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Review

PIK3CA Mutation Assessment in HR+/HER2− Metastatic Breast Cancer: Overview for Oncology Clinical Practice

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Abstract: Activation of the PI3K–AKT–mTOR pathway occurs in several human cancers, including hormone receptor (HR)-positive breast cancer (BC) where is associated with resistance to endocrine therapy and disease progression. In BC, the most common PI3K–AKT–mTOR pathway alteration is represented by PIK3CA oncogenic mutations. These mutations can occur throughout several domains of the p110α catalytic subunit, but the majority are found in the helical and kinase domains (exon 9 and 20) that represent the "hotspots". Considering the central role of the PI3K–AKT–mTOR pathway in HR-positive BC, several inhibitors (both pan-PI3K and isoform-specific) have been developed and tested in clinical trials. Recently, the PI3Kα-selective inhibitor alpelisib was the first PI3K inhibitor approved for clinical use in HR-positive metastatic BC based on the results of the phase III SOLAR-1 trial. Several methods to assess PIK3CA mutational status in tumor samples have been developed and validated, including real-time polymerase chain reaction (PCR), digital droplet PCR (ddPCR), BEAMing assays, Sanger sequencing, and next-generation sequencing (NGS) panels. Several new challenges will be expected once alpelisib is widely available in a clinical setting, including the harmonization of testing procedures for the detection of PI3K–AKT–mTOR pathway alterations. Herein, we provide an overview on PI3K–AKT–mTOR pathway alterations in HR-positive BC, discuss their role in determining prognosis and resistance to endocrine therapy and highlight practical considerations about diagnostic methods for the detection of PI3K–AKT–mTOR pathway activation status.

Keywords: PIK3CA; breast cancer; alpelisib; diagnostic; PCR; next-generation sequencing

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1. Introduction

The phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) cascade is one of the major downstream signaling pathways in human cells and is involved in essential cell processes such as metabolism, survival, proliferation, growth, and motility [1]. The PI3K–AKT–mTOR pathway can be triggered by the activation of various tyrosine kinase receptors (TKRs) or G protein-coupled receptors. Mechanistically, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5,)-trisphosphate (PIP3) at the plasma membrane. Consequently, AKT kinases are activated by PIP3 being able to phosphorylate tuberous sclerosis protein 1 (TSC1) and TSC2, and thereby dissociate the TSC1–TSC2 complex. The TSC1–TSC2 complex negatively regulates the activity of the kinase mTOR. Therefore, AKT activity results in the activation of mTOR complex 1 (mTORC1) and ultimately promotes cell growth and proliferation. Notably, mTORC1 is involved in a negative feedback loop that serves to prevent the overactivation of AKT. The cascade is also antagonized mainly by the tumor suppressor phosphatase and tensin homolog (PTEN), which converts PIP3 to PIP2.

Dysregulation of the PI3K–AKT–mTOR pathway occurs in a large variety of human cancers and has been proven to be implicated in tumor development and progression [2]. Such dysregulation can derive from different mechanisms that include overactivation of growth factor receptors, activating mutations in the PI3K subunits, PTEN loss of function, and mutations in other genes including AKT. PI3K is a heterodimer with a regulatory and a catalytic subunit. The four catalytic isoforms of class I PI3K have different tissue expression patterns. While the α and β (*PIK3CB*) isoforms are ubiquitously expressed in human tissues, the expression of γ (*PIK3CG*) and δ (*PIK3CD*) isoforms is limited to white blood cells [3]. PI3Kα is a heterodimeric protein complex comprising the catalytic subunit p110a (encoded by the *PIK3CA* gene located on chromosome 3) and the regulatory subunit p85a (encoded by the *PIK3R1* gene located on chromosome 5). Mechanistically, p110a binds to the regulatory subunit p85a, which inhibits p110a, and catalyzes the phosphorylation of PIP2 to PIP3 [1]. PI3Kα is the most frequently altered PI3K isoform in solid tumors, playing a prominent role in the aberrant activation of the PI3K-AKT-mTOR pathway [4].

In breast cancer (BC), aberrant activation of this pathway has been well-documented in estrogen receptor (ER)-positive tumors, being associated with resistance to endocrine therapy and disease progression [5]. Figure 1 schematically summarizes the PI3K-AKT-mTOR pathway in ER-positive BC.

