



Palbociclib Rechallenge for Hormone Receptor-Positive/HER-Negative Advanced Breast Cancer: Findings from the Phase II BioPER Trial

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ABSTRACT

Purpose: To assess the efficacy and exploratory biomarkers of continuing palbociclib plus endocrine therapy (ET) beyond progression on prior palbociclib-based regimen in patients with hormone receptor-positive/HER2-negative (HR⁺/HER2⁻) advanced breast cancer (ABC).

Patients and Methods: The multicenter, open-label, phase II BioPER trial included women who had experienced a progressive disease (PD) after having achieved clinical benefit on the immediately prior palbociclib plus ET regimen. Palbociclib (125 mg, 100 mg, or 75 mg daily orally for 3 weeks and 1 week off as per prior palbociclib-based regimen) plus ET of physician's choice were administered in 4-week cycles until PD or unacceptable toxicity. Coprimary endpoints were clinical benefit rate (CBR) and percentage of tumors with baseline loss of retinoblastoma (Rb) protein expression. Additional endpoints included safety and biomarker analysis.

Results: Among 33 patients enrolled, CBR was 34.4% [95% confidence interval (CI), 18.6–53.2; $P < 0.001$] and 13.0% of tumors (95% CI, 5.2–27.5) showed loss of Rb protein expression, meeting both coprimary endpoints. Median progression-free survival was 2.6 months (95% CI, 1.8–6.7). No new safety signals were reported. A signature that included baseline mediators of therapeutic resistance to palbociclib and ET (low Rb score, high cyclin E1 score, *ESR1* mutation) was independently associated with shorter median progression-free survival (HR, 22.0; 95% CI, 1.71–282.9; $P = 0.018$).

Conclusions: Maintaining palbociclib after progression on prior palbociclib-based regimen seems to be a reasonable, investigational approach for selected patients. A composite biomarker signature predicts a subset of patients who may not derive a greater benefit from palbociclib rechallenge, warranting further validation in larger randomized controlled trials.

Introduction

Endocrine therapy remains the backbone of treatment in patients with hormone receptor-positive/HER2-negative (HR⁺/HER2⁻) advanced breast cancer (ABC; ref. 1).

Four phase III studies have shown that the addition of cyclin-dependent kinases (CDK) 4 and 6 inhibitors palbociclib, ribociclib, or abemaciclib, to a nonsteroidal aromatase inhibitor in patients with endocrine-sensitive, HR⁺/HER2⁻ ABC, significantly improves median progression-free survival (PFS) and overall response rate (ORR)

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Translational Relevance

Cyclin-dependent kinases (CDK) 4 and 6 inhibitors (CDK4/6i) plus endocrine therapy (ET) improve outcomes of hormone receptor-positive/HER2-negative (HR⁺/HER2⁻) advanced breast cancer (ABC). However, patients invariably experience disease progression because of acquired resistance and the optimal treatment after progression on a CDK4/6i remains unknown.

The phase II BioPER study explored the value of maintaining palbociclib beyond progression but changing the ET in patients with HR⁺/HER2⁻ ABC who experienced progression after having achieved clinical benefit on the immediately prior palbociclib-containing regimen.

The study achieved both clinical and biological coprimary endpoints with a tolerable safety profile. A signature that included baseline mediators of therapeutic resistance to CDK4/6is and ET (low Rb score, high cyclin E1 score, *ESR1* mutation) strongly predicted worse outcome, representing a promising biomarker to identify those patients who may not derive particular benefit from this strategy. These encouraging findings support further investigation in larger randomized controlled trials.

without a negative impact on global quality of life (2–5). Two of these trials have also shown an improvement in overall survival (OS; refs. 6, 7). On the basis of these results, the combination of CDK4/6 inhibitors plus nonsteroidal aromatase inhibitor has become the preferred first-line regimen for this patient population. Moreover, three phase III trials have also confirmed the benefit of adding a CDK4/6 inhibitor to fulvestrant among endocrine-pretreated patients in terms of PFS and ORR (8–10). Two of these trials have also demonstrated significant OS benefits (11, 12).

