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Biomarkers of palbociclib response in hormone receptor-positive advanced breast cancer from the PARSIFAL trial

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Currently, there are no clinically actionable biomarkers to predict patient to cyclin-dependent kinases 4 and 6 inhibitors (CDK4/6i) plus endocrine therapy for hormone receptor (HR)[+]/ human epidermal growth factor receptor 2 (HER2)[-] advanced breast cancer (ABC). Herein, we report an exploratory biomarker substudy (transFAL) from a subset of patients included in PARSIFAL, a phase II randomized clinical trial that evaluated first-line palbociclib plus fulvestrant or letrozole for HR[+]/HER2[−] ABC. No definitive biomarkers were discovered, however, worse outcomes were found with CDK6 postivity ($p = 0.008$), ER negativity ($p = 0.008$), high Ki67 ($p = 0.04$), and *TP53* mutation ($p = 0.04$). ctDNA density ($p = 0.036$) and number of mutations ($p = 0.033$) at baseline were significantly higher for resistant patients. Our study reveals future directions to explore in the goal to determine biomarkers of response to CDK4/6i.

Palbociclib is a selective inhibitor of cyclin dependent kinases 4 and 6 (CDK4/6)[1]. In randomized clinical trials, palbociclib improved progression-free survival (PFS) when combined with endocrine therapy for patients with hormone receptor-positive/human epidermal growth factor-2-negative (HR[+]/HER2[−]) advanced breast cancer[2,3]. Multiple phase III trials have further confirmed the benefit of CDK4/6 inhibitors for this patient population[3–7]. While CDK4/6 inhibitors have transformed the treatment of HR[+]/HER2[−] advanced breast cancer and are the first-line standard of care for this patient population, there is still a need to identify predictive biomarkers for of sensitivity and resistance, particularly for identifying patients who have a high probability of an early progression.

Retinoblastoma tumor suppressor protein (Rb) phosphorylation status has been indicated as a a potential biomarker[8], with preclinical studies demonstrating that high expression of cyclin D1and Rb and low p16 expression is associated with greater palbociclib effect[9,10]. However, clinical studies have been unsuccessful in translating preclinical findings to clinically useful biomarkers. The PALOMA-1 trial initially selected patients based on molecular biomarkers but failed to find a relationship between *CCND1* gene amplification or p16 loss with palbociclib efficacy[2]. Subsequently, none of these biomarkers were used for enrollment in PALOMA-2[3]. Other biomarkers including copy number of genes and expression/activation levels changes of multiple proteins involving the cyclin D1-CDK4/6-Rb axis have been also explored, but none of them have been robustly validated as specific biomarkers that are capable of predicting response[11–14]. To date, therefore, patient selection for CDK4/6 inhibitor-based therapy only includes HR positivity coupled with a HER2 negative status.

The PARSIFAL clinical trial was a randomized, open-label, phase II clinical trial that aimed to compare fulvestrant or letrozole as the best endocrine partner for palbociclib in treating endocrine-sensitive, HR[+]/HER2[−] advanced breast cancer. The study determined that both treatments had comparable efficacy and safety results[15]. Recently, extended follow-up of the PARSIFAL trial found that a PFS < 12 months on CDK4/6 inhibitors was associated with a worse clinical outcome[16], which align with prior findings from the EMERALD study[17].

Herein, we report the some of the results of transFAL, which was a series of exploratory substudies of the PARSIFAL clinical trial designed to

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uncover predictive biomarkers of sensitivity and resistance to palbociclib-based regimens for HR[+]/HER2[−] advanced breast cancer.

Results

Patient samples and patient characteristics

A subset of patients from PARSIFAL (NCT02491983) were included in the transFAL study (Fig. 1). Thirty-three baseline tumor specimens (fresh or archived tissue) and 72 blood samples were available for analysis. Twenty-two patients had paired baseline and progression blood samples. Twenty-four tissue biopsy samples of the 33 available were evaluable for protein expression by IHC. Nine samples were excluded due to no valid values. For DNA analysis, 16 samples passed the quality control, however, all samples had low cellularity. For RNA analysis, 18 samples passed the quality control with five samples low in cellularity. Hybrid capture-based genomic profiling was carried out on ctDNA from 72 patients at baseline. Patient characteristics of transFAL were consistent with the full PARSIFAL trial population (Supplementary Table 3).

IHC analysis

For the 24 samples available for IHC analysis, nine samples were considered resistant (PFS ≤ 12 months) and 15 were classified as sensitive (PFS > 12 months). Overall, 45.5%, 25.0%, 83.3%, 81.0%, 81.8%, 100.0%, and 39.1% of tumors presented a high IHC score of CDK4, CDK6, total Rb, pRb, ER, PR, and Ki67, respectively (Supplementary Fig. 1), but none demonstrated a statistically significant association with CBR (Supplementary Fig. 2).

Patients were then stratified by high/low IHC score for each biomarker to determine the correlation between protein expression with PFS and OS. High expression of CDK6 (hazard ratio, 0.26; 95% CI: 0.09–0.75; $p = 0.008$ for PFS; HR = 0.07; 95% CI: 0.00–0.63; $p = 0.002$ for OS) and Ki67 (hazard ratio, 0.33; 95% CI: 0.11–1.02; $p = 0.04$ for PFS; hazard ratio, 0.00; 95% CI: 0.00-Inf; $p = 0.008$ for OS) at baseline were associated with a statistically worse PFS (Fig. 2A) and OS (Fig. 2B), respectively. Low ER expression was also associated with a statistically worse PFS ($p = 0.008$), but not OS. No significant associations were found with the other biomarkers evaluated.

Targeted exome sequencing in tumor samples and gene expression profiling by RNA-seq

Eleven samples of the 16 samples available for DNAseq were classified as sensitive (PFS > 12 months) and five (PFS ≤ 12 months) were considered resistant. Full exon coverage included 1425 cancer-related genes and different genetic alterations such as missense, truncating and in-frame mutation, amplifications, and deep deletions were identified in the resistant and sensitive samples. Alterations that were relatively enriched in resistant tumors compared to sensitive patients included genes involved in transcription, such as *AT-rich interaction domain 1A(ARID1A)* and *T Cell Leukemia Homeobox 1*(*TLX1)*, and in cancer (*GLI3* and *FLYWCH1*) (Supplementary Fig. 3).