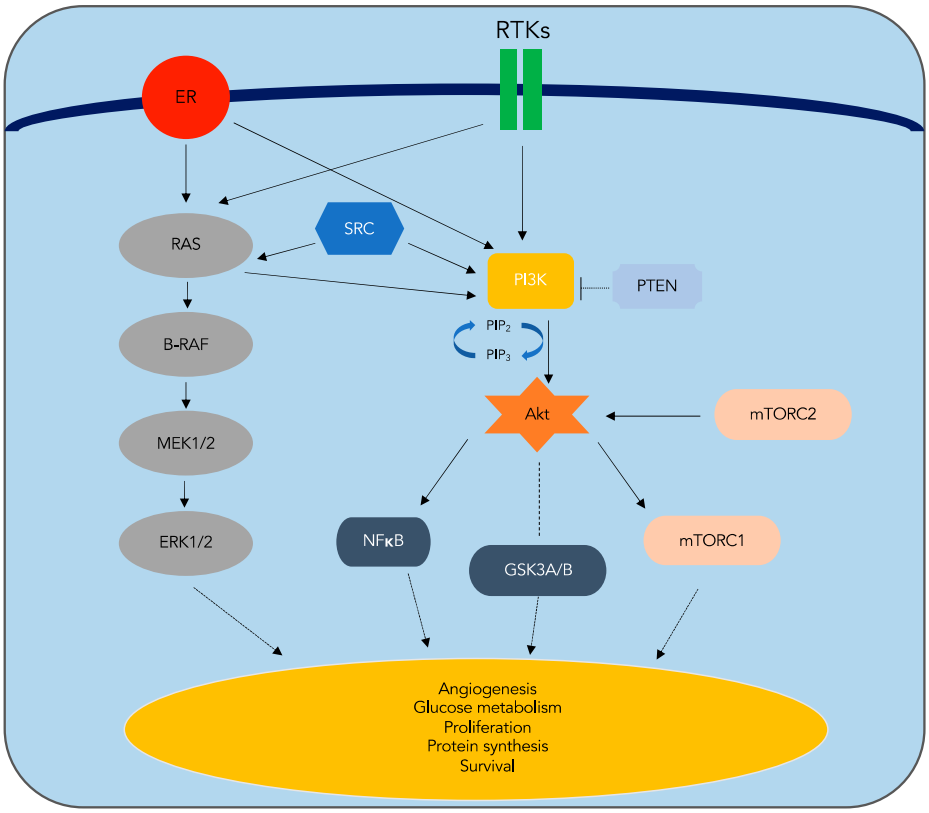

Figure 1. Phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT)-mammalian target of rapamycin (mTOR) in ER-positive/HER2-negative breast cancer (ER+/HER2− BC). Abbreviations: Akt, protein kinase B; B-RAF, murine sarcoma viral oncogene homolog; ER, estrogen receptor; ERK1/2, extracellular-signal regulated kinase 1/2; GSK3A/B, glycogen synthase kinase-3 α/β; HR, hormone receptor-positive; MEK, Mitogen-activated protein kinase; mTOR(C), mammalian target of rapamycin (complex); NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; PI3K, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol phosphate; PTEN, phosphatase and tensin homolog; RTKs, receptor tyrosine kinases; SRC, rous sarcoma.

Considering the central role of the PI3K pathway in ER-positive BC, several inhibitors have been developed and tested in clinical trials. If the use of pan-PI3K inhibitors, such as buparlisib and pictilisib, may ensure broad activity with a range of molecular drivers, isoform-specific inhibitors, including taselisib and alpelisib, may reduce off-target toxicity [6–8]. Indeed, high levels of treatment-related adverse events have been one of the major limitations for the clinical development of these drugs, especially for pan-PI3K inhibitors that have never received approval from regulatory agencies for clinical use [9]. More recently, the PI3Kα-selective inhibitor alpelisib was the first PI3K inhibitor approved for the treatment of ER-positive BC based on the results of the phase III SOLAR-1 trial [10]. At this regard, several new challenges will be expected once alpelisib is widely available in a clinical setting, including the harmonization of testing procedures for the detection of PI3K pathway alterations.