The optimal treatment after progression on a CDK4/6 inhibitor remains unknown in women with HR⁺/HER2⁻ ABC. Preliminary findings have revealed drivers of adaptive resistance more frequently related to endocrine therapy than to CDK4/6 inhibitors in this patient population (13). However the antitumor activity of classical endocrine drugs as single agents is limited (14) and for this reason, the combination of endocrine therapy with a PI3K/ mTOR inhibitor, everolimus (15–17), or alpelisib (18, 19) according to *PIK3CA* mutational status, or even chemotherapy-containing regimens, are commonly used options in this scenario. Accordingly, new therapeutic strategies are being developed in this patient population such as novel endocrine agents, mainly selective estrogen receptor degraders (14), or the maintenance of CDK4/6 inhibition beyond progression changing the endocrine therapy and continuing the same CDK4/6 inhibitor, maintaining the same endocrine therapy and switching to a different CDK4/6 inhibitor, or changing both endocrine therapy and CDK4/6 inhibitor (20–25).

Here, we report the results of BioPER, an investigator-initiated phase II proof-of-concept trial designed to explore the efficacy and safety of continuing palbociclib in combination with a different endocrine therapy agent beyond progression on prior palbociclib-based regimen, and to identify patients who are most likely to benefit from this clinical approach.

Patients and Methods

Patients

Eligible women were 18 years or older with any menopausal status with locally confirmed HR⁺/HER2⁻ unresectable locally advanced

breast cancer not amenable to surgical resection or radiotherapy with curative intent, or metastatic breast cancer. Patients must have had a radiologic or objective evidence of progressive disease (PD) immediately prior to palbociclib plus endocrine therapy-based treatment after having achieved clinical benefit to this regimen (response or stable disease ≥ 24 weeks). Last dose of palbociclib must have been administered no later than 8 weeks and not earlier than 3 weeks from study entry and patients must have been treated with a stable dose of palbociclib (100 mg/day or 125 mg/day) during the last 4 weeks in the previous palbociclib regimen. After a protocol amendment, the enrollment of patients treated with the lowest dose of palbociclib (75 mg/day) during at least 8 weeks and without any grade 3 or 4 adverse events related to palbociclib was permitted. Up to two prior endocrine therapy lines and not more than one line of prior chemotherapy for ABC were allowed. Measurable disease as defined by Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 that was amenable to biopsy, Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, and adequate organ function were also required. Key exclusion criteria were visceral crisis, the use of a CDK4/6 inhibitor other than palbociclib, and the exhaustion of all reasonable endocrine therapy options. Full eligibility criteria are described in the Supplementary Table S1.

The study protocol and supporting documents were approved by the institutional review board at each site. All patients provided written informed consent prior to participation in any study-related activities. This study was performed in accordance with ethical principles consistent with the Declaration of Helsinki and International Council of Harmonization/Good Clinical Practice as well as all applicable regulatory requirements.

Study design and oversight

This was a multicenter, noncontrolled, open-label, phase II trial (NCT03184090), conducted across 21 study sites in Spain and Italy between July 2017 and April 2019, that assessed the antitumor activity, safety, and clinical and molecular predictive biomarkers of palbociclib rechallenge in patients with HR⁺/HER2⁻ ABC. Supplementary Tables S2 and S3 includes the recruiting sites and study members.

All patients included were treated with palbociclib at the same dose level received when completing the previous palbociclib-based regimen [125 mg, 100 mg, or 75 mg administered orally in 4-week cycles (3 weeks of treatment followed by 1 week off)] in combination with endocrine therapy (physician's choice based on prior administered agent including tamoxifen, exemestane, fulvestrant, anastrozole, or letrozole at their corresponding approved dosages). Endocrine therapy should be different from previous treatment line. In addition, pre- and peri-menopausal women must receive gonadotropin-releasing hormone agonist.

Treatment continued until PD, unacceptable toxicity, death, or patient withdrawal for any reason. Dose interruptions and reductions were allowed for palbociclib as defined by prespecified guidelines in the protocol but were not applicable for endocrine therapy. Patients were allowed to discontinue palbociclib and continue with endocrine therapy alone.

