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Review

Next-generation sequencing methodologies to identify patients for targeted therapy: focus on HR+/HER2– metastatic breast cancer

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Summary

Alterations in the phosphoinositide 3-kinase (PI3K)/AKT/PTEN signaling pathway are a well-recognized mechanism of resistance in hormone receptor-positive, HER2-negative metastatic breast cancer (HR+/HER2- mBC). These alterations are present in approximately half of patients with HR+/HER2- mBC. The major alterations in the pathway are somatic mutations in the *PIK3CA* (40–45%) and *AKT1* (5%) genes, and loss-of-function alterations in *PTEN* (5–10%). New targeted agents that act against these alterations have been developed. Therefore, it is important to determine the mutational status of genes in this pathway to potentially offer a therapeutic alternative for these patients. In this review, we discuss the clinical and biological significance of PI3K pathway alterations in HR+/HER2– mBC, focusing on tumors that progress following endocrine therapy and CDK4/6 inhibitor treatment. We then highlight how different diagnostic strategies, including sample type, testing methodology, and timing, can improve the identification of patients who are eligible for targeted therapies and promote the effective integration of molecular diagnostics into routine clinical care.

Key words: Next-generation sequencing, PI3K pathway, metastatic breast cancer, somatic mutation

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Introduction

Dysregulation of the phosphoinositide 3-kinase (PI3K)/AKT/PTEN signaling pathway is a well-recognized mechanism of resistance in hormone receptor-positive, HER2-negative (HR+/HER2–) metastatic breast cancer (mBC), affecting approximately 50% of patients [1,2]. To counteract this resistance, several targeted therapies have been developed, including PI3Kα-specific inhibitors such as alpelisib and inavolisib, and the pan-AKT inhibitor capivasertib, some of which have been approved by

both the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) [3-5]. Accurate molecular testing is essential for personalizing treatment strategies and guiding optimal clinical management in this population.

Pathway activation is commonly driven by somatic mutations in *PIK3CA* (~40–45%) or *AKT1* (~ 5%) as well as by loss-of-function alterations in *PTEN* (~5-10%) [6-8]. Next-generation sequencing (NGS) remains the gold standard for detecting these alterations, offering the sensitivity and breadth required to identify both common and rare variants [9,10]. Broad NGS panels provide comprehensive molecular profiling, including co-occurring mutations and loss of heterozygosity (LOH), while smaller targeted panels or PCR-based assays may offer faster turnaround and lower costs but can miss non-hotspots or complex alterations. In the case of PTEN, integrative diagnostic strategies are often required, as immunohistochemistry (IHC) alone is insufficient to reveal the underlying genetic or epigenetic causes of protein loss [11-13].

In terms of specimen choice, tissue biopsies are considered the reference standard, yielding high-quality DNA [14, 15]. Notably, the CAPItello-291 trial used a tissue-based NGS platform (FoundationOne® CDx) to detect alterations in *PIK3CA*, *AKT1*, and *PTEN* [16, 17]. In contrast, liquid biopsy, primarily via circulating tumor DNA (ctDNA), offers a non-invasive and dynamic method for real-time molecular monitoring. However, its sensitivity depends on ctDNA fraction and prior treatments. In the INAVO120 trial, *PIK3CA* mutations were assessed using ctDNA-based NGS assay (FoundationOne® Liquid CDx). Reflex tissue testing is recommended in cases with low ctDNA yield to minimize the risk of false-negative results [18, 19]. Together, these trials illustrate the complementary roles of tissue and plasma in assessment of the PI3K pathway.

Herein, we examine the clinical and biological significance of PI3K pathway alterations in HR+/HER2- MBC, particularly in tumors that progress after endocrine therapy and CDK4/6 inhibitor treatment. We highlight how diagnostic strategies, including sample type, testing methodology, and timing, can improve the identification of patients eligible for targeted therapies and promote the effective integration of molecular diagnostics into routine clinical care.

Mechanisms of resistance

In HR+/HER2- breast cancer, mechanisms related to the use of both CDK4/6 inhibitors and endocrine therapy (ET) involve complex interactions targeting the cell cycle machinery and hormone signaling pathways [20, 21]. Combination therapy works synergistically by inhibiting cell cycle progression through CDK4/6 blockade, while simultaneously suppressing estrogen-dependent tumor growth. Despite the significant improvements in progression-free survival (PFS) demonstrated in clinical trials and real-world settings [22], resistance inevitably develops through multiple mechanisms, including alterations in cell cycle regulators (*RB1* loss and *CCNE1* amplification), *CDK4/6* mutations, and activation of alternative signaling pathways, such as PI3K/AKT/mTOR [23]. These resistance mechanisms can be attributed specifically to endocrine therapy, CDK4/6 inhibitors, or both treatments, often based on underlying genetic features and molecular markers [24].

CDK4/6 INHIBITOR-RELATED MECHANISMS OF RESISTANCE

Resistance associated with CDK4/6 inhibitors encompasses a complex network of molecular alterations that affect cell cycle regulation and alternative signaling pathways. These mechanisms can be categorized into primary alterations in cell cycle machinery components and secondary activation of compensatory signaling cascades [25, 26].