In this review article, we provide an overview on PI3K pathway alterations in ER-positive BC, discuss their roles in determining prognosis and resistance to endocrine therapy and highlight practical considerations about diagnostic methods for the detection of PI3K pathway activation status.

2. PI3K Pathway Alterations in ER-Positive BC

As aforementioned, dysregulation of the PI3K–AKT–mTOR pathway has been demonstrated to be implicated in BC development and progression [5]. The most common PI3K pathway alteration in BC is represented by *PIK3CA* oncogenic mutations [11]. The frequency of *PIK3CA* mutations range between 18–40%, being more frequently detected in ER-positive rather than ER-negative tumors [12–14]. Several pieces of evidence suggest that *PIK3CA* oncogene alterations are implicated in PI3K pathway activation in ER-positive BC [11], including both somatic mutations and gene amplifications [15]. Even if *PIK3CA* gene amplification has been correlated to an increased PI3K pathway activity, its frequency is relatively rare and detected in less than 10% of all BCs [15]. On the other hand, mutations of the *PIK3CA* gene are the most common mechanism responsible for deregulation of the PI3K pathway in ER-positive BC. Importantly, several preclinical models demonstrated that *PIK3CA*-activating mutations confer an aggressive phenotype and growth-factor-independent tumor growth [16,17]. Although *PIK3CA* mutations resulted as oncogenic in several in vitro models, these alterations are weak oncogenes in vivo with different degrees of estrogen dependence [18]. The discordance in growth and proliferation between single *PIK3CA* mutations in different preclinical models implies that additional mechanisms (either intrinsic or extrinsic to the PI3K pathway) are needed for the full PI3K oncogenic phenotype [11]. The clinical corollary to this observation is the lack of benefit from PI3K inhibitor monotherapy in several solid tumors [2].

In BC, activating tumor mutations of *PIK3CA* may occur in several domains of the p110α catalytic subunit; however, the most common mutations occur in the helical and kinase domains (exon 9 and 20), which represent the "hotspots" [19,20]. The highest frequency of missense mutations corresponds to the following amino acids: E542K and E545K in exon 9 (helical domain), and H1047R and H1047L in exon 20 (kinase domain) [19,20]. E545K and H1047R are activated by distinct mechanisms: E545K mimics activation by RTK phosphopeptides and is dependent on Ras, and H1047R increases lipid membrane binding, promoting access to PIP2 substrate in a Ras-independent manner [21]. Figure 2 summarizes prevalence and distribution of *PIK3CA* hotspots mutations in ER-positive BC. *PIK3CA* mutations are not the only genetic alterations that can affect the activity of the PI3K pathway, but the frequency of the other alterations is much lower than *PIK3CA* mutations [13]. For instance, inactivation of the *PTEN* gene can result in abnormal PI3K pathway activation via loss of function mutations, gene deletions, or transcriptional down-regulation [22]. However, *PTEN* mutations are rare in ER-positive BC and are mutually exclusive with *PIK3CA* mutations in untreated breast tumors [12,23]. Conversely, *PTEN* mutations can emerge during tumor evolution and have been associated with resistance to PI3Ka-selective inhibitors in ER-positive BC [24,25].

Furthermore, activating mutations in AKT isoforms are rarely identified in ER-positive BC, with the AKT1 E17K hotspot mutation that can be found in 3% of cases [12].