Endpoints

Coprimary endpoints were the clinical benefit rate (CBR) according to RECIST version 1.1 as assessed by investigator review (efficacy coprimary endpoint), and the percentage of patients with loss of retinoblastoma (Rb) protein expression in tumor cells at baseline after progression on palbociclib and endocrine therapy (biological coprimary endpoint). CBR was defined as best overall response of complete

response, partial response, or stable disease ≥ 24 weeks. The loss of Rb protein expression was defined as $<1\%$ of tumor cells with positive nuclear staining.

Secondary endpoints included PFS (time from initiation of therapy until PD or death from any cause), ORR (best overall response of either complete response or partial response), duration of response (time from initial response to PD or death from any cause), OS (time from initiation of therapy until death from any cause), and toxicity as determined by the NCI Common Terminology Criteria for Adverse Events (CTCAE) v.4.0.3.

Exploratory endpoints included the assessment of the association between baseline clinical characteristics, molecular alterations in the cyclin D–CDK4/6–Rb pathway, PAM50 intrinsic subtypes, genomic alterations in circulating tumor DNA (ctDNA), and dynamic changes of ctDNA with sensitivity to study treatment.

Assessments

Study visits occurred on day 1 of each 28-day cycle, with a follow-up for 30 days after discontinuation of treatment, then every 6 months until the end of study.

Tumor assessments were carried out by computed tomography or magnetic resonance imaging according to RECIST version 1.1 at baseline and every 8 weeks up to 6 months of study treatment start. Thereafter, disease assessment was performed every 12 weeks until PD, initiation of a new anticancer therapy, or withdrawal from the study, whichever came first. Bone scans were carried out at baseline and every 24 weeks until the end of the study for patients with bone lesions identified at baseline, unless clinically or biochemically suspected bone progression.

Laboratory tests were performed on days 1 and 14 of the first two cycles and on day 1 of subsequent cycles. Vital signs, weight, and ECOG performance status were assessed on day 1 of every cycle.

Safety was evaluated on day 1 of every cycle in all patients who received at least one dose of study treatment by assessment of adverse events, clinical laboratory tests, physical examinations, and vital signs.

Biomarker analysis

Representative formalin-fixed and paraffin-embedded (FFPE) tumor tissue biopsies from relapsed metastatic or locally advanced disease were collected at baseline—after the documented PD to the prior palbociclib-containing regimen—and the end of treatment (EOT) or PD in the current regimen if applicable. Protocol-specified exploratory endpoints on tumor tissue biopsies at EOT/PD are still being analyzed and are not included in this report.

Sequential blood samples for ctDNA analysis were obtained at baseline, day 15 of cycle 1 (C₁D₁₅), day 1 of cycle 4 (C₄D₁), and at the EOT/PD.

IHC

Serial, adjacent, 3-micrometer-thick FFPE tissue sections of collected samples were cut from the tumor block using a microtome and mounted onto histologic glass slides before undergoing IHC. Sections were first deparaffinized, then rehydrated and incubated in 0.3% hydrogen peroxidase. Heat-induced epitope retrieval and incubation with the primary antibodies (clones, dilutions, and conditions are shown in Supplementary Table S4) was performed on-board an automated platform (Autostainer Link 48, Dako). After incubation with a polymer with peroxidase (EnVision FLEX, Dako), the reaction was visualized using 3,3'-diaminobenzidine tetrahydrochloride, then hematoxylin as counterstain. Negative controls were used by excluding the primary antibody. Whole slides were automatically scanned at 20 $\times$

using the Aperio CS2 scanner (Aperio Inc.) and analyzed with QuPath program version 0.2.0 (26). Scoring was based on the blinded independent central review performed quantitatively by an expert pathologist (L. Comerma). Immunoreactivity was considered evaluable when a tumor core contained at least 10% tumor cells and graded quantitatively according to the percentages of positive immunolabeled cells over the total cells per section. For CDK6, phosphorylated Rb (pRb), and Rb, high IHC score was defined as $\geq 1\%$ of tumor cells with positive nuclear staining. For CDK4, cyclin D1, and cyclin E1, high IHC score was defined as $\geq 10\%$ of tumor cells with positive nuclear staining (Supplementary Table S5).