RB1 gene alterations as a primary resistance mechanism

In the canonical pathway, CDK4/6 complexed with D-type cyclins phosphorylates the retinoblastoma (RB) protein, which subsequently releases E2F transcription factors from inhibitory binding, enabling the transcription of genes that are essential for G1/S phase transition [27]. Given that functional RB is the primary substrate and mediator of CDK4/6 inhibitory activity, its disruption represents a fundamental resistance mechanism. Clinical evidence demonstrates that patients harboring *RB1* loss-of-function mutations exhibit significantly decreased PFS following CDK4/6 inhibitor therapy, compared to those without such alterations [28]. Longitudinal genomic analyses of tumor samples revealed enrichment of *RB1* alterations in post-treatment specimens, including various splicing site substitutions, insertions, deletions, and point mutations, not detected in pre-treatment biopsies. These alterations can emerge as polyclonal mutations after exposure to CDK4/6 inhibitors, suggesting convergent evolution under therapeutic pressure [29]. Intriguingly, CDK4/6 inhibitor treatment itself can promote βTrCP1-mediated ubiquitination and proteasomal degradation of the RB protein, contributing to treatment resistance through post-translational mechanisms independent of genetic alterations [30]. Even heterozygous *RB1* loss has been identified as a biomarker of acquired resistance and poor clinical outcome, suggesting that a partial reduction in RB function may be sufficient to confer therapeutic resistance [28].

Cyclin E-CDK2 axis activation

In addition to *RB1* alterations, amplification or overexpression of cyclin E1 (CCNE1) represents another prevalent resistance mechanism [31]. Elevated CCNE1 level enables its binding partner CDK2 to phosphorylate RB, effectively bypassing CDK4/6 inhibition and restoring cell cycle progression [26]. This mechanism has been validated through multiplex single-cell imaging analyses, demonstrating concurrent upregulation of CDK2 and cyclin E1 in resistant cells [32].

Recent evidence indicates that CDK2 activation occurs in a two-step process: initial RB protein degradation, followed by c-Myc-mediated amplification of E2F transcriptional activity [30]. The cyclin E-CDK2 complex is particularly crucial for cell survival in cancers with concurrent MYC and CCNE1 overexpression, establishing a synthetic lethal interaction that represents a potential therapeutic vulnerability [33].

Alternative signaling pathway activation

Resistance to CDK4/6 inhibitors frequently involves the activation of alternative signaling pathways that circumvent cell cycle arrest. Activation of the interferon (IFN) signaling pathway is strongly associated with both intrinsic and acquired resistance [34]. Mechanistically, downregulation of the splicing regulator NSRP1 contributes to CDK4/6 inhibitor resistance by mediating alternative splicing of NSD2 mRNA and subsequent activation of the IFN pathway [35]. Aberrant FGFR signaling represents another major resistance mechanism, with FGFR inhibition capable of reversing CDK4/6 inhibitor resistance in preclinical models. Additional resistance pathways include the activation of the MAPK and PI3K/AKT signaling cascades [25].

Mechanisms associated to changes in signal transduction pathways

The PI3K/AKT/PTEN signaling axis represents a critical mediator of acquired and intrinsic resistance to CDK4/6 inhibitors in HR+/HER2- breast cancer through complex molecular interactions with the cell cycle machinery. Dysregulation of this pathway enables cancer cells to circumvent CDK4/6 inhibition via multiple interconnected mechanisms. Genomic alterations affecting this pathway are quite common. Specifically, across 9,598 breast cancer cases, including both primary and metastatic cohorts, from studies available in cBioPortal (MSK-2018, MSK-2022, MSK-2025, METABRIC, and TCGA), mutations in *PIK3CA*, *AKT1*, or *PTEN* were detected in 4,700 tumors (~49%) (36-40). These alterations typically involve recurrent hotspot mutations (e.g., *PIK3CA* H1047R, E545K; *AKT1* E17K) or loss-of-function events (*PTEN*), and represent clinically actionable targets for pathway-directed therapies (Fig. 1).

