breast cancer.[1,4,6,7,10,12,16,17]

Important for testing: *PIK3CA* mutations are found not only in metastatic lesions but also in primary ones (in contrast to for example *PIK3CA* mutations in colon cancer). It has been generally accepted that *PIK3CA* mutations in breast cancer are clonal, early events. While in other cancers, the subclonal nature of mutations in the PI3K pathway is suggested by the higher frequency of mutations in metastatic *versus* primary lesions from the same patient.[6,18]

ROLE OF PIK3CA MUTATION IN HORMONE SENSITIVE BREAST CANCER

The aforementioned mutations in hotspot regions of *PIK3CA* are found in over a third of oestrogen receptor (ER)-positive breast cancers, *i.e.* presenting the most common genomic alterations in this molecular subtype of breast cancer1,[4,7,9,10,13,15,16,19] and is linked to promotion of resistance to endocrine therapy[1,7 13,15,16,19]. This fact makes it possible to hypothesise that both pathways can drive proliferation and survival in these cancer cells and are interconnected. Translational research indeed proves that inhibition of the PI3K-pathway in ER-positive breast cancer results in induction of ER-dependent transcriptional activity. These effects on the transcriptome were not restricted to a few selected ER target genes but rather resulted in expression of hundreds of genes controlled by oestrogen response elements (ERE)-containing promotors. Importantly, the causative role of ER in rewriting gene expression upon PI3K inhibition was underscored by its complete prevention when Fulvestrant, a direct ER antagonist, is added. It is also shown that suppression of the PI3K pathway leads to a consistent increase in transcription of ER itself.[4,13]

Compensatory activation of ER-dependent genes occurring early upon PI3K inhibition decreases the antitumour efficacy of PI3K inhibitors. This may explain the limited activity of PI3K inhibitors when used in monotherapy in patients with ER positive breast cancers. It also explains why the dual blockade (PI3K and ER) is effective, even in patients who have progressed on previous endocrine therapies. The current possible targeted therapy against PI3K includes pan-class I PI3K inhibitors, isoform specific PI3K inhibitors and dual-specific PI3K/mTOR inhibitors. An overview of these inhibitors can be found in *Table 2*. In breast cancer, alpelisib, taselisib and GDC-0077 are the most investigated PI3K inhibitors.[4,13,20]

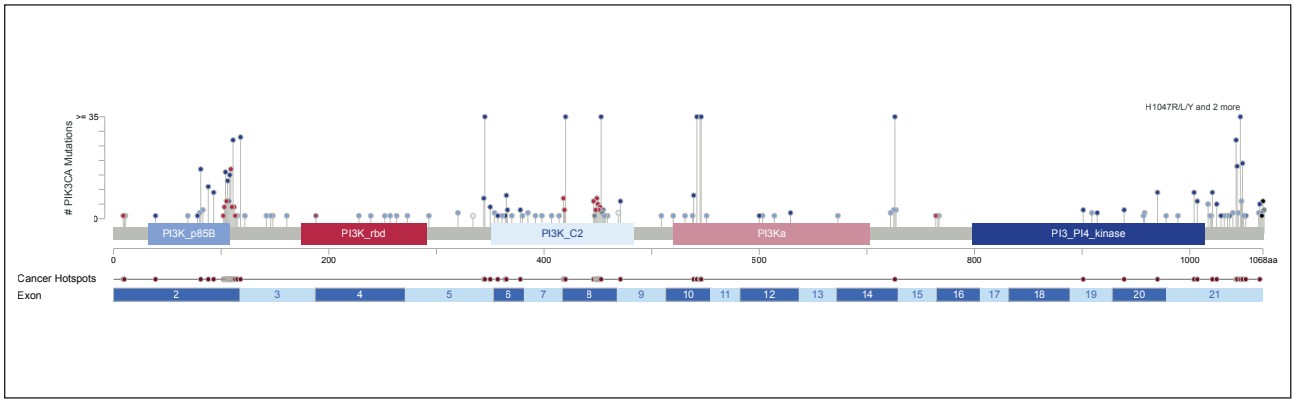

FIGURE 2. Lollipop plot of the different domains of PIK3CA (according to the PFAM-database) and localisation of the most frequent mutations (based on all available open source datasets). Exported from cBioPortal.org.