PAM50 intrinsic subtyping

A 3- μ m-thick FFPE breast tissue was stained with hematoxylin and eosin to confirm the presence of invasive tumor cells and determine the tumor area by an expert pathologist (L. Comerma). For RNA isolation (RNeasy FFPE Kit, Qiagen), 1–6 ten-micron-thick FFPE slides were used for each tumor specimen and, if needed, tumor area was macrodissected to avoid contamination of normal breast tissue. After sample quality control using the 2200 TapeStation Software (Agilent Technologies), a minimum of approximately 150 ng of total RNA was used to measure the expression of 50 breast cancer-related genes plus 5 housekeeping genes using the nCounter platform (NanoString Technologies; ref. 27). Data were log₂ transformed and normalized using the housekeeping genes. Intrinsic subtyping (luminal A, luminal B, HER2-enriched, basal-like, and normal-like) was performed according to the research-based PAM50 intrinsic subtype predictor (27).

Plasma samples and DNA isolation

Venous blood was extracted at each timepoint and collected in STRECK Cell-Free DNA BCT tubes. Plasma was first separated from the peripheral blood cells by centrifugation at 2,800 rpm for 10 minutes at 4°C, then aliquoted in a 1.5 mL tube, and immediately stored in a deep freezer at -80°C . Cell-free circulating DNA (cfDNA)—that is released into the peripheral blood due to apoptosis, necrosis, or active release (28)—was extracted from plasma using the AVENIO cfDNA isolation kit (Roche Sequencing) and quantified by Qubit fluorometer (Thermo Fisher Scientific). Purity of cfDNA was assessed by electrophoresis (4200 TapeStation system, Agilent) to discard the presence of genomic DNA contamination.

Libraries were prepared with approximately 20 to 50 ng of cfDNA extracted from plasma samples using a broad targeted next-generation sequencing-based 77-gene panel (Avenio ctDNA Expanded Kit, Roche Sequencing), including coverage of the most prevalent tumor suppressor genes in human cancers (Supplementary Table S6). Libraries were sequenced in a NextSeq platform (Illumina) and the Avenio Oncology Analysis Software version 2.0.0 was used for FASTQ trimming, alignment to the reference genome, generation of variant calling files, and variant annotation.

Statistical analysis

Clinical data were assessed in the efficacy analysis set, which included all the patients who received at least one dose of study drug and fulfilled all selection criteria. Safety data were evaluated in the safety analysis set, which included all patients who received at least one dose of study drug (Fig. 1A). Biomarker data were assessed in the biomarker analysis set, which included all the patients who had evaluable samples for exploratory analyses. Signature analyses were conducted in all patients with evaluable samples for those specific biomarkers included in the composite predictive signature (Fig. 1B and C).