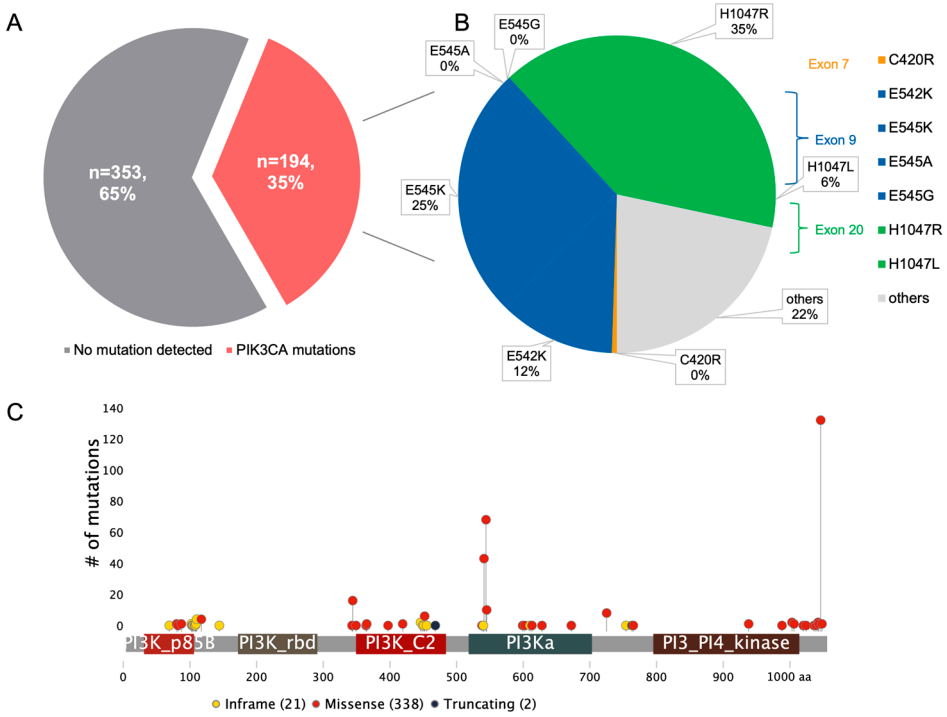

Figure 2. Prevalence and distribution of *PIK3CA* hotspots mutations in ER-positive/HER2−negative breast cancer (ER+/HER2− BC). (**A**) PIK3CA mutation prevalence in ER+/HER2− BC (n = 547 screened from the Catalogue of Somatic Mutations in Cancer [COSMIC]). (**B**) Variant frequency in PIK3CA mutated ER-positive/HER2−negative BC (n = 194). COSMIC data are filtered to ER-positive/HER2−negative BC taken from the Catalogue of Somatic Mutations in Cancer (COSMIC). Available at: www.sanger.ac.uk/genetics/CGP/cosmic (accessed on 10 January 2021). (**C**) Lollipop plot representing the distribution of PIK3CA mutations positioned by their amino acid coordinates across the protein domain. Data were retrieved from The Cancer Genome Atlas (TCGA) breast cancer (n = 825) genomic dataset through cBioportal and reanalyzed by RStudio software (version 3.6.3). Available at: https://www.cbioportal.org (accessed on 10 January 2021).

In ER-positive BC, convincing evidence supports the key role of the Pi3K/Akt/mTOR pathway activation in mediating endocrine resistance. ER signaling fosters tumor growth and survival by different molecular mechanisms, including both genomic and non-genomic functions [26]. At nuclear level, estrogens bind ER protein leading to the creation of a transcription complex that exerts the so-called ER classical genomic activity, by directly binding specific promoter regions of target genes known as ER elements (EREs) [27]. Endocrine agents, including tamoxifen and selective estrogen receptor degraders (SERDs), effectively inhibit ER classical genomic activity. In addition, estrogen/ER complexes can also interact with other transcription factors, such as AP-1/SP-1 family members, modulating alternative transcriptional programs [28,29]. Conversely, the non-nuclear ER mechanism of action is mediated by an extended molecular crosstalk between ER and other intracellular pathways, in which estrogen/ER complexes directly interact with other molecules and their downstream signaling pathways, including the Pi3K/Akt/mTOR pathway [30]. Importantly, an aberrant activation of this pathway can promote the development of endocrine resistance, acting as escape pathway and bypassing the ER signaling blockade [30]. Moreover, it can also modulate both alternative genomic and non-genomic ER functions and ultimately sustains resistant cell growth.

Interestingly, hyperactivation of the PI3K signaling is associated with low ER levels, which have been correlated with resistance to endocrine therapy [31]. Notably, significant crosstalk between ER and PI3K pathways exists. Inhibition of PI3K signaling results in an induction of the ER-dependent transcriptional activity by increased expression of genes containing ER-binding sites, occupancy by the ER of promoter regions of up-regulated genes, and higher expression of estrogen receptor 1 (ESR1) messenger RNA (mRNA) levels [32]. In addition, PI3K inhibition is able to induce an open chromatin state at the ER target loci in breast cancer models. Mechanistically, the histone-lysine N-methyltransferase 2D (KMT2D), a histone H3 lysine 4 methyltransferase, is required for forkhead box protein A1 (FOXA1), pre-B-cell leukemia transcription factor 1 (PBX1), and ER recruitment and activation. AKT binds and phosphorylates KMT2D, attenuating methyltransferase activity and ER function, whereas PI3Kα inhibition enhances KMT2D activity [33]. These observations provided a strong rationale to combine PI3K inhibition with endocrine therapy.