For the 18 samples available for RNA-seq, 12 samples were characterized as sensitive (PFS > 12 months) and six (PFS ≤ 12 months) were classified as resistant. The analysis of hypervariable genes (HV-genes) demonstrated the presence of at least two subgroups in both the sensitive and resistant cohort (Supplementary Fig. 4). Nevertheless, initial comparison of gene expression profiles did not show any statistically significant difference in expression pattern. Functional analysis of the identified genes performed with Ingenuity Pathway Analysis did not show any consistent pathway differences.

ctDNA analysis in baseline and paired samples

Among the 72 samples available for ctDNA analysis, thirty-three patients were considered resistant (PFS < 9 months) and 23 patients were classified as sensitive (PFS > 31 months), with 16 having an intermediate sensitivity. The most frequent mutations identified were phosphatidylinositol 3-kinase, catalytic, alpha *(PIK3CA)* (28.0%), tumor protein p53 (*TP53)* (28.0%), Ataxia Telangiectasia Mutated (ATM) (15.0%), epidermal growth factor receptor (*EGFR)* (11.0%), *ARID1A* (10.0%), cadherin 1 (*CDH1)* (8.0%), and phosphatase and tensin homolog deleted on chromosome 10 (*PTEN)* (8.0%). There were 12 patients without a somatic mutation (17.0%). The heatmap of somatic mutations is depicted in Supplementary Fig. 5. Resistant samples had significantly more mutations than both moderately sensitive ($p = 0.035$) and sensitive ($p = 0.033$) samples (Fig. 3). As for the number of patients with mutations, there was no significant differences between de novo (28/34 with mutations) and non-de novo (35/39 with mutations) patients. Baseline ctDNA density and its relationship with clinical response was also evaluated. ctDNA density at baseline was higher for resistant patients as compared with those considered as sensitive ($p = 0.049$) (Fig. 4).

Patients were analyzed by mutational status for *TP53*, *mTOR*, *GNAS*, *ARID1A*, and *ESR1* to determine a correlation with PFS (Fig. 5). A significantly shorter PFS ($p = 0.04$) was associated with *TP53*. There were no

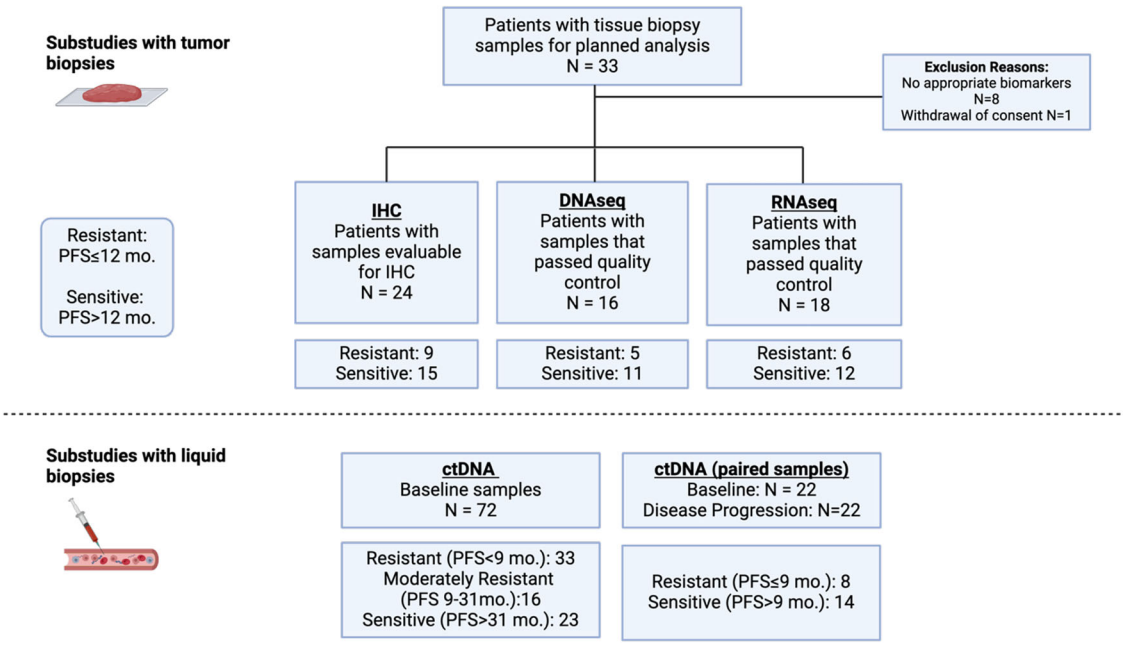


Fig. 1 | Diagram of samples analyzed for substudies. ctDNA circulating tumor DNA, IHC immunohistochemistry, PFS progression-free survival.