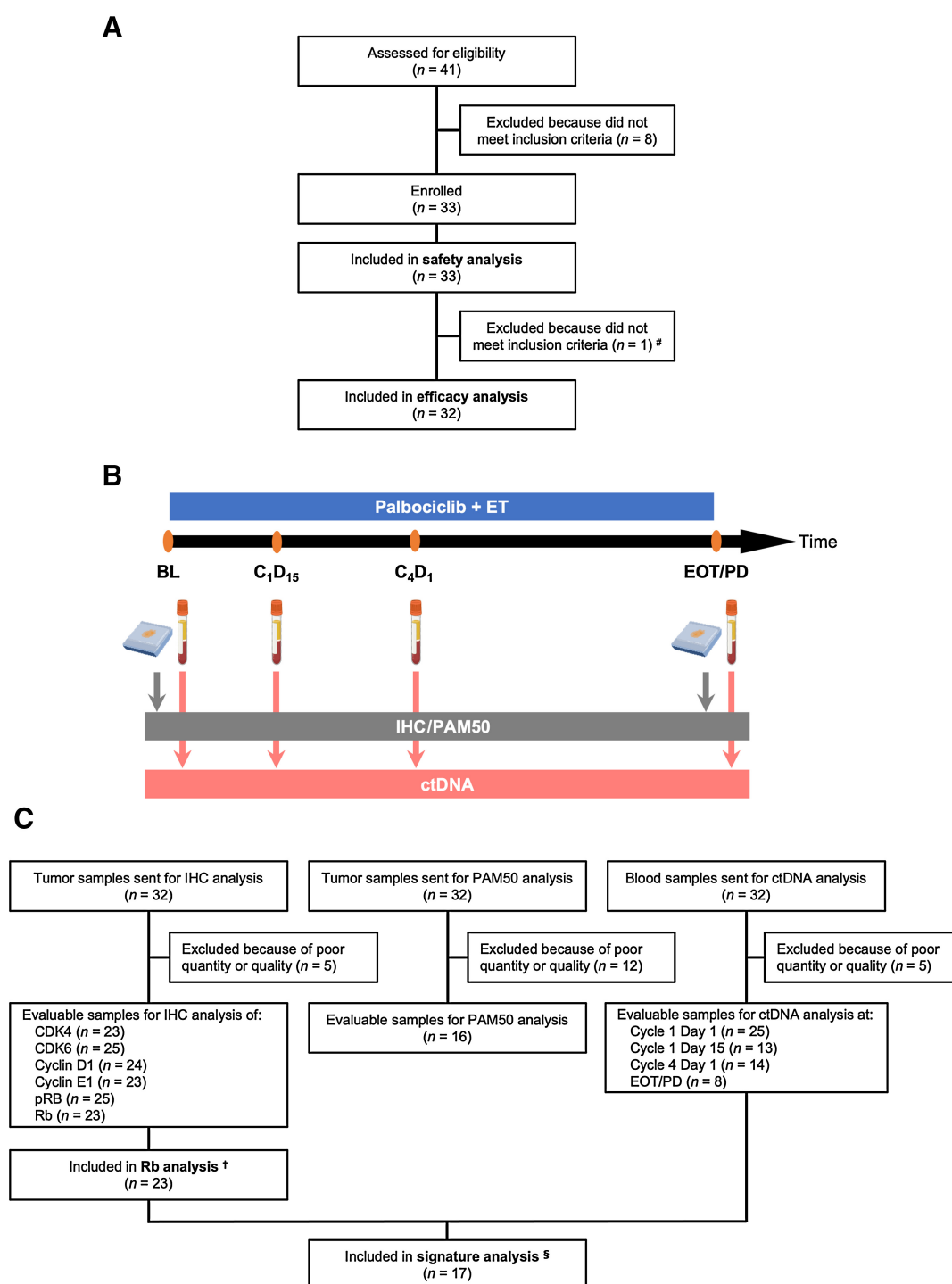


Figure 1.

A, CONSORT diagram of the BioPER trial. **B**, Study schema of tumor and blood samples collection. **C**, Flowchart of tumor and blood samples used for biomarker studies. BL, baseline; C1D15, day 15 of cycle 1; C4D1, day 1 of cycle 4; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; ctDNA, circulating tumor DNA; EOT/PD, end of treatment or progressive disease; ET, endocrine therapy; pRb, phosphorylated retinoblastoma; Rb, retinoblastoma. [#], One patient was excluded from the efficacy analysis because of lack of clinical benefit on prior palbociclib-based regimen. [†], All patients who had evaluable Rb expression by IHC. [§], All patients who had contemporaneously evaluable Rb and cyclin E1 expression by IHC, and ESR1 mutation status on ctDNA.

The total one-sided type I error was divided into 0.025 for each of the two coprimary endpoints. The CBR and loss of Rb protein expression were analyzed with one-sided exact binominal method and Wilson score confidence intervals (CI), respectively. The two coprimary endpoints were sequentially analyzed: the CBR before and the loss of Rb protein expression thereafter. It was predetermined that loss of Rb protein expression would be analyzed at a nominal α level of one-sided 0.025 if CBR was not met. Alternatively, if CBR was met, the loss of Rb protein expression would be analyzed with a one-sided 0.05 nominal α level, corresponding to a 90% two-sided Wilson CI. The study was designed to test the null hypotheses that the true CBR and loss of Rb protein expression rate were $\leq 5\%$. The alternative hypotheses were that the true rate for both primary endpoints in these patients was

Table 1. Baseline clinical and pathologic characteristics of trial participants included in the efficacy population.