Molecular crosstalk between PI3K/AKT and cell cycle machinery

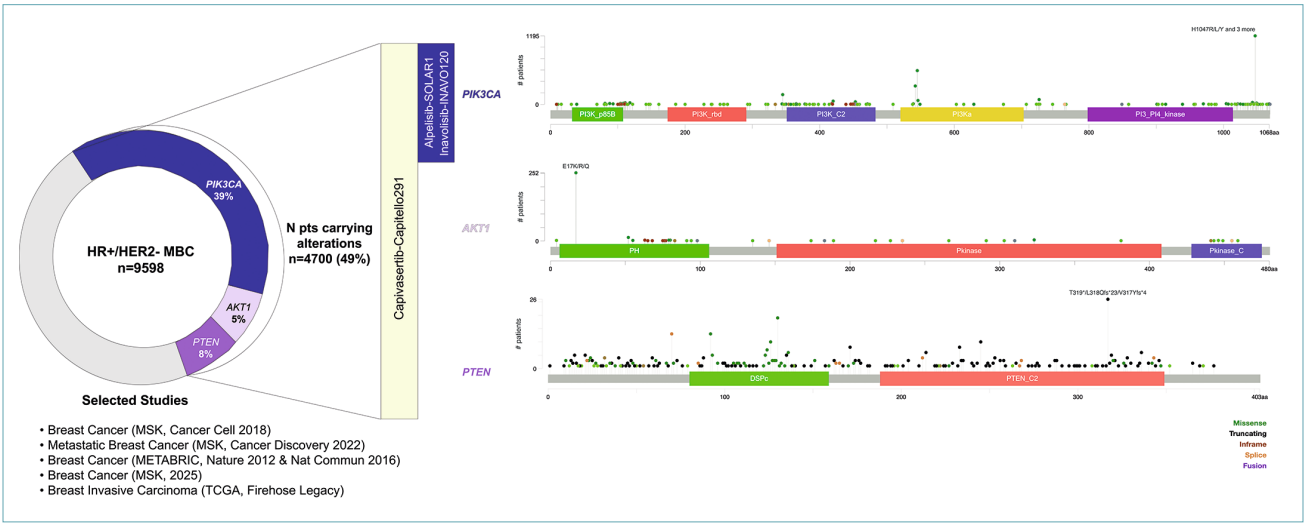

Figure 1. PI3K/AKT/PTEN signaling pathway and associated genomic alterations in HR+/HER2– metastatic breast cancer. Data from 9,598 cases across selected cBioPortal studies (MSK-2018, MSK-2022, MSK-2025, METABRIC, TCGA) show that ~49% of tumors harbor alterations in *PIK3CA*, *AKT1*, and/or *PTEN*. Lollipop plots indicate the distribution and domain localization of recurrent mutations; *PTEN* deletions are not included. Approved and emerging targeted therapies directed against these alterations are shown at their respective sites of action.

The PI3K/AKT pathway exhibits extensive and bidirectional crosstalk with the cell cycle regulatory machinery, creating a sophisticated network that enables cancer cells to develop resistance to CDK4/6 inhibitors. This molecular interplay occurs at multiple levels and involves direct protein-protein interactions, post-translational modifications, and transcriptional regulation. At the protein level, resistant cells characteristically display concurrent upregulation of multiple cyclins (A, E, and D1), activated phospho-CDK2, and elevated phospho-AKT levels [41]. This coordinated activation pattern suggests a mechanistic link between AKT signaling and cell cycle regulation. AKT directly phosphorylates and inactivates p27Kip1, a CDK inhibitor that normally inhibits CDK2 activity [42]. By neutralizing p27Kip1, activated AKT releases CDK2 from inhibition, allowing the formation of active Cyclin E-CDK2 complexes that drive S-phase entry, despite CDK4/6 blockade. AKT promotes cyclin D1 expression through multiple mechanisms. AKT phosphorylates and inhibits glycogen synthase kinase 3β (GSK3β), which normally targets cyclin D1 for proteasomal degradation [25]. By stabilizing the cyclin D1 protein, AKT enhances the formation of cyclin D1-CDK4/6 complexes, potentially overwhelming the inhibitory capacity of CDK4/6 inhibitors through a mass-action effect. Furthermore, AKT activates mTORC1-mediated protein synthesis of cyclins and other cell cycle regulators, maintaining their expression despite CDK4/6 inhibition [43].

PTEN loss as a driver of cross-resistance

PTEN loss represents a critical mechanism for both intrinsic and acquired resistance to CDK4/6 inhibitors in HR+/HER2- breast cancer (7, 44). The molecular consequences of *loss of PTEN* are multifaceted. First, PTEN deficiency leads to constitutive activation of the PI3K/AKT/mTOR signaling cascade, which serves as a bypass pathway when CDK4/6 is inhibited [26]. This hyperactivation of AKT signaling promotes cell survival by inhibiting pro-apoptotic factors, such as BAD and caspase-9, while simultaneously driving cell cycle progression despite CDK4/6 inhibition. Second, PTEN loss facilitates reactivation of the AKT-mTORC1 pathway in breast cancers treated with CDK4/6 inhibitors, enabling the translation of key cell cycle proteins even when canonical CDK4/6 activity is blocked [45]. Notably, PTEN loss has been identified as a mediator of clinical cross-resistance to both CDK4/6 and PI3Kα inhibitors, suggesting shared resistance mechanisms between these therapeutic classes [44]. This cross-resistance phenomenon has significant implications for treatment sequencing strategies for breast cancer management.

PIK3CA mutations in resistance development

PIK3CA mutations, which encode the p110α catalytic subunit of class I PI3K, represent a significant mechanism for both intrinsic and acquired resistance to CDK4/6 inhibitors in HR+/HER2- breast cancer. These activating mutations, predominantly occurring in hotspot regions within the helical (p.E542K, p.E545K) and kinase (p.H1047R) domains, lead to constitutive activation of PI3K signaling, independent of upstream receptor tyrosine kinase activity [46].

Liquid biopsy analyses have revealed that *PIK3CA* mutations are associated with reduced sensitivity to CDK4/6 inhibitors in metastatic breast cancer patients, with mutated patients showing significantly shorter progression-free survival compared to wild-type counterparts (7.44 vs 12.9 months) [47]. This clinical observation is supported by mechanistic studies demonstrating that *PIK3CA*/*AKT1* mutations reduce the efficacy of palbociclib in breast cancer cells through constitutive activation of downstream AKT signaling [48]. The resistance mechanism involves amplification of the mutant *PIK3CA* allele in some cases, leading to upregulation of PI3K signaling, which enables cells to regain proliferative capacity despite CDK4/6 inhibition [49]. This amplification phenomenon suggests an adaptive response under therapeutic pressure, wherein cancer cells selectively enhance oncogenic signaling pathways to circumvent drug-induced growth inhibition.