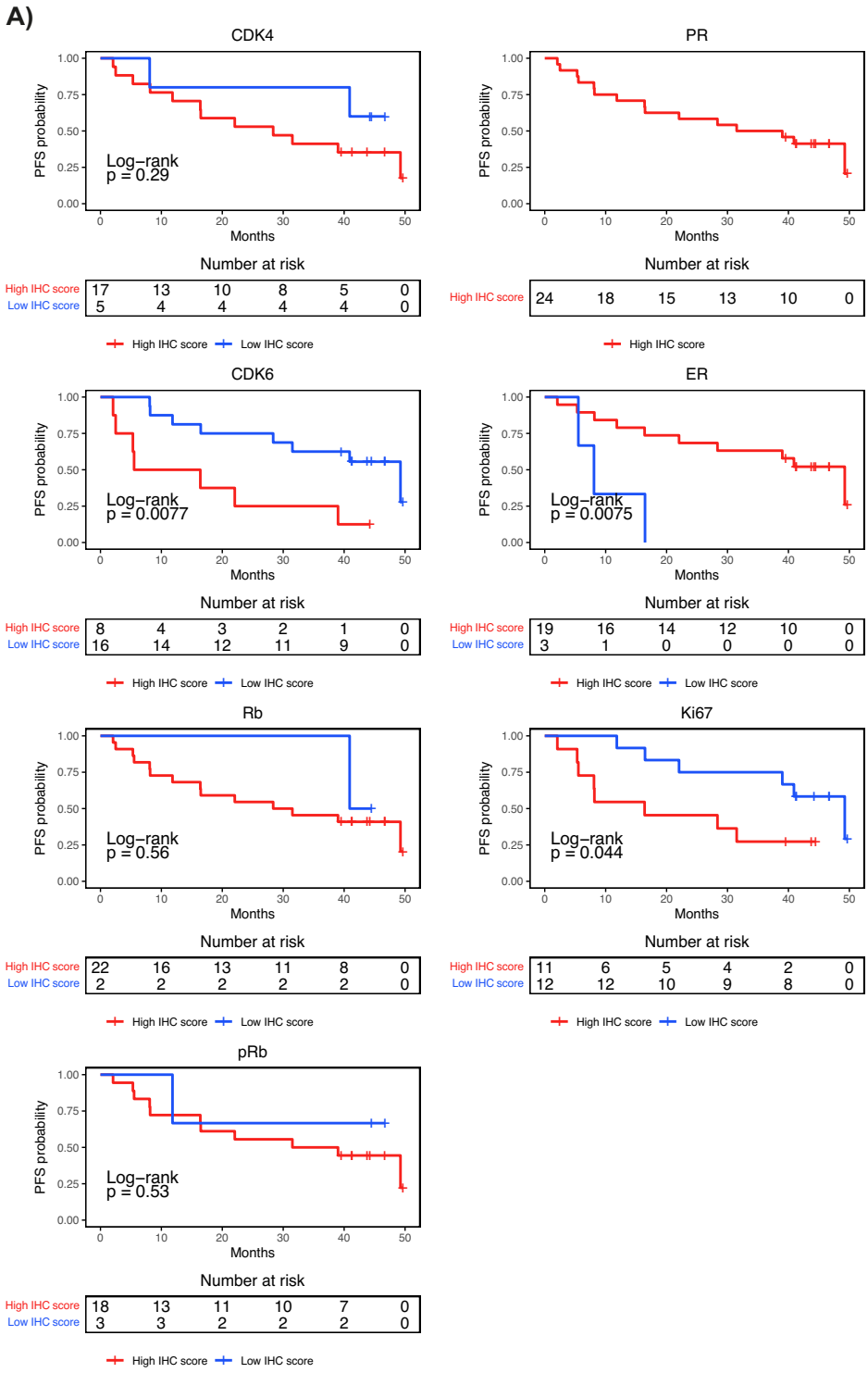
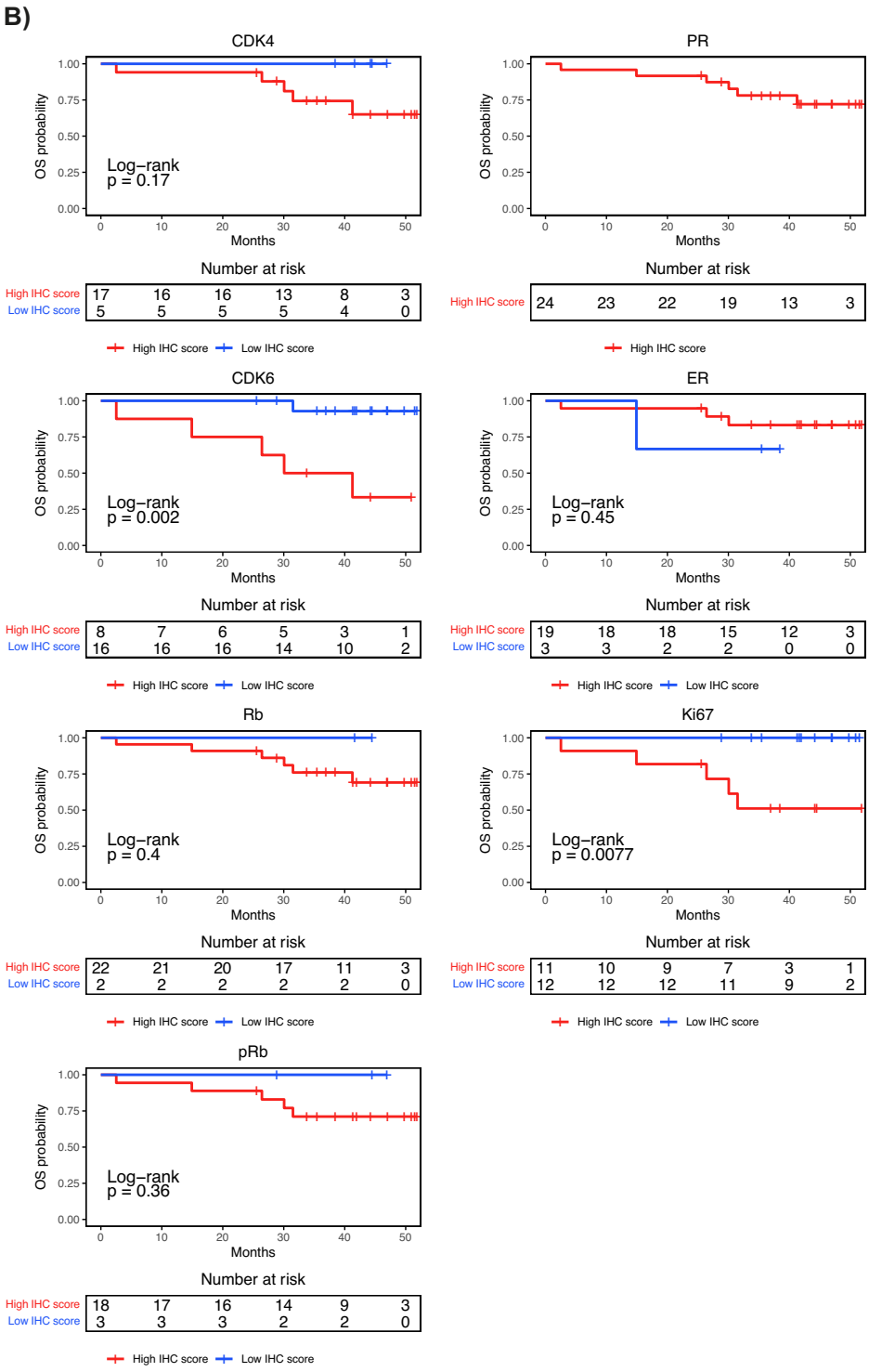


Fig. 2 | Correlation of baseline protein expression with efficacy. A Correlation of baseline protein expression with median progression-free survival. **B** Correlation of baseline protein expression with median overall survival. CDK Cyclin dependent kinase, IHC immunohistochemistry, pRb phosphorylated retinoblastoma, RB Retinoblastoma, PFS progression-free survival, PR progesterone receptor, ER estrogen receptor, OS overall survival.

statistically significant differences for other mutations. When analyzed dichotomously, *TP53* mutation was associated with shorter PFS ($p = 0.031$), whereas *mTOR* and *GNAS* mutations were correlated with longer PFS ($p = 0.03$ and $p = 0.032$, respectively) (Supplementary Fig. 6a–c).There were no significant differences for *ARID1A* (Supplementary Fig. 6d) or *ESR1*(Supplementary Fig. 6e) mutations.