ROLE OF PIK3CA MUTATIONS IN HER2-AMPLIFIED BREAST CANCER

PIK3CA mutations are not limited to hormone sensitive BCs. In fact, they can be seen in all types of breast cancers, therefore also in *HER2*-amplified breast cancers and in triple negative breast cancers. Mutations are seen in the same regions and at the same codon as seen in hormone sensitive BCs.[21] HER2 is a RTK. Upon binding to a ligand and dimerisation, HER2 recruits and activates PI3K. It is anticipated that *PIK3CA* mutations affect response to anti-HER2 therapy, reaching lower rate of pathologic complete response (pCR) in the neoadjuvant setting and disease/progression free survival (DFS/PFS). A meta-analysis by *Loibl et al.* in 2016, showed indeed a lower rate of pCR, yet significant differences in DFS and PFS were not observed. However, significant differences in pCR rates are not seen in all clinical trials.[22,23] The role of *PIK3CA* mutations in resistance to anti-HER2 therapy is therefore still under debate, but more recent *in vitro* studies do support the hypothesis. The latter have shown that anti-PI3Kα can overcome this resistance to anti-HER2 therapy in case of proven *PIK3CA* mutation.[21] Currently there is an ongoing phase II/III trial examining the efficacy of anti-HER2 monoclonal antibody therapy in combination with alpelisib in *PIK3CA* mutated *HER2*-amplified breast cancers.[24] Awaiting these results, there isn't yet an indication for administering alpelisib in *HER2*-amplified BC in Belgium, nor is there an indication for testing *PIK3CA* mutations in this type of breast cancers in the clinical setting.

ROLE OF PIK3CA MUTATIONS IN TRIPLE NEGATIVE BREAST CANCER

As triple negative breast cancer (TNBC) is not oncogenetically driven by activation of the oestrogen receptor pathway or the activation of the HER2 pathway, these types of cancers are not eligible for hormone therapy or anti-HER2 therapy. Standard of care systemic treatment is therefore limited to chemotherapeutical agents. There is a lack of targeted therapeutic options. Currently, only PARP inhibitors and atezolizumab (an anti-PD-L1 monoclonal antibody) are used in clinical practice, given the indication of germline *BRCA1/2* mutations or PD-L1 expression on immunohistochemistry (SP142 assay, cut-off inflammatory cell proportion >1%). The search for new targeted therapeutic options continues. *PIK3CA* mutations are also found in TNBC and might act as a potential target for anti PI3K/AKT inhibition. The first clinical trials suggest that this role may be ill-fated, but these studies did not select for *PIK3CA* mutated patients. At this moment, there are still ongoing trials researching the efficacy of addition of anti-PI3Kα to chemotherapy for selected patients with *PIK3CA* mutated TNBC.[25] Because of the lack of sufficient data, there is also no indication for the use of alpelisib and for testing *PIK3CA* in TNBC in the clinical setting.

On a side note, the activation of the PI3K pathway has also an important, though negative role in response to checkpoint inhibitors. In finding the most adequate biomarker for predicting response to anti-PD(L)1 therapy, it has become clear that *PIK3CA*-mutated tumours evade tumour immunity, as they promote infiltration of immune inhibitory myeloid cells in the tumour micro-environment (TME) on one hand. On the other hand, the TME contains very few CD8+ cytotoxic T cells. As such, there might be a role for using *PIK3CA* mutations, alongside other more validated biomarkers, in predicting nonresponse to checkpoint inhibitors.[5]

CLINICAL TRIALS

When clinical trials in *PIK3CA* mutated breast cancer are mentioned, there usually is a direct referral to the SOLAR-1 study, one of the first phase III studies researching combined inhibition of PI3Kα (alpelisib) and the ER pathway

TABLE 2. Overview of inhibitors of PI3K. Adapted from Araki K, et al. (2018), Collins D. (2017) and Arafeh R, et al. (2019).[1,5,6]