ESR1 GENE ALTERATIONS AS MECHANISM OF ENDOCRINE THERAPY RESISTANCE

ESR1 mutations represent a predominant mechanism of acquired endocrine therapy resistance, occurring primarily in the ligand-binding domain (LBD) of estrogen receptor alpha [50]. These mutations lead to constitutive ligand-independent receptor activation, allowing tumor cells to proliferate despite estrogen deprivation [51]. While rare in primary treatment-naïve tumors (~1%), *ESR1* mutations are detected in approximately 10-50% of metastatic endocrine-resistant breast cancers, suggesting their emergence under the selective pressure of endocrine therapy [52]. The most common *ESR1* mutations (p.Y537S, p.Y537N, p.Y537C, and p.D538G) stabilize the receptor in an agonist conformation, promoting coactivator recruitment, even in the absence of estrogen [53]. Notably, these mutations occur more frequently following aromatase inhibitor therapy than selective estrogen receptor modulators (SERMs) or selective estrogen receptor degraders (SERDs) and are associated with more aggressive disease behavior and worse clinical outcomes [54]. In addition to point mutations, alterations in ER expression, including *ESR1* amplification and transcriptional upregulation, can drive resistance by increasing the overall ER signaling ca-

pacity, potentially overwhelming the inhibitory effects of endocrine therapies [55]. Additionally, changes in the balance of ER co-regulators, including increased expression of coactivators (e.g., SRC-1, SRC-3) or decreased expression of corepressors (e.g., NCoR, SMRT), can enhance ER transcriptional activity despite endocrine therapy [56].

Therapeutic strategies to address drug resistance

Targeting PI3K/AKT/mTOR pathway

Activation of the PI3K/AKT/PTEN pathway can be targeted with specific PI3Kα inhibitors, such as alpelisib and inavolisib, and the pan-AKT inhibitor capivasertib [57]. These alterations are mainly caused by activating mutations in the *PIK3CA* gene encoding the alpha isoform of the catalytic subunit (p110) of PI3K [10].

The most common mutations in *PIK3CA* (ENSG00000121879) are located in the helix domain (exon 9, E542 and E545 residues) and at position H1047 in exon 20 [58]. The two mutations in the helix domain cause a substitution from glutamic acid (E - Glu) to lysine (K - Lys), whereas in exon 20, the mutation changed the amino acid from histidine (H - His) to arginine (R - Arg). In all three cases, the final effect is a hyperactivation of the PI3K enzyme and constitutive activation of downstream AKT [59]. Another potentially relevant is *AKT1 gene,* which is altered in approximately 10% of HR+/HER- tumors. Interestingly, *AKT1* mutations are rare in *PIK3CA* mutated HR+/HER- breast cancer, suggesting that the prevalence of pathway mutations is even more quantitatively relevant[21]. Inactivating mutations in the *PTEN* gene, which result in loss of its tumor suppressor function, further increase the percentage of patients with alterations in the PI3K/AKT/PTEN pathway [14,16,23]. In terms of clinical actions, alterations in the PI3K/AKT/PTEN pathway render the tumors potentially susceptible to treatment with specific inhibitors of the pathway, as demonstrated in different trials

In the initial evaluation of the mTOR inhibitor everolimus, targeting theraptor/mTOR complex 1 (mTORC1), in the phase III randomized BOLERO-2 trial showed that everolimus plus exemestane almost doubled PFS compared to exemestane and placebo in patients with HR+/HER- advanced breast cancer relapsing or progressing on ET, although the subsequent overall survival (OS) analysis did not show any significant improvement [60]. The use of a pan-PI3K inhibitor (buparlisib) in combination with fulvestrant was investigated in the BELLE-2 trial. The results of the trial showed potential benefits in PFS but not in OS with the use of PI3K inhibitors; toxicity issues associated with the use of a pan inhibitor precluded further investigations [61]. Significant benefits were observed when an alpha-specific PI3K inhibitor was used. The phase III randomized SOLAR-1 trial was conducted in patients with *PIK3CA*-mutated, HR+/HER- advanced breast cancer relapsing to previous ET. Patients were randomized to receive the specific alpha PI3K inhibitor alpelisib in combination with fulvestrant or placebo plus fulvestrant. Alpelisib significantly improved PFS (11.0 months vs 5.7 months, HR = 0.65; 95% CI, 0.50 to 0.85) and induced significant benefits also in terms of OS (39.3 months versus 31.4 months in the placebo group); HR = 0.86; 95% CI, 0.64-1.15) [3]. Based on these results, the combination was approved by the FDA for the treatment of *PIK3CA*-mutant, ET-resistant, HR+/HER- advanced breast cancer. The benefit of the combination alpelisib and fulvestrant was further confirmed in a real-life setting [62].

Another PI3K inhibitor (inavolisib) was recently approved by the FDA in combination with palbociclib and fulvestrant for treatment of endocrine-resistant, *PIK3CA*-mutated, HR+/HER2-negative advanced breast cancer [18, 19]. The pivotal trial INAVO120 tested inavolisib in the first-line setting in patients with *PIK3CA*-mutated, HR+/HER- advanced breast cancer who had had relapse during or within 12 months after the completion of adjuvant endocrine therapy. INAVO120 showed clinical efficacy of the addition of first-line inavolisib to CDK4/6i and ET in endocrine-resistant patients with metastatic progression or within 1 year of adjuvant ET, in term of PFS (15.0 vs 7.3 months, HR = 0.43; 95% CI, 0.32 to 0.59; P < 0.001) and trended toward better OS (stratified HR = 0.64, 95% CI, 0.43 to 0.97; P = 0.03, which did not cross the predefined boundary for significance of < 0.0098)) [18].