Additional hybrid capture-based genomic profiling was carried out on ctDNA from paired samples obtained at baseline ($n = 22$) and at the time of disease progression ($n = 22$). The most frequent baseline mutations were *PIK3CA* (39.0%), *TP53* (20.0%), *ESR1* (14.0%), *EGFR* (14.0%), *CDH1* (14.0%), *ARID1A* (11.0%), *ATM* (11.0%), and *BRCA2* (9.0%). For progression samples, the most frequent mutations were *PIK3CA* (41.0%), *ESR1* (23.0%), *TP53* (18.0%), *EGFR* (18.0%), *ARID1A* (14.0%), *NF1* (9.0%), *BRCA2* (9.0%), *CDH1* (9.0%), and *ATM* (9.0%). There were three patients without mutations (3/22; 14.0%). No significant differences were found between the number of mutations in progression vs. baseline or between resistant and sensitive patients. Among patients with paired samples treated with palbociclib-letrozole, 12.5% had *ESR1* mutations at screening and 37.5% at disease progression (Fig. 6A), and for palbociclib-fulvestrant, none had *ESR1* mutations at screening and 14.3% at disease progression (Fig. 6B).

Fig. 2 | Continued



Discussion

CDK4/6 inhibitors have transformed the landscape of HR[+]/HER2[−] advanced breast cancer, becoming the preferred first-line therapy when combined with endocrine therapy[18]. However, there remains a critical need for biomarkers that can predict sensitivity and resistance, with particular emphasis on identifying early progression. Utilizing a biorepository of patients treated with first-line palbociclib and endocrine therapy from the PARSIFAL study, we conducted an exploratory biomarker study to that aimed to identify potential biomarkers in HR[+]/HER2[−] advanced breast cancer patients that could predict clinical benefit. Our analysis determined that worse outcomes were found with CDK6 positivity, ER negativity, high Ki67, *TP53* mutation and increased ctDNA density and number of mutations at baseline.

Both preclinical and clinical studies have been largely unsuccessful in identifying a validated biomarker of response. While preclinical studies have suggested a role of pRB, *RB1* mutations, *CCDN1* gene amplifications, and CCNE1 was retrospectively validated[19], none have translated to the clinical setting. However, these results have led to an explosion of extensive and additional molecular analyses to find novel biomarkers of CDK4/6 inhibitors sensitivity and resistance.

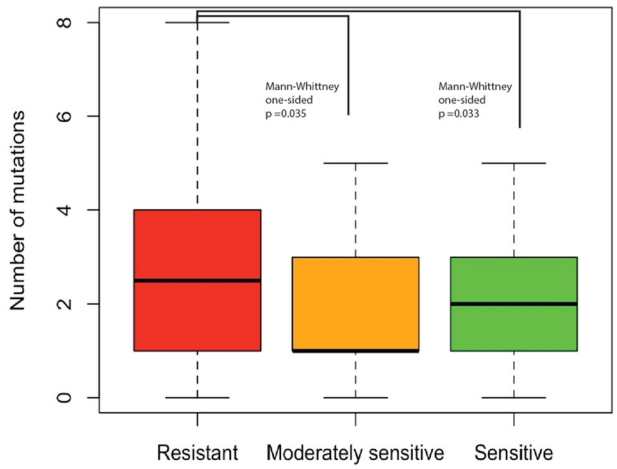


Fig. 3 | Correlation of number of mutations and resistance/sensitivity.

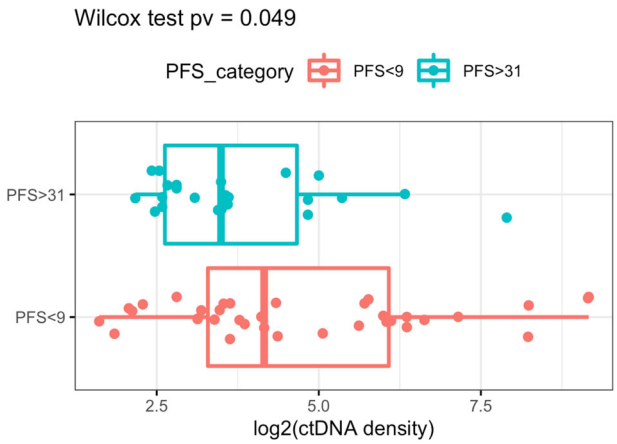


Fig. 4 | ctDNA status and its correlation with progression-free survival. PFS progression-free survival, ctDNA circulating tumor DNA.

Our results are consistent with other studies that have not been able to elucidate a single biomarker that is predictive a response. The PALOMA-2 trial was unable to find relevant biomarkers associated with a clinical benefit[20]. Additionally, the BioPER study, which aimed to discover biomarkers for maintaining palbociclib after progression on prior palbociclib-based regimens, identified a biomarker signature that may be associated with worse PFS but needs further validation[21]. Several studies have identified potential biomarkers such as CCNE1 amplification, however translation to clinical practice has been difficult as none have been definitive enough to influence treatment decisions.

While hormone receptor status remains the most important determinant for therapy, several prognostic markers for HR[+]/HER2[−] advanced breast cancer have already been identified and include mutational status, progesterone receptor negativity, high tumor grade, number and site of metastases, circulating tumor cell count and higher Ki67 level, shorter time to progression to advanced breast cancer, and poor performance status[22]. Furthermore, multiple mechanisms of resistance to CDK4/6i have been uncovered, such as Cyclin E, CDK6, RAS-pathway, and HIPPO pathway[19,23,24]. However, despite all these efforts, there are still no predictive markers of response that are currently validated for the clinical setting.