3. Prognostic and Predictive Value of PIK3CA Mutational Status in ER-Positive BC

The influence of PIK3CA mutational status on survival outcomes of patients with ER-positive BC is still debated. In early-stage ER-positive breast cancer, PIK3CA mutations represent a good prognostic factor for invasive disease-free survival (iDFS), but not for distant disease-free survival (DDFS) nor overall survival (OS) [34]. Conversely, patients with PIK3CA-mutated ER-positive metastatic BC seem to derive less benefit from chemotherapy and present a worse OS when compared with the PIK3CA wild-type counterparts [35]. Similarly, controversial results have been reported about the predictive role of PIK3CA mutations on response to PI3K inhibitors (Table 1). In the early setting, no predictive value has been documented. In the phase II LORELEI trial, which evaluated neoadjuvant therapy with letrozole +/− the PIK3 inhibitor taselisib in patients with ER-positive/HER2−negative BC, similar benefit from the addition of taselisib was observed in the overall population as compared to the PIK3CA-mutated subgroup [36]. Similar results were obtained in a phase II, window-of-opportunity trial where PIK3CA mutation was not predictive of Ki67 response to the pan-PI3K inhibitor pictilisib combined with anastrozole [37]. On the other hand, contradictory results have been obtained in the metastatic setting where the BELLE-2 and BELLE-3 trials demonstrated a significant benefit in progression-free survival (PFS) with the administration of the pan-PI3K inhibitor buparlisib plus endocrine therapy in patients with PIK3CA mutations [38,39]. In contrast, the FERGI and PEGGY trials did not identify any predictive value related to the PIK3CA mutational status [40,41]. Table 1 reports the main findings from phase II/III clinical trials on the predictive role of PIK3CA mutational status in ER-positive, HER2−negative advanced or metastatic BC.

Table 1. Main results on the predictive role of PIK3CA mutational status from phase II/III clinical trials testing PI3K inhibitors in ER-positive/HER2−negative advanced or metastatic breast cancer.

Trial	Phase	Population	Treatment	Tested Tissue	HR in PIK3CA Mutated	HR in PIK3CA WT/ITT Pop	Conclusion
BELLE-2 [38]	III	ER+/HER2−, after AI	Fulvestrant ± buparlisib	Archived tissue	0.78 (0.67–0.89)	0.76 (0.60–0.97)	No predictive value
				Blood (ctDNA)	0.58 (0.41–0.82)	1.02 (0.79–1.30)	Benefit only in PIK3CA mutated
BELLE-3 [39]	III	ER+/HER2−, after ET + everolimus	Fulvestrant ± buparlisib	Archived tissue	0.39 (0.23–0.65)	0.81 (0.59–1.12)	Benefit only in PIK3CA mutated
				Blood (ctDNA)	0.46 (0.29–0.73)	0.73 (0.53–1.00)	
FERGI [40]	II	ER+/HER2−, after AI	Fulvestrant ± pictilisib	Tissue (not specified)	0.73 (0.42–1.28)	0.74 (0.52–1.06)	No predictive value
PEGGY [41]	II	ER+/HER2−, 1st/2nd line CT	Paclitaxel ± pictilisib	Tissue (not specified)	1.06 (0.52–2.12)	0.95 (0.62–1.46)	No predictive value
SOLAR-1 [10,42]	III	ER+/HER2−, after ET	Fulvestrant ± alpelisib	Archived tissue	0.65 (0.50–0.85)	0.85 (0.58-1.25)	Benefit only in PIK3CA mutated
				Blood (ctDNA)	0.55 (0.39–0.79)	0.80 (0.60–1.06)	
SANDPIPER [43]	III	ER+/HER2−, after AI	Fulvestrant ± taselisib	Archived tissue	0.70 (0.56–0.89)	0.69 (0.44–1.08)	Benefit only in PIK3CA mutated
				Blood (ctDNA)	0.62 (0.47–0.83)	0.86 (0.57–1.27	