The AKT inhibitor (capivasertib) was tested in the phase III CAPItello-291 trial [63]. Capivasertib is an oral kinase inhibitor of all AKTs [1-3] that blocks the phosphorylation of its downstream substrates of AKT. At the preclinical level, the drug has demonstrated differential activity in different models, including ER–positive breast cancer models with alterations in *PIK3CA*, *AKT1*, and *PTEN* [5]. In phase III trials, capivasertib showed significant benefits by improving PFS in patients with HR+/HER2- breast cancer harboring mutations in *PIK3CA*, *AKT1*, or *PTEN,* and was subsequently approved by the FDA, and subsequent by EMA in combination with fulvestrant in patients progressing from first-line therapy [16, 17].

Use of novel endocrine therapies

Relative to ET, one of the major resistance mechanisms (mainly related to the use of aromatase inhibitors) is related to the presence of mutations in the *ESR1* that confer ligand-independent and constitutive activation of the receptor. This constitutive ER activation-associated resistance can be bypassed by drugs that degrade the ER. Fulvestrant was one of the first drugs to act with this mechanism; however, it did not induce a significant increase in PFS [64]. Oral SERDs represent a significant advancement in the treatment of HR+/HER2- breast cancer, with elacestrant leading as the first FDA- and EMA-approved oral SERD specifically for ESR1-mutated disease [65]. Elacestrant functions by competitively displacing estradiol and inducing conformational changes in estrogen receptor alpha (ERα) that are incompatible with transcriptional activation, forming critical interactions with helices H3, H5, H6, and H11 [66]. In the pivotal phase III EMERALD trial, elacestrant demonstrated significant improvement in PFS compared with standard endocrine therapy, particularly in patients with ESR1 mutations who had received CDK4/6 inhibitors for ≥12 months [67]. Several other oral SERDs are in various stages of clinical development, with mixed results: camizestrant showed a superior PFS benefit versus fulvestrant in the phase II SERENA-2 [68] and proved to improve outcomes also in the upfront use in the phase III SERENA-6 trials [69], while giredestrant and amcenestrant failed to demonstrate PFS improvement in their respective pivotal trials [70]. Additional compounds in development include imlunestrant and other novel SERDs, that have been shown to improve PFS in the EMBER-3 clinical trial, with or without the combination with abemaciclib, with ongoing trials evaluating combinations of targeted therapies and potential applications in early-stage breast cancer [65]. Notably, different SERDs employ distinct molecular mechanisms for ERα degradation, suggesting that their sequential use may offer therapeutic advantages in treatment strategies [71]. The spectrum of compounds under development is broad, and include novel agents that function as inhibitors of ER, including Complete Estrogen Receptor Antagonist (CERAN, e.g., palazestrant), selective estrogen receptor-α covalent antagonist (SERCA, e.g., H3B-6545), and (PROTAC, es., vepdegestrant). Collectively, these oral new endocrine agents represent a promising therapeutic approach that can potentially overcome the limitations of existing treatments, particularly in patients with *ESR1* mutations that drive resistance to conventional endocrine therapies.

Methodological aspects

In the era of precision medicine, the advent of next-generation sequencing (NGS) platforms has revolutionized the analytical paradigm to select tumor patients who can benefit from targeted treatment [72]. In particular, NGS systems allow the detection of low-frequency molecular alterations in a wide range of biological samples, including scant diagnostic specimens, such as small biopsies, cytological preparations, and liquid biopsies [73]. These sampling approaches are affected by the low abundance of nucleic acids, which impacts the clinical stratification of patients with tumor [74]. Unlike singleplex technologies that can identify a few referenced alterations, NGS platforms comprehensively evaluate the molecular landscape of target regions, improving the detectability of clinically actionable molecular alterations [75]. Moreover, NGS systems demonstrate higher technical sensitivity and specificity for detecting low-abundance molecular alterations in scant biological sources (tissue and liquid biopsy samples) than conventional techniques [76]. Given these advantages, NGS strategies have been successfully adopted in diagnostic algorithms to identify mutations/alterations in the PI3K/AKT pathway in advanced breast cancer ER+/HER2-relapsing after endocrine and CDK inhibitors, dynamically fingerprinting the molecular landscape of tumor patients [10]. Despite these advantages, several limitations drastically impact the widespread use of NGS techniques in the genomic profiling of advanced BC patients. NGS systems are affected by high technical costs and require highly trained personnel to easily administer analytical procedures and clinically interpret the molecular records [10]. Moreover, NGS assays may be grouped into targeted and comprehensive genomic profiling (CGP) depending on the reference region covered by the panel [77]. If target NGS panels are designed to cover actionable alterations across clinically informative genes, CGPs enable a profiles of wide genome regions, by calculating complex genomic hallmarks (TMB – Tumor Mutational Burden, HRD – Homologous Recombination Deficiency, and MSI – MicroSatelllite Instability - status) [78]. These strategies show integrative technical advantages in detecting clinically relevant alterations in the PI3K/AKT pathway [7]. Target NGS panels highlight higher technical sensitivity, for detecting clinically informative molecular alterations in scant diagnostic samples, while CGP procedures do not successfully detect molecular alterations in low-quality/quantity diagnostic samples. Conversely, target NGS strategies may be affected by a limited reference range compared with the CGPsC Assay, which covers all genomic regions with actionable alterations [79].