Although transFAL did not identify a conclusive biomarker, several results could help in further investigations. We determined that dysregulation of the CDK4/6 pathway may be a critical factor in predicting response given that high CDK6 expression (≥1% positive cells) at baseline was associated with a significantly worse median PFS ($p = 0.0077$). Indeed, previous reports indicate that high CDK6 expression could have a role in resistance mechanisms[23,24]. We also found that high Ki67 levels (≥10% positive cells) at baseline were associated with a shorter median PFS ($p = 0.044$). This is in alignment with the fact that Ki67 is known as a prognostic factor for breast cancer. A higher Ki67 index for patients with HR[+]/HER2[−] has been correlated with more aggressive tumors and a higher risk of recurrence[25,26].

ctDNA has been used for treatment tailoring, tracking mechanisms of drug resistance, and early prediction response[27]. In transFAL we determined that high ctDNA density at baseline, TP53 mutations, and a larger number of mutations were associated with poorer outcomes, consistent with other studies[28]. Exploratory analysis from PALOMA-3 similarly demonstrated a worse PFS and OS for patients with either a baseline ctDNA fraction >10%, *TP53* mutations, or *FGFR1* gene amplification[29,30]. In MONARCH-3, a shorter PFS was also noted for patients with one or more of 70 cancer-related gene alterations in baseline ctDNA[31]. Pooled analysis of the MONALEESA studies identified potential genes but none were statistically significant[32]. Furthermore, the BioltaLEE trial noted that the presence of a detectable mutation in baseline liquid biopsy samples was associated with poorer prognosis[33]. The BioltaLEE study also identified serum thymidine kinase I (TK1) activity as a promising prognostic marker of early resistance to ribociclib, which could be identified within 15 days on treatment[34]. Interestingly studies have demonstrated serum TK1 levels are highly concordant with Ki-67 IHC tumor tissue biopsies[35,36]. Given that TK1 would reduce the need for tumor tissue, this could be a more convenient biomarker than Ki67. Interestingly, there appeared to be more *ESR1* mutations at disease progression in palbociclib-letrozole patients, suggesting there may be a lower rate of endocrine therapy escape via *ESR1* mutations in the fulvestrant arm.

In the transFAL study sample collection and availability was a major challenge. The number of patients included in each substudy was limited because the our goal was to identify promising biomarkers that would need validation in other clinical studies. Additionally, in PARSIFAL, tumor and plasma samples at the start of protocol treatment or following disease progression were not mandatory, therefore only a small subset of patients could be evaluated in this exploratory analysis. Furthermore, the study lacked having samples that were collected at multiple timepoints throughout treatment, which could have provided a better understanding of ctDNA dynamics. Given the limited samples available it was also necessary to have difference criteria for resistance and sensitive samples between the substudies, which further limited the ability to draw larger conclusions.

Determining biomarkers of resistance remains a universal problem. Around 20–25% of patients will rapidly progress on CDK4/6 inhibitors with poor outcomes. Biomarkers of resistance could help identify these patients and help in tailoring therapeutic approaches. While the transFAL substudies failed to determine a clinically promising biomarker, our findings that high Ki67 levels and CDK6 expression in the tumor and high ctDNA density were associated with poorer outcomes could help guide future studies. The mechanisms and/or biomarkers of resistance might be diverse among patients, and our data suggests that a single biomarker may not be sufficient. The pursuit of predictive biomarkers is crucial for the advancement of personalized medicine and will likely require a collaborative effort to pool of resources, expertise, and data to address these challenges.

Methods

Study design and patients population

transFAL was a series of exploratory substudies utilizing tumor and blood samples from the PARSIFAL trial and performed immunohistochemistry (IHC) analysis, exome and RNA-sequencing (RNA-seq), and circulating tumor DNA (ctDNA) analysis (Fig. 1). PARSIFAL (NCT02491983) was a randomized, open-label, phase II clinical trial evaluating palbociclib plus either fulvestrant or letrozole for endocrine-sensitive, HR[+]/HER2[−] advanced breast cancer. Briefly, patients were 18 years or older with any menopausal status and locally confirmed hormone receptor–positive, HER2-negative, unresectable, locally advanced, or metastatic breast cancer