Several reasons may explain such discrepancy. Compensatory mutational mechanisms can be involved in intrinsic and adaptive resistance to PI3K inhibition [44]. *PIK3CA*-mutated BCs display a higher frequency of mitogen-activated protein kinase kinase kinase 1 (MAP3K1) mutations, which are involved in the activation of the MEK pathway [35]. Preclinical evidence showed that MAP3K1 loss of function could promote resistance to α-selective PI3K inhibition by activating insulin receptor substrate 1 (IRS1) [45], even if this association needs to be confirmed in the clinical setting. Accordingly, targeting PI3K and other co-drivers, including MEK pathway components, could increase PI3K inhibitors antitumor activity. Furthermore, *PIK3CA* mutations do not lead to pathway activation in a subset of patients [46,47], potentially resulting in a null or limited biological effect of PI3K inhibition on tumor cells. Conversely, double *PIK3CA* mutations on the same allele (also referred as in cis) are associated with an augmented PI3K pathway activity and downstream signaling through increased p110α membrane lipid binding [48]. This biological finding has been confirmed in the clinical setting, where patients with BC and double PIK3CA mutations had an increased sensitivity and clinical benefit to PI3Kα-selective inhibitors as compared to single-hotspot mutations. In this way, the use of more specific and higher bioactive drugs, such as the PI3Kα-selective alpelisib, can produce better results as highlighted in the SOLAR-1 trial [10].

SOLAR-1 is a phase 3, randomized study that investigated the addition of alpelisib to fulvestrant in patients with ER-positive/HER2−negative advanced BC who had received previous endocrine therapy [10]. Of note, patients were enrolled into two different cohorts based on tumor-tissue *PIK3CA* mutation status. The study met its primary endpoint demonstrating a statistically significant improvement in PFS in patients carrying PIK3CA mutations (median PFS 11.0 vs. 5.7 months, hazard ratio (HR) 0.65; 95% confidence interval (CI), 0.50–0.85; $p < 0.001$) [10]. However, this consistent benefit in PFS did not translate in a statistically significant improvement in OS, while the numeric improvement of 7.9 months in median OS in the alpelisib arm might be considered as clinically relevant [49]. Notably, patients with lung and/or liver metastases reported an improvement in median OS of 14.4 months, supporting the concept that some patient subgroups may derive considerable clinical benefit with addition of alpelisib to fulvestrant.

Several mechanisms of secondary resistance, such as PTEN mutations and deletions, can dampen the efficacy of PI3Kα-selective inhibitors [24,25], providing a rationale to combine PI3K and AKT/mTOR inhibitors. Similarly, CDK4 activation has been identified as a mechanism of primary resistance to alpha-selective PI3K inhibition [50], prompting the development of combinations of PI3K and CDK 4/6 inhibitors. Lastly, the development of new mutations-specific PIK3CA inhibitors, such as GDC-0077, may improve bioactivity and guarantee a strongest inhibition of cancer-cell-carrying *PIK3CA* mutations [51].

4. Diagnostic Methods for the Detection of PIK3CA Mutations in Breast Cancer

Several methods to assess *PIK3CA* mutations in tumor samples have been developed and validated. These include real-time polymerase chain reaction (PCR), digital droplet PCR (ddPCR), BEAMing assays, Sanger sequencing, and next-generation sequencing (NGS) panels.

As every diagnostic test, PIK3CA tests are commonly evaluated in terms of key analytical and clinical criteria [52]. Analytical criteria include reagent stability, precision of the assay, sensitivity and specificity, and the applicability of the test to different tumor specimens. On the other hand, clinical criteria encompass the correlation between test results and clinical outcome, its utility in the contest of other available tests, its reproducibility, and, importantly, the labor and time required to perform the analysis. Obviously, each method displays some advantages and limitations (Table 2). Generally, when we decide to use a highly sensitive method, such as real-time PCR or ddPCR, genomic coverage is reduced, and we can focus only on one or a limited number of genes. Conversely, we can adopt NGS platforms to look at hundreds or thousands of genes but, in that case, sensitivity to detect an alteration that is present at a very low level is dampened.