International guidelines

International guidelines have indicated a panel of mandatory testing genes to optimize the clinical stratification of advanced BC patients [80]. Because of the rapidly evolving molecular landscape, NGS systems are recommended as upfront testing strategies to evaluate the molecular assessment of real-world clinical patients [81]. Notably, the ESMO Precision Medicine Working Group recommends NGS-based strategies to assess molecular hallmarks in BC patients, covering ESCAT 1 alterations able to select BC patients for personalized treatments [82]. Not surprisingly, the diagnostic workflows designed for NGS systems is affected by the lack of analytically validated and harmonized procedures, which drastically affect diagnostic implementation to guide clinical decision-making processes for BC patients [77]. Consequently, a plethora of commercially available NGS assays may be adopted to analyze clinically relevant biomarkers in BC patients [52]. On this basis, sequencing strategies may be distinguished in terms of the reference range (target panel, CGP – Comprehensive Genomic Profiling - assay), analytical sensitivity (limit of detection, - LOD) and biological input (genomic DNA, circulating nucleic acids) [83]. International and national societies have focused on technical limitations in the clinical application of NGS systems to assess the genomic profile of BC patients [84]. Remarkably, harmonized pre-analytical (from sample collection to nucleic acid purification), analytical (from the selection of the most adequate NGS panel for data analysis), and post-analytical procedures (interpretation and sharing of molecular records with oncologists) are recommended to fill the gap between clinical trials and routine diagnostic practice in selecting BC patients eligible for target treatments [84]. In the same scenario, a consensus panel of experts was established to clarify the challenging points in the technical evaluation of molecular fingerprints of BC patients. In particular, NGS testing has emerged as the most useful diagnostically available solution to comprehensively cover all actionable alterations across clinically relevant biomarkers in BC patients, but singleplex technologies may also play a crucial role in the diagnostic setting [85]. In particular, the vast majority of *PIK3CA* hotspot activating mutations can be detected using RT-PCR platforms, whereas HER2 analysis could also be approached via IHC, supporting the integrative role of technical strategies to perform molecular analysis of BC patients [46]. Additionally, it has been suggested that mBC patients should be included in trials when eligible for targeted treatment in accordance with *AKT1*, *PTEN*, and *ESR1* genomic alterations [86]. Consequently, updated recommendations heavily support NGS platforms as the most appropriate diagnostic strategy to analyze both tissue and liquid biopsy samples from patients with advanced ER+/HER2- BC [82]. In this scenario, the clinical frame to elicit *ESR1* resistance mutations after endocrine relapse is also crucial for successfully adopting highly sensitive NGS platforms [53]. Conversely, *PIK3CA*/*AKT1*/*PTEN* actionable alterations are recommended to be found by NGS systems in basal settings, demonstrating the pivotal role of NGS in detecting clinically informative alterations in different clinical frames [63, 87]. These recommendations were derived from pivotal clinical trials using gene sequencing to inform treatments, including EMERALD and CAPItello-291.

Given the PI3K/AKT/PTEN pathway, *PIK3CA* actionable mutations occur in 40.0% of advanced ER+/HER2- BC patients [88], whereas *PTEN* and *AKT1* alterations are less frequent in the target population (5.0-10.0 and 2.0-5.0%, respectively), demonstrating significant clinical implications. Considering the heterogeneous landscape of clinically relevant *PIK3CA* mutations, technologies (able to capture all targeted *PIK3CA* variants) play a central role in the clinical management of ER+/HER2- BC patients [46]. FDA approved Therascreen® PIK3CA RGQ Real-time PCR assay (able to cover 11 hotspot mutations across exons 7, 9, and 20) as a companion diagnostic test to detect *PIK3CA* hot-spot alterations starting from FFPE (Formalin-Fixed and Paraffin-Embedded) and liquid biopsy samples [89]. Despite being a less time-consuming and easily interpretable system, Therascreen® PIK3CA RGQ real-time PCR assay is affected by a scant reference range excluding from target treatment a non-negligible percentage of BC patients (16.0-21.0%). Moreover, negative results on liquid biopsy need to be confirmed in paired tissue samples, clinically impacting the turnaround time (TAT) of clinical reporting. Conversely, NGS platforms comprehensively evaluate *PIK3CA* druggable mutations in tissue and blood specimens using targeted NGS panels in routine diagnostic settings [10]. As a scalable and time-saving strategy, NGS systems are selected as an upfront testing approach in the vast majority of institutions involved in molecular tests [90]. In addition, activating mutations in the PI3K/AKT/PTEN pathway may also be derived from *AKT1* and *PTEN,* supporting the clinical implementation of NGS platforms to administer ER+/HER2- BC patients [91]. Consequently, heterogeneous activating mutations (point mutations/deletion/amplification) can be simultaneously detected using NGS systems. In this scenario, identification of *PTEN* alterations still represents an opening challenge due to the “PTEN loss” phenotype, depending on several molecular hallmarks affecting

protein function. IHC could represent an integrative, feasible, and cost-saving method to detect PTEN loss in FFPE samples [92]. Although IHC is highly standardized in the diagnostic routine stage, genomic analysis is recommended to comprehensively capture all genomic hallmarks of "PTEN loss" and other clinically actionable alterations in the *PI3K*/*AKT*/*PTEN* pathway.