Inhibitor type	Code name	Drug name	Pharmaceutical company	Target	Dev. Stage
Pan-class I inhibitors	BKM120	Buparlisib	Novartis	Pan-PI3K	III
	GDC-0941	Pictilisib	Genentech	Pan-PI3K	II
	BAY 80-6946	Copanlisib	Bayer AG	Pan-PI3K	III
	SF1126	CH5132799	Semaphore Pharmaceuticals	Pan-PI3K	II
	XL147	ilaralisib	Exelixis/Sanofi	Pan-PI3K	II
	PX866	ZSTK474	Oncothyreon	Pan-PI3K	NA
Isoform-specific class I inhibitors	GDC-0032	Taselisib	Genentech	p110α (β sparing)	III
	BYL719	Alpelisib	Novartis	p110α	III
	GDC-0077		Genentech	p110α	II-III
	MLN1117	Serabelisib	Takeda	p110α	II
	MEN1611			p110α	I-II
	BAY 1082439		Bayer	p110α/β	NA
	CH5132799		Chugai Pharmaceuticals	PI3Kα/γ	I
	GSK2636771		GlasxSmithKline	p110β	II
	AZD8186		AstraZeneca	p110β	I-II
	SAR260301		Sanofi	p110β	NA
	CAL-101	Idelalisib	Gilead Sciences	p110δ	IV
	IPI-145	Duvelisib	Infinity	p110δ	III
	AMG319		Amgen	p110δ	NA
Dual-specific PI3K class I/ mTOR inhibitors	BEZ235	Dactolisib	Novartis	PI3K/mTOR	III
	GDC-0980	Apitolisib	Genetech	PI3K/mTOR	II
	PF-05212384	Gedatolisib	Pfizer	PI3K/mTOR	II
	PF-0691502		Pfizer	PI3K/mTOR	NA
	GSK-2126458	Omipalisib	GlaxoSmithKline	PI3K/mTOR	I
	XL765	Voxtalisib	Exelixis/Sanofi	PI3K/mTOR	II

Dev. Stage, stage of drug development according to clinicaltrials.gov.; NA, not available.

(selective oestrogen receptor degrader [SERD], fulvestrant) *versus* fulvestrant monotherapy in hormone sensitive (HR+), HER2-negative (HER2-) advanced breast cancer, previously treated with endocrine therapy. Patients were sub-divided into two cohorts based on their *PIK3CA* mutational status. In the *PIK3CA* mutated cohort, the median progression-free survival (PFS) in the combination therapy group was 11.0 months (95% confidence interval [CI]: 7.5-14.5 months) in comparison to 5.7 months in the fulvestrant monotherapy group (95%CI: 3.7-7.4 months). The hazard ratio for progression or death was 0.65 (95%CI: 0.50-0.85, p< 0.0001). Overall response (OR) in the group with combination the-

rapy was greater (26.6%), compared to the group with fulvestrant monotherapy (12.8%).[11,26]

Based on the results of the SOLAR-1 study, the European Medicines Agency (EMA), the Food and Drugs Administration (FDA) and the Belgian Federal Agency for Medicines and Health Products (FAMHP) has authorised the use of Alpelisib.[10,27-29] Before January 2021, the use of alpelisib was in Belgium approved by means of a medical need program for patients whose clinical setting matches the inclusion criteria of the SOLAR-1 trial: metastatic or advanced HR+ BC with progressive disease treatment with an aromatase inhibitor and harbouring a *PIK3CA* hotspot mutation.[27] It is important to mention that the SOLAR-1 study used the Therascreen PCR test for detecting *PIK3CA* mutations. This test is limited to eleven specific mutations in the *PIK3CA* gene.[30] Based on this evidence, the FDA approved the use of the Therascreen PCR test as a companion diagnostic tool for alpelisib.[29] In January 2021, an amendment on the medical need program for the use of alpelisib in metastatic or advanced HR+ BC with progressive disease after at least three lines of systemic treatment was approved in Belgium. This approval extended the indication from PIK3CA hotspot mutations to all activating PIK3CA mutations.[31] This approval stresses out the importance of testing for more possible mutations than the ones included in the Therascreen PCR test. Indeed, response to alpelisib has been shown in HR+, HER2- breast cancer harbouring mutations other than the hotspots included in the Therascreen PCR test.[32]