Characteristics	Efficacy population (n = 32)
Age (years), median (range)	59.5 (42.0–80.0)
Menopausal status	
Premenopausal or perimenopausal	0
Postmenopausal	32 (100)
ECOG performance status	
0	14 (43.8)
1	18 (56.2)
Visceral involvement	
Yes	25 (78.1)
No	7 (21.9)
Number of disease sites	
<3	13 (40.6)
≥ 3	19 (59.4)
Previous regimens	
(Neo)Adjuvant ET	15 (46.9)
(Neo)Adjuvant CT	15 (46.9)
CT for MBC	4 (12.5)
Lines of prior ET for MBC ^a	
1	27 (84.4)
2	5 (15.6)
ET agent used in prior palbociclib-based regimen	
Letrozole	15 (46.9)
Fulvestrant	14 (43.8)
Exemestane	3 (9.4)
PFS for the prior palbociclib-based regimen (months), median (range)	13.8 (5.5–47.1)
Lines of previous systemic therapy for MBC, n median (range) ^a	
1	1 (1–4)
2	24 (75.0)
3	6 (18.8)
4	1 (3.1)
ET agent used in the current regimen	
Fulvestrant	18 (56.3)
Letrozole	9 (28.1)
Others	5 (15.6)
Starting dose of palbociclib in the current regimen	
125 mg	23 (71.9)
100 mg	6 (18.8)
75 mg	3 (9.4)

Note: Data are n (%), unless otherwise specified.

Abbreviations: CT, chemotherapy; ECOG, Eastern Cooperative Oncology Group; ET, endocrine therapy; MBC, metastatic breast cancer.

^aIncluding the prior palbociclib-based regimen.

$\geq 20\%$. We estimated a sample size of 33 patients to attain an 80% power at a nominal one-sided α level of 0.025.

For all secondary endpoints, we used two-sided *P* values with an $\alpha \leq 0.05$ level of significance and 95% CI. The associations with a *P* < 0.1 have been described. Survival curves were plotted using the Kaplan–Meier method.

Exploratory endpoints included correlation of CBR and PFS with baseline clinical characteristics, expression of markers involved in the cyclin D1-CDK4/6-Rb axis, PAM50 intrinsic subtypes, prevalence, and dynamic changes of genomic alterations in ctDNA. Mean differences were compared with the *t* test. The OR between two prognostic groups according to patients' characteristics or biomarker status was estimated using a logistic regression model. The analysis was based on the Wald test. The HR between the two prognostic groups was estimated using a Cox proportional hazards model, after assessment of the proportionality of hazards using log–log plots. The analysis was based on the Wald test and Breslow method for handling ties.

The predictive markers considered as clinically relevant or shown to be significant in a univariate model were included in the multivariate regression model. The subset with the lowest value of bias corrected through the Akaike Information Criteria was selected.

Results from overall correlative analyses should be considered descriptive because of the small number of samples and unadjusted sequential testing. *P* values and 95% CI do not represent a confirmatory measure of clinical or statistical relevance, but a rough reference for designing future, hypothesis-driven studies and informing investigators about the uncertainty of our exploratory data.

Data analysis was carried out using R statistical software version 4.0.2.

Data availability

Data collected within the BioPER study will be made available to researchers upon revision and approval based on scientific merit by the trial management group (which includes a qualified statistician) of a detailed proposal for their use. The data required for the approved, specified purposes and the trial protocol will be provided after the completion of a data-sharing agreement that will be set up by the study.

Table 2. Tumor best response according to RECIST version 1.1 of trial participants included in the efficacy population.

Characteristics	Efficacy population (n = 32)	
	N (%)	95% CI
CBR	11 (34.4)	18.6–53.2
Best response		
CR	0	—
PR	2 (6.3)	0.1–20.8
SD ≥ 24 weeks	9 (28.1)	13.7–46.7
PD	20 (62.5)	43.7–78.9
NE	1 (3.1)	0.08–16.2
ORR	2 (6.3)	0.1–20.8
DoR, median (range)	22.9 (9.2–36.7)	—
PFS, median (months)	2.6	1.8–6.7

Note: Clinical response was evaluated in patients with measurable disease at baseline as per RECIST version 1.1 who received at least one cycle of study treatment. Clinical responses were confirmed at the subsequent tumor assessment as per RECIST version 1.1. Data are n (%), unless otherwise specified.

Abbreviations: CBR, clinical benefit rate; CR, complete response; DoR, duration of response; NE, not evaluable; ORR, overall response rate; PD, progressive disease; PFS, progression-free survival; PR, partial response; RECIST, Response Evaluation Criteria in Solid Tumors; SD, stable disease.