Table 2. Comparison of methods for PIK3CA mutation assessment.

	Real-time PCR	ddPCR	BEAMing	Sanger seq	NGS
Pros	Sensitive: can detect mutant DNA present at 1–5%	High level of sensitivity and specificity	High level of sensitivity	Unknown mutations can be detected	Sensitive for low-abundance mutations
	Relatively inexpensive	Relatively inexpensive		Can detect gene fusions using RNA	Can detect a wide range of genetic changes in numerous genes
Cons	Only detects known targeted mutations	Only detects known targeted mutations	Only detects known targeted mutations	Labor intensive	Expensive and requires different DNA preparation method than other molecular mutation assays
		Limited in the types of mutations detected	Limited in the types of mutations detected	Not as sensitive: requires mutant DNA to be present at 20%–25%	Requires more tumor tissue and sophisticated bioinformatics
		Limited multiplexing capability	Limited multiplexing capability	Cannot detect changes in exon or gene copy number	
Most common sample type	Tumor tissue and plasma	Plasma	Plasma	Tumor tissue	Tumor tissue and plasma

In the SOLAR-1 trial, tissue biopsy samples were collected by investigator sites and sent to a single central laboratory for PIK3CA testing [10]. For SOLAR-1 enrollment, PIK3CA mutation testing initially utilized a validated PCR-based clinical trial assay and transitioned to the QIAGEN therascreen® PIK3CA RGQ PCR Kit [53]. PIK3CA tests were multiplex qualitative real-time PCR assays for the detection of specific mutations in the PIK3CA gene. PIK3CA mutation testing was performed on formalin-fixed paraffin-embedded (FFPE) tumor specimens using tissue obtained at initial diagnosis or at the most recent biopsy. Patients enrolled in the PIK3CA-mutant cohort were those with ≥1 mutation in exons 7, 9, and 20, which include mutations common in patients with ER-positive BC. As expected, the majority of tissue samples used for PIK3CA screening at enrollment in SOLAR-1 were from primary tumors rather than metastases (77% vs. 22%) [53]. Distribution of PIK3CA mutation status was similar for primary as compared to metastatic tumors. Even if mutational heterogeneity between primary tumor and metastases suggests that biomarker assessment should be performed in multiple sites to account for genomic clonal evolution [54,55], it might be very challenging and poorly reliable in daily clinical practice. Moreover, a comparative genomic analysis comparing primary tumors and matched metastases from patients with BC highlighted that concordance was elevated (92%) for highly recurrent gene variants [56]. On the other hand, other studies reported that acquired driver mutations after treatment with cyclin-dependent kinase 4 and 6 (CDK 4/6) inhibitors and endocrine therapy can be found in up to 30% of patients, including 6% of new emerging *PIK3CA* mutations [57]. In this view, tissue samples from primary tumor can be used to test patients eligible for alpelisib in clinical practice, even if a re-biopsy of the metastatic lesions if feasible is generally preferable. Furthermore, a retrospective analysis applied the NGS FoundationOne CDx™ gene panel on SOLAR-1 tissue samples [53]. Of 404 patients with valid NGS results, 229 (59%) had a PIK3CA alteration and 165 (41%) had no detectable PIK3CA alteration. Among 239 patients with a PIK3CA alterations, 238 (99%) presented *PIK3CA* mutations. Overall, 208 (87%) had a mutation detectable by PCR, while 30 (13%) more patients were detected with a mutation not included in the probes used for PCR.