Another important phase III trial regarding *PIK3CA* mutations and administration of a PI3K inhibitor is the SANDPIPER trial, of which preliminary results are available. This trial has the same indicative conditions as the SOLAR-1 trial. The authors researched the efficacy of combined inhibition of PI3Kα (taselisib) and a SERD (fulvestrant) *versus* SERD monotherapy in HR+ HER2- locally advanced or metastatic BC, showing progression on endocrine therapy and harbouring *PIK3CA* mutations. Unlike the SOLAR-1 study, no comparison is made with a non-PIK3CA-mutated cohort. In this study, the results show the same tendency as seen in the SOLAR-1 study, although rather modest. The mean PFS in the combination therapy arm was 7.43 months (95%CI: 7.26-9.07 months) in comparison to 5.39 months (95%CI: 3.68-7.29 months) in the fulvestrant monotherapy arm. The hazard ratio for PFS was 0.70 (95%CI: 0.56-0.89). The objective response rate was 28.0% and 11.9%, respectively (p-value 0.0002). The overall survival in the combination therapy arm was 26.81 months (95%CI: 21.29 months-N/A) compared to 23.56 months (95%CI: 18.00 months-N/A). The hazard ratio for overall survival was 0.85 (95%CI: 0.58-1.25).[33]

The SOLAR-1 and SANDPIPER studies are of course not the only available clinical trials regarding the administration of PI3K inhibition in breast cancer. Searching registered clinical trials at clinicaltrials.gov, seventeen active studies are found, six of which are phase III (including the SOLAR-1 study). The four other phase III studies do not have any results yet. An overview of all phase III studies can be found in *Table 3*. Interestingly, it is seen that some of these phase III trials cover other clinical settings or other molecular subtypes of breast cancer, as mentioned before. This could mean that in the near future the clinical indication for testing *PIK3CA* mutation might expand to triple negative breast cancer depending on the results of this clinical trial.[35] But awaiting this data, administering alpelisib and PIK3CA testing in this particular molecular subtypes of breast cancers is not yet indicated in Belgium. Testing PIK3CA mutations is limited to the context of these clinical trials for now.

PIK3CA IN OTHER CANCERS

The presence of *PIK3CA* mutations is not limited to breast cancer. Mutations of *PIK3CA* are also found in 24-46% of endometrial cancers, 20-27% of bladder cancers, 14-23% of cervical cancers and 13-28% of colorectal cancers. It is also seen in squamous cell carcinomas of the lung and the head-and-neck region, accounting for 12-15%. Importantly, besides in breast, p110α is also the most frequently mutated protein in endometrial cancers.[1,6] As kinase domain mutations account for >50% of the *PIK3CA* mutations in breast cancer, helical mutations pre-dominate in head-and-neck and lung squamous carcinomas. For a few of these other types of cancer, clinical trials do exist, but none of them have reached phase III so far.

RECOMMENDATIONS TOWARDS PIK3CA MUTATION ANALYSIS

INDICATION

At this moment, the SOLAR-1 trial is the only phase III trial with results confirming the efficacy of a PI3K inhibitor (alpelisib) in combination with fulvestrant. In this trial, only patients with locally advanced or metastatic ER+ HER2- breast cancers were included. The *PIK3CA* mutational status is a predictor of response to this dual blockade of the PI3K and the ER pathway.[11] The results of this trial has led to the current situation in Belgium, starting January 2021, requiring testing activating mutations in the *PIK3CA* gene as one of the absolute indications for the administration of alpelisib in combination with fulvestrant for this specific breast cancer type and clinical setting. The efficacy of the PI3K inhibitor, nor the PIK3CA testing has been validated in other clinical settings, other intrinsic subtypes of breast cancer or other cancer types.

TABLE 3. Overview of phase III clinical trials regarding PI3K inhibition in breast cancer (source clinical-trials.gov).