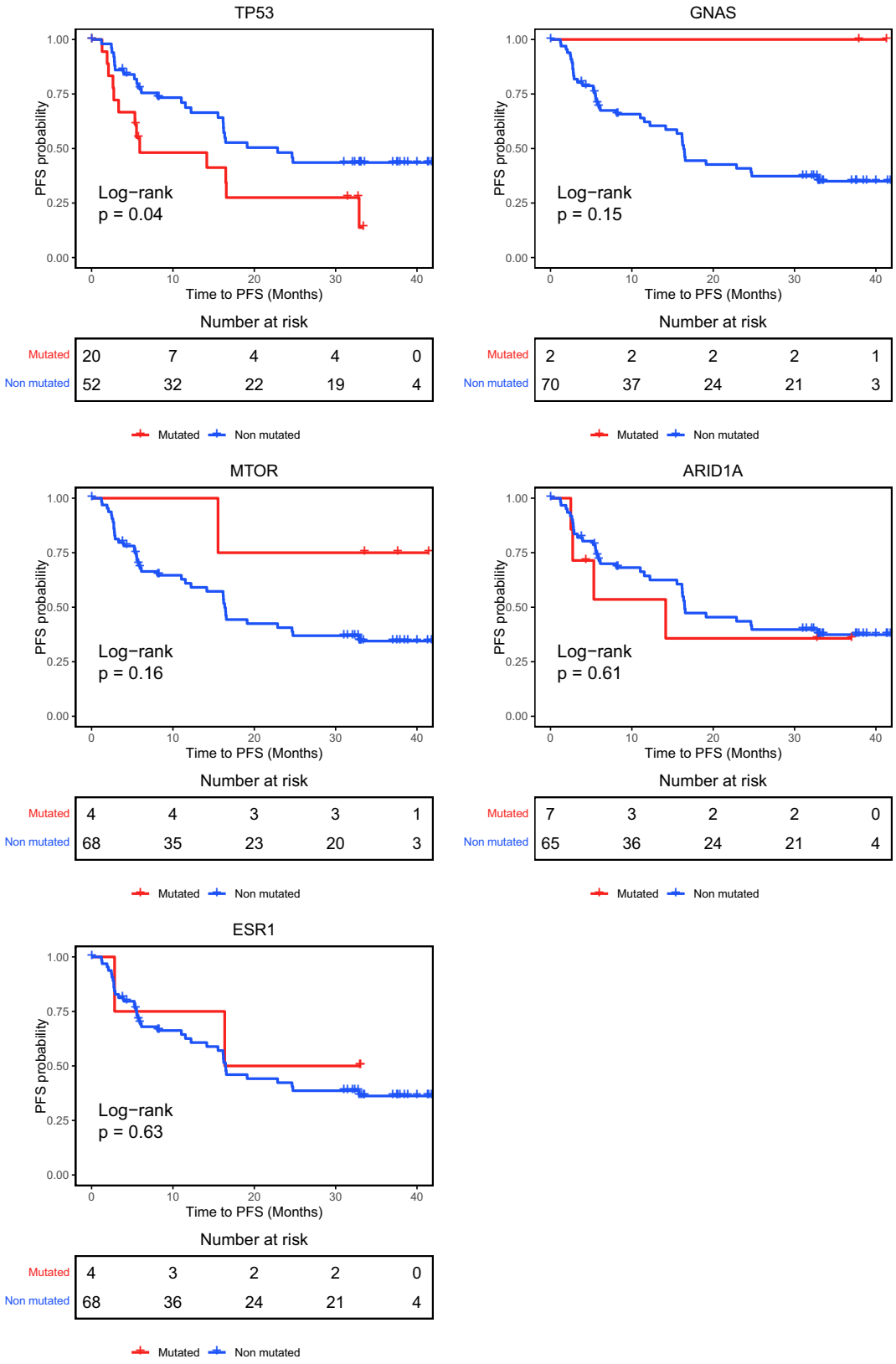


Fig. 5 | Correlation of baseline mutational status and progression-free survival. ARID1A AT-rich interactive domain 1A, ESR1 estrogen receptor 1, GNAS guanine nucleotide-binding protein, alpha stimulating, mTOR Mammalian target of rapamycin, PFS progression-free survival, TP53 tumor protein 53.

not amenable to surgical resection or radiotherapy with curative intent. Patients had not received systemic therapy for advanced disease, had an Eastern Cooperative Oncology Group performance status score of 0–2, and adequate organ function[15].

Samples were obtained from patients in the PARSIFAL study and gave their consent for samples to be used for future breast cancer research for analyses not strictly related to the aims of the current research. Therefore informed content was exempt in transFAL. The study protocol was carried

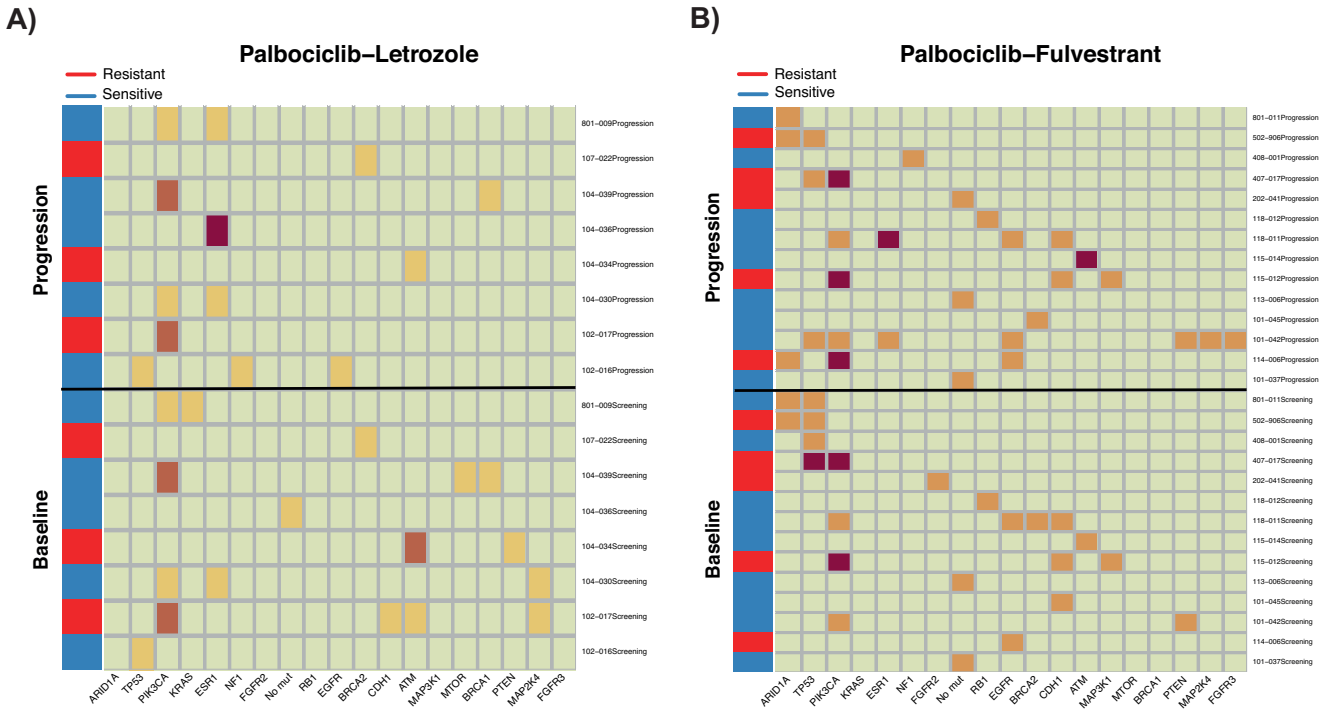


Fig. 6 | Somatic mutations in progression vs. baseline mutations. A Somatic mutation in patients treated with palbociclib-letrozole and **B** Somatic mutation palbociclib-fulvestrant treated patients.

out following the Ethical Guidelines for Biomedical Research on Human Subjects, respecting the Declaration of Helsinki and the Code of Good Clinical Practice. The transFAL study was approved by the Ethics Committee of Hospital del Mar Research Institute (2024/11413/I).

Given the exploratory nature of transFAL, substudies used PFS based cutoffs of sensitivity or resistance that were adapted in each substudy. In the studies with tumor biopsies, samples from "resistant" patients were from those with a PFS ≤ 12 months versus "sensitive" from those with a PFS > 12 months. For substudies using baseline liquid biopsies, more distinct patient outcomes were used: resistance was defined as PFS < 9 months, intermediate as PFS 9–31 months, and sensitive as a PFS > 31 months. For substudies utilizing ctDNA paired samples, resistance was defined as PFS ≤ 9 months and sensitive as PFS > 9 months.

IHC procedures

Seven consecutively unstained, 3-micrometer–thick tissue slides were obtained from each tumor block and stained. Sections were dewaxed in xylene and then rehydrated by immersion in ethanol. Endogenous peroxides were blocked, heat induced epitope retrieval was performed, and endogenous peroxidase was quenched. Sections were stained for CDK4, CDK6, total Rb, phosphorylated Rb (pRb), endocrine receptor (ER), progesterone receptor (PR), and Ki67, (Supplementary Table 1) followed by incubation immunoglobulin dextran polymer. Sections were visualized with 3,30-diaminobenzidine and counterstained with hematoxylin in an Agilent Link platform.