For the primary analysis of SOLAR-1, mutation status was determined from a tumor tissue sample. Moreover, plasma samples were also collected at baseline and analyzed by PCR to retrospectively assess PFS by circulating tumor DNA (ctDNA)-based PIK3CA mutation status as a secondary endpoint [42]. In line with the results observed when PIK3CA mutational status was assessed on tumor samples, there was a 45% risk reduction in PFS for patients with ctDNA PIK3CA mutations (HR 0.55; 95% CI 0.39–0.79), while there was only 20% risk reduction for patients without (HR 0.80; 95% CI 0.60–1.06). Concordance between tissue and ctDNA was 55%, with fewer patients with PIK3CA-positive status in plasma than in tumor tissue. Potential explanations for the lack of concordance include tumor heterogeneity, different sequencing techniques, spatial and temporal factors, and potential plasma DNA contamination [58]. Several biomarker analyses of clinical trials compared *PIK3CA* mutation status from tissue and plasma obtained from patients with ER-positive/HER2−negative advanced BC [38,39,58]. Although each study utilized a different PIK3CA testing method, concordance rates ranged from 70% to 83%. Interestingly, in the BOLERO-2 trial a higher concordance between ctDNA and tumor tissue from metastatic lesions was observed [59]. Several issues on plasma to tissue concordance exist. Many cancer patients shed insufficient tumor DNA and, therefore, ctDNA levels vary greatly due to several factors such as disease burden, non-tumor shedding, and clearance. ctDNA and tissue DNA are often collected at different times, being representative of different diseases. Indeed, ctDNA and tissue DNA should represent the same biological entity but often differ due to biological, clinical, and technical reasons [60]. If plasma is positive for PIK3CA, this may obviate the need for a biopsy given that this predicts for excellent outcome on alpelisib (median PFS: 10.9 months), similar to that observed when alpelisib is given based on tumor tissue results. In contrast, if plasma genotyping for PIK3CA is negative, this result cannot fully obviate need for a tumor biopsy. As the plasma PIK3CA-negative population is a

mixture of true and false negatives, biopsy to further investigate the presence of PIK3CA positive tumor tissue is warranted.

Importantly, the U.S. Food and Drug Administration (FDA) approved alpelisib along with *therascreen* PIK3CA RGQ PCR Kit (QIAGEN GmbH), FoundationOne® CDx and FoundationOne® Liquid CDx as companion diagnostics. Conversely, the European Medicines Agency (EMA) generally recommended the use of a validated test for PIK3CA mutation assessment.

5. Conclusions

PIK3CA mutations are highly represented in ER + BC and are of relevant clinical interest due to the possibility of using targeted therapies. It is critical to adopt the best methodology to assess all the possible gene alterations, in order to increase the number of patients potentially benefiting from such treatments. In daily practice, the PCR-based companion diagnostics can be generally sufficient to identify patients eligible for treatment with alpelisib. However, the use of larger NGS panels, which are even more easily available at considerably reduced costs, might allow to identify alterations in genes related to therapeutic resistance [25] as well as less-common PIK3CA mutations [53]. NGS panels allow covering numerous alterations at once, even starting from low input DNA. Available diagnostic assays for the evaluation of PIK3CA mutation status may be used on both tumor tissue and ctDNA. The latter is particularly valuable in case of unavailable or inaccessible tissue material. Assessment of PIK3CA mutation status on primary tumor is generally acceptable when a new biopsy of the metastatic site is not feasible, while the profiling of metastatic sample is recommended to handle tumor heterogeneity and clonal evolution. In this way, the use of assays for ct-DNA-based evaluation of PIK3CA mutation status might reduce the number of invasive procedures and allow serial monitoring of tumor evolution. However, liquid biopsy suffers from several issues, in particular low ctDNA content. Even if NGS analysis may overcome this problem, it should be considered that patients with negative results on ctDNA analysis should undergo a second liquid biopsy test or, if possible, a tissue biopsy to exclude false-negative results. In this scenario, the molecular report is crucial to guarantee that all relevant information is available to the clinicians [61,62]. Molecular reports should be brief, easy to interpret, and include the most interesting information to clinicians. In particular, any misinterpretation should be carefully avoided. Basic requirements for all molecular reports should include patients' unique identifiers, ward or service, date, sample type, and the name of the clinician ordering the molecular analysis. Information on any sample information issue (e.g., any fixation problem, presence of contaminants, the amount of neoplastic cells) and on the mutational status of the analyzed biomarkers have to be reported, as well as the methodology used, the reference range, detection limits, and the run parameters.

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Conflicts of Interest: G.C. served as consultant or advisor for Roche, Lilly, and Bristol-Myers Squibb, served on the speaker's bureau for Roche, Pfizer, and Lilly, received travel funding from Pfizer

and Roche, and received honoraria from Roche, Pfizer, Lilly, Novartis, and SEAGEN. A.M. has no competing interests to disclose. C.C. has received honoraria for consultancy/advisory role/speaker bureau from Pfizer, Lilly, Novartis, Roche, and MSD all outside the submitted work.

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