Source of samples

Another challenge is the selection of the most insightful biological material for patients with BC. Of note, both tissue and liquid biopsy samples may be collected to successfully perform molecular analysis of predictive biomarkers selecting patients with BC for the best therapeutic option. In particular, tissue and liquid biopsies may be considered complementary biosources for collecting genomic data at different time points [93]. On this basis, tissue samples (small biopsy, surgical resections) are routinely considered the gold standard materials for molecular tests, evaluating the neoplastic cell percentage of each sample [94]. Not surprisingly, patients with advanced BC may be affected by scant FFPE samples, decreasing the detection rate of actionable alterations in routine diagnostic practice. Conversely, actionable alterations may be found in 30.0-40.0% of BC patients who relapse after the first line of endocrine therapy [95]. Given the dynamic collection of liquid biopsies, peripheral blood is recommended to identify actionable *ESR1* alterations in patients with advanced BC or to integrate genomic profiles when tissue samples are not sufficient for molecular analysis. Liquid biopsy has emerged as a dynamic and insightful source of nucleic acids complementing molecular analysis when scant samples are the only biological source eligible for molecular testing [96]. As suggested, pre-preanalytical handling procedures for liquid biopsy samples should be accurately carried out while preserving ccfDNA (circulating cell-free DNA). In particular, plasma separation (containing ccfDNA affected by a half-life of 15 min) is mandatory within 1 h after peripheral blood withdrawal (in EDTA tubes). When shipping procedures require a longer period, dedicated collection tubes may stabilize ccfDNA up to 7 days [97].

Timing of molecular test

In first-line treatment of ER+/HER2- advanced BC patients, PI3K/AKT/PTEN molecular assessment is considered a negative prognostic factor that does not impact first-line treatment options [88]. Not surprisingly, a combination strategy based on PI3K inhibitor (PI-3Ki) plus palbociclib and fulvestrant prolongs PFS in ET-resistant, HR+/HER2- patients [19], suggesting that upfront genomic profiling may significantly modify the clinical paradigm of BC patients. Considering that approximately 30.0% of patients with HR+/HER2- metastatic BC are endocrine-resistant at first-line, a relevant percentage of patients with *PIK3CA*-mutant could benefit from an upfront testing strategy [98].
In addition, genetic alterations may be present at diagnosis; therefore, molecular fingerprinting of tumor patients guides the treatment decision-making process of the second-third line in patients relapsing after ET and CDK4/6 treatment [37]. However, sub-clonal druggable molecular alterations at diagnosis may be enriched in relapsing patients, paving the way for molecular monitoring of BC patients owing to high-throughput technologies. Despite this significant evidence, if molecular tests are not performed before implementing first-line treatment, the most adequate biological source (tissue or peripheral blood) should be genotyped after first-line relapse to optimize clinical stratification of BC patients [99].

Conclusions

In the era of precision medicine, patients with ER +/HER2-advanced BC may benefit from targeted drugs that can improve clinical benefit compared with conventional treatments. On this basis, molecular assessment of tumor patients is mandatory to guide therapeutic decision-making procedures for patients who relapse after standard-of-care therapy. In 2024, the European Society for Medical Oncology (ESMO) Precision Medicine Working Group updated previously designed practical recommendations guiding the clinical implementation of NGS-based testing strategies for patients with metastatic cancers [84]. Notably, clinical reporting of actionable alterations should be supported by a ranking system based on the ESMO Scale of Clinical Actionability of Molecular Targets (ESCAT), which easily shares all clinically relevant molecular records with medical oncologists. Moreover, the widespread diffusion of NGS systems affects the detectability of non-clinically significant molecular alterations, stressing the need for harmonized technical protocols in the molecular profiling of BC patients. A prospective trial on HR+/HER2-advanced BC patients evaluated the impact of targeted therapy matched with genomic profiling (and hence with specific genomic alterations) on the clinical outcome of BC patients. Not surprisingly, target therapy/genomic alterations in matched predictive biomarkers showed better PFS when genomic alterations were ESCAT I/II (100). In 70.0% of ER+/HER2- BC cases, genomic analysis significantly modifies clinical outcome under targeted treatment. Of note, technical limitations

depending on scant reference and/or low sensitivity techniques combined with the heterogeneous distribution of subclonal clinically actionable mutations dramatically impact the identification of BC patients who could benefit from targeted therapy at diagnosis. Consequently, misidentification of these clinically relevant mutations may affect the rapid shift of treatment in relapsing BC patients. The rapidly evolving scenarios of predictive biomarkers will optimize the clinical stratification of advanced BC patients, demonstrating how genomic profiles will progressively become a key player in the clinical management of these patients. In this scenario, NGS remains the most adequate technical solution, which simultaneously covers all the actionable mutations in driver genes. On this basis, the widespread diffusion of NGS platforms in upfront diagnostic workflows is supported by the commercial availability of NGS systems that are more scalable, easier to use, and less expensive than the older-generation sequencing systems. Moreover, the lack of adequate tissue material in 20.0-30.0% of patients with advanced BC requires integrative tools to carry out molecular analysis. Liquid biopsy is a dynamic, less invasive, and easily to-collect source of nucleic acids that integrates genomic profiles. Despite these advantages, tissue specimens remain the gold standard for molecular testing because the low abundance of cfDNA in blood torrent requires highly sensitive technologies to detect clinically relevant molecular alterations in real-world settings. Given the rapid diffusion of NGS systems in diagnostic practice, multidisciplinary teams including oncologists, pathologists, molecular biologists, surgeons, nurses, technicians, bioinformatics, and geneticists are needed to successfully administrate all challenging procedures of the testing journey of BC patients. Molecular records shared on clinical reports should be clearly interpretable by clinicians, containing only clinically relevant data in the main page (for example, ESCAT I/II actionability level). In addition, analytical parameters (tested genes, run quality checks) supporting analytical validation of testing procedures should also be defined. The discovery of new potential targets, and/or new drugs specifically blocking tumors with genomic alterations, is likely to increase in the next future, requiring comprehensive technical platforms able to identify clinically significant genomic signatures starting from diagnostic routine samples (Fig. 2).