Whole slides were scanned at 200x using an Aperio CS2 Scanner (Leica Biosystems) and analyzed with QuPath Quantitative Pathology and Bioimage Analysis Software. Scoring was done by an expert pathologist blinded to clinical data.

Tumors were classified according to its IHC score as previously described[21]. High IHC score was defined as ≥1% of tumor cells with positive nuclear staining for CDK6, Rb, pRb, ER, PR. For CDK4 and Ki67 high IHC was defined as ≥10% of tumor cells with positive nuclear staining.

Exome sequencing and RNA-seq analysis

DNA libraries for targeted exome sequencing were prepared by fragmenting the extracted DNA and ligating adapters. Target enrichment was achieved using a custom-designed exome capture panel. The enriched DNA libraries were subjected to next-generation sequencing technology to generate paired-end sequencing reads of 100 to 300 base pairs on an Illumina® platform.

Variant calling was performed, and the identified variants were annotated using public databases and bioinformatics resources. Common single nucleotide variants, small insertions/deletions, and other genetic alterations were filtered and prioritized based on their potential functional impact and known associations with cancer.

RNA libraries for RNA-seq were generated by converting total RNA into complementary DNA (cDNA) through reverse transcription. Subsequently, adapters were ligated, and the cDNA was amplified to create the final RNA-seq libraries. The RNA libraries were subjected to next-generation sequencing technology using an Illumina platform to generate paired-end sequencing reads. The read length was 100 to 300 base pairs.

For both targeted exome sequencing and RNA-seq data, the raw sequencing reads underwent quality control and preprocessing to remove adapter sequences and low-quality reads. The processed reads were then aligned to the human reference genome HG38. Differential gene expression analysis was conducted to identify genes that were significantly upregulated or downregulated in the tumor samples compared to normal tissue[37].

ctDNA analysis

From 2–4 mL of plasma, a 70-gene panel (ALTUM, detailed in Supplementary Table 2) was analyzed at baseline and progression blood samples when available. Plasma aliquots were centrifuged were stored at −80 °C. Plasma samples were processed using QIAmp® Circulating Nucleic Acid Kit following the manufacturer´s protocol. Plasma aliquots from the sample were collected in the same tube before ctDNA extraction.

The total amount of ctDNA was eluted in a final volume of 25 µL. 1 µL of eluted cfDNA was quantified using Qubit™ dsDNA HS Assay kit following the manufacturer´s protocol.

The Agilent® 2100 Bioanalyzer System with the High Sensitivity DNA Kit was used to evaluate the quality and purity of the extracted cfDNA. 1 µL of eluted cfDNA was employed following the manufacturer protocol.

Statistical analysis

We used two-sided p values with an $\alpha \leq 0.05$ level of significance and 95% Confidence Interval (CI). The associations with a p value < 0.1 have been described. The relationship between biomarker expression and PFS/overall survival (OS) was analyzed using Kaplan–Meier curves and Cox regression. Clinical benefit rate (CBR) was defined as best overall response of complete response, partial response, or stable disease ≥24 weeks. PFS was defined as the time from initiation of therapy until disease progression or death from any cause and OS as the time from initiation of therapy until death from any cause. p values were calculated using the long-rank test for survival analysis.

Data availability

Data collected within this study will be made available to researchers after contacting the corresponding author and upon revision and approval based on scientific merit by the transFAL study management group (which includes a qualified statistician) of a detailed proposal for their use. The data required for the approved, specified purposes, the trial protocol, and the statistical analysis plan will be provided after the completion of a data sharing agreement that will be set up by the study sponsor, beginning 1 month and ending 5 years after article publication. All data provided are anonymized to respect the privacy of patients who have participated in the trial in line with applicable laws and regulations. Estimate timeframe for response will be within 30 days. Please, address requests for data to the corresponding author.

Code availability

The underlying code for this study is not publicly available but may be made available to qualified researchers on reasonable request from the corresponding author.