NCT	Title	Acronym	Status	Conditions	Interventions	Enrol-ment	Study design	Outcome measure-ments
NCT02437318	Study Assessing the Efficacy and Safety of Alpelisib Plus Fulvestrant in Men and Post-menopausal Women with Advanced Breast Cancer Which Progressed on or After Aromatase Inhibitor Treatment.[11,26]	SOLAR-1	Active, not recruiting, has results	HR+ HER2- advanced breast cancer previously treated with endocrine therapy, *PIK3CA* mutation cohort	Fulvestrant + Alpelisib *vs.* Fulvestrant + placebo	572	Allocation: Randomise Intervention Model: Parallel Assignment Masking: Triple (Parti-cipant, Care Provider, Investigator) Primary Purpose: Treatment	OR, PFS, OS
NCT02340221	A Study of Tase-lisib + Fulvestrant Versus Placebo + Fulvestrant in Parti-cipants With Advanced or Metastatic Breast Cancer Who Have Disease Recurrence or Progression During or After Aromatase Inhibitor Therapy.[33]	SANDPI-PER	Active, not recruiting, has results	HR+ HER2- advanced breast cancer showing progres-sion after previously treated with endocrine therapy and *PIK3CA* mutation enriched	Fulvestrant + Taselisib *vs.* Fulvestrant + placebo	631	Allocation: Randomi-sed Inter-vention Model: Parallel Asignment Masking: Double (Participant, Investigator) Primary Purpose: Treat-ment	PFS, OR, Percentage of patients with clinical bene-fit, duration of response, percentage of patients with adverse even-ts, QoL
NCT04208178	Study of Alpeli-sib (BYL719) in Combination With Trastuzumab and Pertuzumab as Maintenance Therapy in Patients With HER2-positive Advanced Breast Cancer With a *PIK3CA* Mutation.[24]		Recruiting	Advanced HER2+ Breast Cancer with a *PIK3CA* mutation	Trastuzumab + Pertuzumab + Alpelisib *vs.* Trastuzumab + Pertuzumab + placebo	548	Allocation: Non-Ran-domised Intervention Model: Sequential Assignment Masking: None (Open Label Primary Purpose: Treatment	PFS, OS, OR
NCT03439046	Study of the Mo-lecular Features of Postmenopausal Women With HR+ HER2-negative aBC on First-line Treatment With Ri-bociclib and Letro-zole and, in Patients With a *PIK3CA* Mutation, on Se-cond-line Treatment With Alpelisib Plus Fulvestrant.[34]	BioItaLEE	Active, not rcruiting	HR+ HER2- advanced breast cancer with *PIK3CA* mutation (second line treatment)	Fulvestrant + Alpelisib	287	Allocation: N/A Intervention Model: Sin-gle Group Assignment Masking: None (Open Label) Primary Purpose: Treatment	Changes from baseline ctDNA alterations, serum TK1 concentrati-ons, tumour mutational burden and percentage of patients with DNA altera-tions, with clinical benefit and OR.

TABLE 3 (CONTINUED). Overview of phase III clinical trials regarding PI3K inhibition in breast cancer (source clinicaltrials.gov).

NCT	Title	Acronym	Status	Conditions	Interventions	Enrolment	Study design	Outcome measurements
NCT04251533	Study Assessing the Efficacy and Safety of Alpelisib + Nab-paclitaxel in Subjects With Advanced TNBC Who Carry Either a *PIK3CA* Mutation or Have PTEN Loss Without *PIK3CA* Mutation.[35]	EPIK-B3	Recruiting	Advanced triple negative breast cancer	Nab-paclitaxel + Alpelisib *vs.* Nab-paclitaxel + placebo	566	Allocation: Randomised Intervention Model: Factorial Assign-mentMasking: Double (Participant, Investigator) Primary Purpose: Treatment	PFS, OR, OS, Clinical benefit rate, time to response, duration of Response, Quality of life, changes from baseline mutational status
NCT04191499	A Study Evaluating the Efficacy and Safety of GDC-0077 + Palbociclib + Fulvestrant vs Placebo + Palbociclib + Fulvestrant in Patients With *PIK3CA*-Mutant, Hormone Receptor-Positive, Her2-Negative, Locally Advanced or Metastatic Breast Cancer.[36]		Recruiting	HR+ HER2- advanced breast cancer with *PIK3CA* mutations	GDC-0077 + Palbociclib + Fulvestrant *vs.* Placebo + Palbociclib + Fulvestrant	400	Allocation: Randomised Intervention Model: Parallel Assignment-Masking: Double (Participant, Investigator) Primary Purpose: Treatment	FS, OR, DOR, CBR, OS, time to deterioration, adverse events

Recommendation A: *PIK3CA* mutation analysis is indicated for the administration of alpelisib with fulvestrant in previously treated, advanced or metastatic HR+ HER2- breast cancer, based on the results of the SOLAR-1 trial.