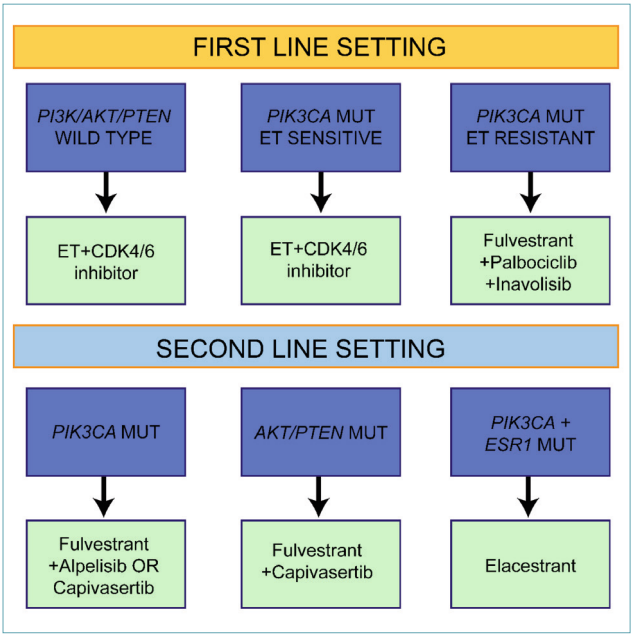

Figure 2. Therapeutic algorithm for advanced ER+/HER2- advanced breast cancer.

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Conflicts of interest statement

Umberto Malapelle reports personal/consulting or speakers' bureau fees from AstraZeneca, Amgen, Boehringer Ingelheim, Diaceutics, Diatech, Eli Lilly & Company, GlaxoSmithKline, Hedra, Janssen Biotech, Merck, Merck Sharp & Dohme, Novartis, Roche Health Solutions Inc., and ThermoFisher Scientific outside the submitted work. Carmen Criscitiello has participated in advisory or consultancy roles and speakers' bureau engagements for Eli Lilly, Pfizer, Novartis, Roche, AstraZeneca, MSD, Daiichi Sankyo, Gilead, and Seagen. Giuseppe Curigliano has received honoraria for speaker engagements from Roche, Seattle Genetics, Novartis, Lilly, Pfizer, Foundation Medicine, NanoString, Samsung, Celltrion, BMS, and MSD; honoraria for consultancy from Roche, Seattle Genetics, and NanoString; honoraria for participation in advisory boards from Roche, Lilly, Pfizer, Foundation Medicine, Samsung, Celltrion, and Mylan; honoraria for writing engagements from Novartis and BMS; and honoraria for participation in the Ellipsis Scientific Affairs Group. He has also received institutional research funding for conducting phase I and II clinical trials from Pfizer, Roche, Novartis, Sanofi, Celgene, Servier, Orion, AstraZeneca, Seattle Genetics, AbbVie, Tesaro, BMS, Merck Serono, Merck Sharp & Dohme, Janssen-Cilag,

Philogen, Bayer, Medivation, and Medimmune. Carmine De Angelis reports advisory role for Roche, Lilly, Novartis, AstraZeneca, Pfizer, Gilead, Daiichi Sankyo and GSK and speaker honoraria from Roche, Lilly, Novartis, Pfizer, and GSK; Travel Grants from Gilead and research support (to the Institution) from Novartis, GILEAD and Daiichi Sankyo outside the submitted work. Dario de Biase has received personal fees as speaker bureau from Menarini Stemline, GSK, Eli Lilly, and Astrazeneca, for work performed outside of the current study. Francesco Pepe reports personal/consulting and/or speakers' bureau fees from Menarini International, Jansen, Themrofisher and Roche outside the submitted work. Giuseppe Perrone has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Diaceutics, Merck, AstraZeneca, Novartis, Daiichi Sankyo, Exact Sciences, Sakura Finetek Europe. Cristian Scatena reports speaker bureau for Astra Zeneca, Daiichi Sankyo, Cerca Biotech, Gilead, MSD; advisory role for Astra Zeneca, Daiichi Sankyo, Exact Sciences, Menarini; consulting for Astra Zeneca, Daiichi Sankyo, Gilead, Menarini, MSD; research grants from Gilead. Maria Scatolini has received advisory fees, honoraria, travel accommodations/expenses, grants from Astra Zeneca, MSD, GSK, Pierre Fabre, Menarini, Roche, Servier. Konstantinos Venetis has received honoraria for speaker bureau from Merck Sharp & Dohme (MSD), Roche, and AstraZeneca. Nicola Fusco has received honoraria for consulting, advisory role, speaker bureau, travel, and/or research grants from Merck Sharp & Dohme (MSD), Merck, Novartis, AstraZeneca, Roche, Menarini Group, Daiichi Sankyo, GlaxoSmithKline (GSK), Gilead, Sysmex, Genomic Health, Veracyte, Sakura, Leica Biosystems, Lilly, Pfizer, ThermoFisher, Abbvie.
The other Authors declare that they have no conflicts of interest.

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Authors' contributions

Study conception and design: UM and NF. Data Collection: SB, IC, CC, GC, GD, CD, DDB, FP, GP, CS, MS, DT, and KV. Manuscript writing: UM, NF, FP, KV Critical revision and editing: ALL THE AUTHORS. Approval of the final version: ALL THE AUTHORS.

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