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References

1. Turner, N. C. et al. Palbociclib in hormone-receptor-positive advanced breast cancer. *N. Engl. J. Med.* **373**, 209–219 (2015).
2. Finn, R. S. et al. The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol.* **16**, 25–35 (2015).
3. Finn, R. S. et al. Palbociclib and letrozole in advanced breast cancer. *N. Engl. J. Med.* **375**, 1925–1936 (2016).
4. Goetz, M. P. et al. MONARCH 3: abemaciclib as initial therapy for advanced breast cancer. *J. Clin. Oncol.* **35**, 3638–3646 (2017).
5. Hortobagyi, G. N. et al. Ribociclib as first-line therapy for HR-positive, advanced breast cancer. *N. Engl. J. Med.* **375**, 1738–1748 (2016).
6. Slamon, D. J. et al. Overall survival with ribociclib plus fulvestrant in advanced breast cancer. *N. Engl. J. Med.* **382**, 514–524 (2020).
7. Sledge, G. W. et al. The effect of abemaciclib plus fulvestrant on overall survival in hormone receptor-positive, ERBB2-negative breast cancer that progressed on endocrine therapy-MONARCH 2: a randomized clinical trial. *JAMA Oncol.* **6**, 116–124 (2020).
8. Arnedos, M. et al. Modulation of Rb phosphorylation and antiproliferative response to palbociclib: the preoperative-palbociclib (POP) randomized clinical trial. *Ann. Oncol. J. Eur. Soc. Med. Oncol.* **29**, 1755–1762 (2018).
9. Sherr, C. J., Beach, D. & Shapiro, G. I. Targeting CDK4 and CDK6: from discovery to therapy. *Cancer Discov.* **6**, 353–367 (2016).
10. Finn, R. S. et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Res. BCR* **11**, R77 (2009).
11. Fribbens, C. et al. Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer. *J. Clin. Oncol. J. Am. Soc. Clin. Oncol.* **34**, 2961–2968 (2016).
12. Cristofanilli, M. et al. Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *Lancet Oncol.* **17**, 425–439 (2016).
13. Cristofanilli, M. et al. Predictors of prolonged benefit from palbociclib plus fulvestrant in women with endocrine-resistant hormone receptor-positive/human epidermal growth factor receptor 2-negative metastatic breast cancer in PALOMA-3. *Eur. J. Cancer Oxf. Engl.* **104**, 21–31 (2018).
14. Dean, J. L., McClendon, A. K., Stengel, K. R. & Knudsen, E. S. Modeling the effect of the RB tumor suppressor on disease progression: dependence on oncogene network and cellular context. *Oncogene* **29**, 68–80 (2010).
15. Llombart-Cussac, A. et al. Fulvestrant-palbociclib vs letrozole-palbociclib as initial therapy for endocrine-sensitive, hormone receptor–positive, ERBB2-negative advanced breast cancer: a randomized clinical trial. *JAMA Oncol.* **7**, 1791–1799 (2021).
16. Antonio L. C. et al. Abstract. RF01-03: PARSIFAL-LONG: extended follow-up of hormone receptor-positive/HER2-negative advanced breast cancer patients treated with fulvestrant and palbociclib vs. letrozole and palbociclib in the PARSIFAL study. *Cancer Res.* **84**, RF01-03 (2024).
17. Bardia, A. et al. Abstract PS17-02: Elacestrant vs standard-of-care in ER+/HER2- advanced or metastatic breast cancer (mBC) with ESR1 mutation: key biomarkers and clinical subgroup analyses from the phase 3 EMERALD trial. *Cancer Res.* **84**, PS17-02 (2024).
18. Gradishar, W. J. et al. Breast cancer, version 3.2022, NCCN clinical practice guidelines in oncology. *J. Natl Compr. Canc. Netw.* **20**, 691–722 (2022).
19. Turner, N. C. et al. Cyclin E1 expression and palbociclib efficacy in previously treated hormone receptor–positive metastatic breast cancer. *J. Clin. Oncol.* **37**, 1169–1178 (2019).
20. Finn, R. S. et al. Biomarker analyses of response to cyclin-dependent kinase 4/6 inhibition and endocrine therapy in women with treatment-naïve metastatic breast cancer. *Clin. Cancer Res.* **26**, 110–121 (2020).
21. Albanell, J. et al. Palbociclib rechallenge for hormone receptor–positive/HER-negative advanced breast cancer: findings from the phase II BioPER Trial. *Clin. Cancer Res.* **29**, 67–80 (2023).
22. Cuyún Carter, G. et al. Prognostic factors in hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR +/HER2–) advanced breast cancer: a systematic literature review. *Cancer Manag. Res.* **13**, 6537–6566 (2021).
23. Li, Z. et al. Loss of the FAT1 tumor suppressor promotes resistance to CDK4/6 inhibitors via the Hippo pathway. *Cancer Cell* **34**, 893–905.e8 (2018).
24. Yang, C. et al. Acquired CDK6 amplification promotes breast cancer resistance to CDK4/6 inhibitors and loss of ER signaling and dependence. *Oncogene* **36**, 2255–2264 (2017).
25. Arima, N. et al. Ki-67 index value and progesterone receptor status can predict prognosis and suitable treatment in node-negative breast cancer patients with estrogen receptor-positive and HER2-negative tumors. *Oncol. Lett.* **17**, 616–622 (2019).
26. Davey, M. G., Hynes, S. O., Kerin, M. J., Miller, N. & Lowery, A. J. Ki-67 as a Prognostic Biomarker in Invasive Breast Cancer. *Cancers* **13**, 4455 (2021).
27. Ignatiadis, M., Sledge, G. W. & Jeffrey, S. S. Liquid biopsy enters the clinic — implementation issues and future challenges. *Nat. Rev. Clin. Oncol.* **18**, 297–312 (2021).

28. Pascual, J. et al. Baseline mutations and ctDNA dynamics as prognostic and predictive factors in ER-positive/HER2-negative metastatic breast cancer patients. *Clin. Cancer Res.* **29**, 4166–4177 (2023).
29. O'Leary, B. et al. Circulating tumor DNA markers for early progression on fulvestrant with or without palbociclib in ER+ advanced breast cancer. *JNCI J. Natl Cancer Inst.* **113**, 309–317 (2021).
30. Cristofanilli, M. et al. Overall survival with palbociclib and fulvestrant in women with HR+/HER2− ABC: updated exploratory analyses of PALOMA-3, a double-blind, phase III randomized study. *Clin. Cancer Res.* **28**, 3433–3442 (2022).
31. Goetz, M. P. et al. Landscape of baseline and acquired genomic alterations in circulating tumor DNA with abemaciclib alone or with endocrine therapy in advanced breast cancer. *Clin. Cancer Res.* **30**, 2233–2244 (2024).
32. André, F. et al. Pooled ctDNA analysis of MONALEESA phase III advanced breast cancer trials. *Ann. Oncol.* **34**, 1003–1014 (2023).
33. Bianchini, G. et al. Abstract GS3-07: circulating tumor DNA (ctDNA) dynamics in patients with hormone receptor positive (HR+)/HER2 negative (HER2-) advanced breast cancer (aBC) treated in first line with ribociclib (R) and letrozole (L) in the BioItaLEE trial. *Cancer Res.* **82**, GS3-07–GS3-07 (2022).
34. Malorni, L. et al. Serum thymidine kinase activity in patients with HR-positive/HER2-negative advanced breast cancer treated with ribociclib plus letrozole: Results from the prospective BioItaLEE trial. *Eur. J. Cancer* **186**, 1–11 (2023).
35. Bagegni, N. et al. Serum thymidine kinase 1 activity as a pharmacodynamic marker of cyclin-dependent kinase 4/6 inhibition in patients with early-stage breast cancer receiving neoadjuvant palbociclib. *Breast Cancer Res.* **19**, 123 (2017).
36. Matikas, A. et al. Prognostic role of serum thymidine kinase 1 kinetics during neoadjuvant chemotherapy for early breast cancer. *ESMO Open* **6**, 100076 (2021).
37. Dozmorov, I. M. et al. Internal standard-based analysis of microarray data2—analysis of functional associations between HVE-genes. *Nucleic Acids Res.* **39**, 7881–7899 (2011).

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Author contributions

Conception and design: J.A., J.M.P.G., J.C., A.L.C. Collection and assembly of the data: J.A., A.G.P., C.L.A., M.B., F.R., A.G., B.B., V.S. Data analysis and interpretation: All authors. Manuscript writing: J.A., P.G., J.A.G., E.S., J.R.M., J.M.P.G., J.C., A.L.C. All authors reviewed the results and approved of the design.

Competing interests

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Additional information

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