TIMING OF THE BIOPSY

As stated earlier, it is generally accepted that PIK3CA mutations are early clonal events in breast cancer, unlike the findings in other cancer types. This evidence is supported by the small difference in prevalence of PIK3CA mutations between early breast cancer and metastatic breast cancer. This means that a PIK3CA mutation in metastatic breast cancer is likely to be present in the primary tumour as well.[6,18] This means that timing of the biopsy used is not of the utmost importance and testing of *PIK3CA* mutations can be done on the resection or biopsy of either the primary focus or a metastatic lesion. Given the sole indication as described above, testing for a *PIK-3CA* mutational status is most useful in the metastatic setting, given the findings of the SOLAR-1 trial.[11] Furthermore, we emphasise to use recent tumour tissue in the context of the most optimal DNA quality. Older samples, especially formalin fixed paraffin embedded (FFPE) tissue, can have worse DNA quality, leading to technical failures.

Recommendation B: *PIK3CA* mutations can be tested in the resection or biopsy of either a metastatic focus or the primary lesion. We emphasise to take the testing indication (metastatic or advanced setting) and optimal DNA quality into account by using a more recent tissue sample.

SAMPLE MATERIAL

As for all molecular diagnostics in the field of solid tumours, formalin fixed paraffin embedded (FFPE) tissue is considered the gold standard for mutational analysis, mainly due to the evidence provided by clinical trials as well as the availability of representative tumour tissue. This is also the case for *PIK3CA* mutation testing.

Yet, taking a biopsy is an invasive intervention and the tumour site might even be inaccessible. In these cases, there

is a role for cell free tumour DNA (ctDNA) from a blood sample or other fluids (liquid biopsy). The use of *PIK3CA* testing on liquid biopsy has been demonstrated before, but there is still insufficient data concerning patient outcome when testing *PIK3CA* mutation on liquid biopsy. Furthermore, there are some important challenges to overcome regarding liquid biopsy. For the time being, it is still impossible to distinguish the absence of a mutation from the absence or a low amount of cell free tumour DNA (ctDNA). Moreover, the low amount of ctDNA might lead to assay errors resulting in false-negative or even false-positive results. Lastly, there is still no consensus on time points for taking liquid biopsies, although the latter challenge is less applicable to *PIK3CA* testing, considering that *PIK3CA* mutation are early clonal events. On the other hand, liquid biopsies enable us to overcome tumour heterogeneity, to capture tumour DNA from multiple metastatic sites at once and to monitor treatment effects based on ctDNA levels.[37] At present, the benefits do not exceed the disadvantages for implementing *PIK3CA* testing in liquid biopsy in clinical practice. Therefore, we recommend the use of FFPE tumour material for PIK3CA mutation analysis. Liquid biopsy should only be used in cases of insufficient tumour tissue and inadequate DNA quality. However, there is a role for testing in liquid biopsies in clinical trials. Results from these trials might solidify a possible utility for liquid biopsy in clinical practice.

Recommendation C: We recommend testing for *PIK3CA* mutations on representative FFPE material whenever available. We acknowledge the possible use of testing *PIK3CA* on liquid biopsy with a role in clinical trials. Further data concerning patient outcome is needed for possible implementation in clinical practice.

TESTING TECHNIQUES AND COVERAGE OF THE PIK3CA GENE

Mutational analysis is most frequently performed by means of next generation sequencing (NGS) or polymerase chain reaction (PCR). There are several commercial PCR-based kits available with the Therascreen PIK3CA RGQ PCR test (Qiagen) and the Cobas PIK3CA Mutation Test (Roche Diagnostics) being the most widely used. The former PCR test was used in the SOLAR-1 trial and was therefore approved by the FDA in the United States of America as a companion diagnostic tool for alpelisib.[37]

While PCR assays are relatively fast, cost-effective and generally require less input DNA, NGS offers the possibility to detect a more comprehensive range of activating *PIK3CA* mutations not limited to the typical hotspots. In fact, the Therascreen PIK3CA RGQ PCR test allows identification of eleven specific hotspot mutations across three exons (exon 8, 10 and 21: C420R, E542K, E545A, E545D, E545G, E545K, Q546E, Q546R, H1047L, H1047R, H1047Y). Yet, response to alpelisib is also seen in HR+ HER- BC, harbouring other PIK3CA mutations.[32] Furthermore, in a retrospective analysis based on the SOLAR-1 trial, NGS identified up to 60 different point mutations in the *PIK3CA* gene across more than the three aforementioned exons. Data from the GENIE project show that not all target mutations from the Therascreen test are the most frequent *PIK3CA* mutations to be found. Following the GENIE trial, there is also a rationale for testing mutations in exon 2, 5 and 14. Moreover, NGS can provide comprehensive analysis of more genes at the same time, *e.g. ESR1*.[37]

Recommendation D: PIK3CA mutational analysis is preferably done with NGS. Based on these findings, the Belgian Working Group of Molecular Pathology recommends NGS for finding activating mutations in the PIK3CA gene with coverage of at least exons 2, 5, 8, 10, 14 and 21.

REIMBURSEMENT

PIK3CA testing is fully reimbursed in Belgium as a molecular test under the NGS convention. This test can be charged with a maximum of one per patient per year. Importantly, the used testing technique should at least cover the proposed exons in agreement with the Compermed expert group on breast cancer. Currently, the minimum required coverage of the *PIK3CA* gene include exons 2, 5, 8, 10, 15 and 21.

Concurrent testing of HER2 amplifications by means of *in situ* hybridisation does not interfere with this reimbursement, as HER2 testing is reimbursed under article 33ter of the RIZIV/INAMI nomenclature regarding pathological and genetical testing modalities. Both tests can be performed and are reimbursed for each patient per year for the same clinical setting.

CONCLUSION

Given the results from the SOLAR-1 trial and the approval of the amendment on the medical need program for the administration of alpelisib, we recommend mutational analysis of the *PIK3CA* gene in metastatic HR+ HER2- BC patients having received three lines of therapy with at least endocrine therapy in order to administer the combination therapy of alpelisib and fulvestrant. Mutational analysis of *PIK3CA* is preferably done on representative FFPE tumour material using NGS, covering at least exons 2, 5, 8, 10, 14 and 21. PCR-based tests represent an alternative, but are generally limited to specific hotspot mutations. There is a role for testing *PIK3CA* mutations in a liquid biopsy, but there is still insufficient data to support widespread use in clinical practice. Therefore, the latter should be used with caution and only when insufficient tumour tissue is available or in case DNA quality from tissue is unsatisfactory. It is important to bear

KEY MESSAGES FOR CLINICAL PRACTICE

1. ***PIK3CA*** **mutations in previously treated advanced or metastatic HR+ HER2- breast cancer can be targeted with alpelisib and fulvestrant combination therapy, according to the SOLAR1-study: effectiveness of dual blockade of the PI3K and ER pathway.**
2. **The most frequent *PIK3CA* mutations are found in exons 2, 5, 8, 10, 14 and 21.**
3. ***PIK3CA*** **mutational analysis is preferably done on FFPE tumour material with NGS.**
4. ***PIK3CA*** **mutations are also found in other types of cancer besides breast cancer: endometrial cancer, bladder cancer, colon cancer, squamous cell carcinoma of head-and-neck, squamous cell carcinoma of lung, *etc*.**
5. **There are ongoing trials for anti-*PIK3CA* therapy in other molecular subtypes of breast cancer and other cancer types.**

in mind, that further (phase III) clinical trials are ongoing of which the results might lead to an expansion of the clinical indication for *PIK3CA* mutational testing.

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