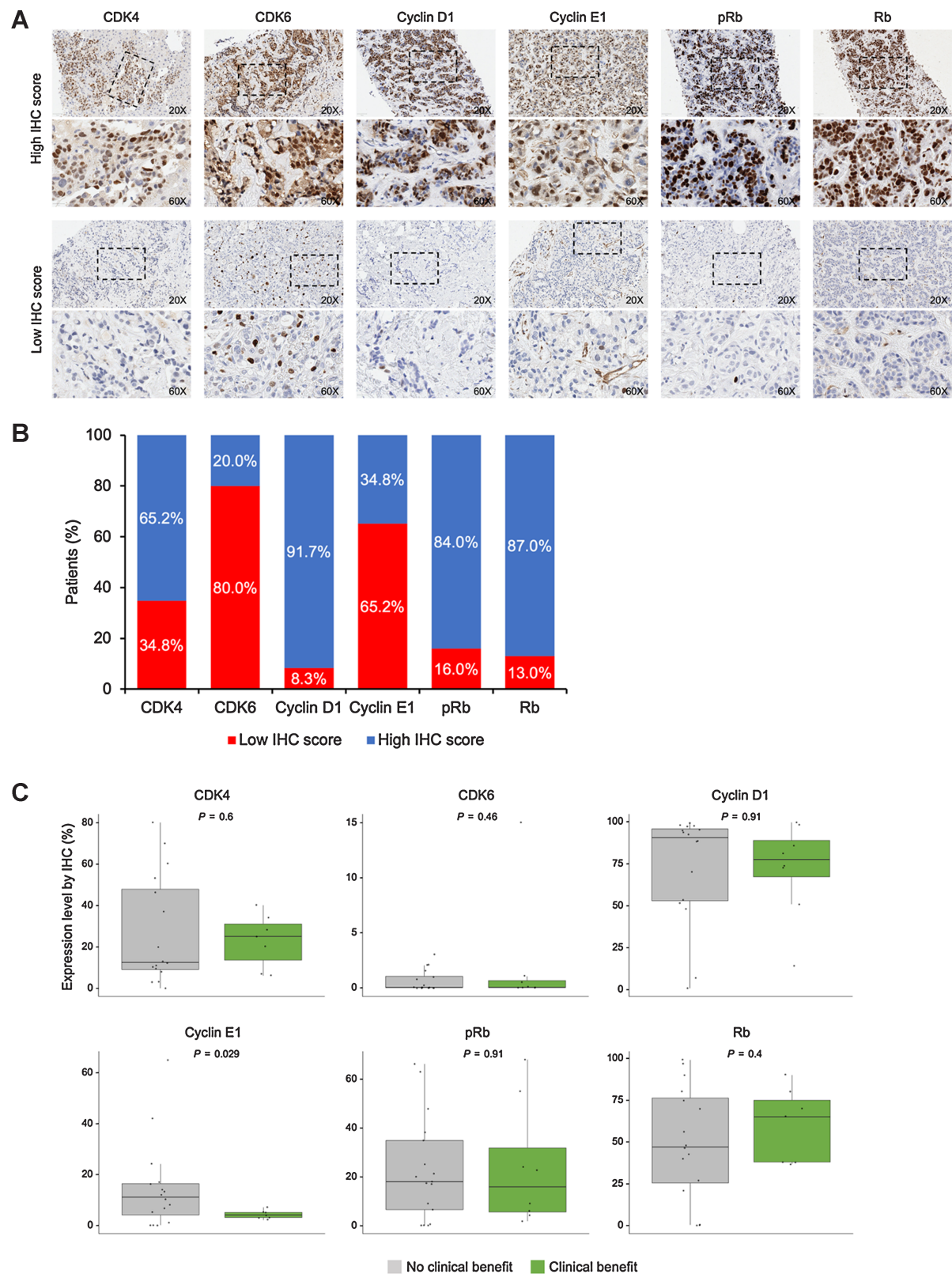


Figure 2. Representative IHC staining (A) and quantification of baseline protein biomarkers involved in the cyclin D1-CDK4/6-retinoblastoma axis (B). Correlation of baseline protein expression with clinical benefit (C) and progression-free survival (Continued on the following page.)

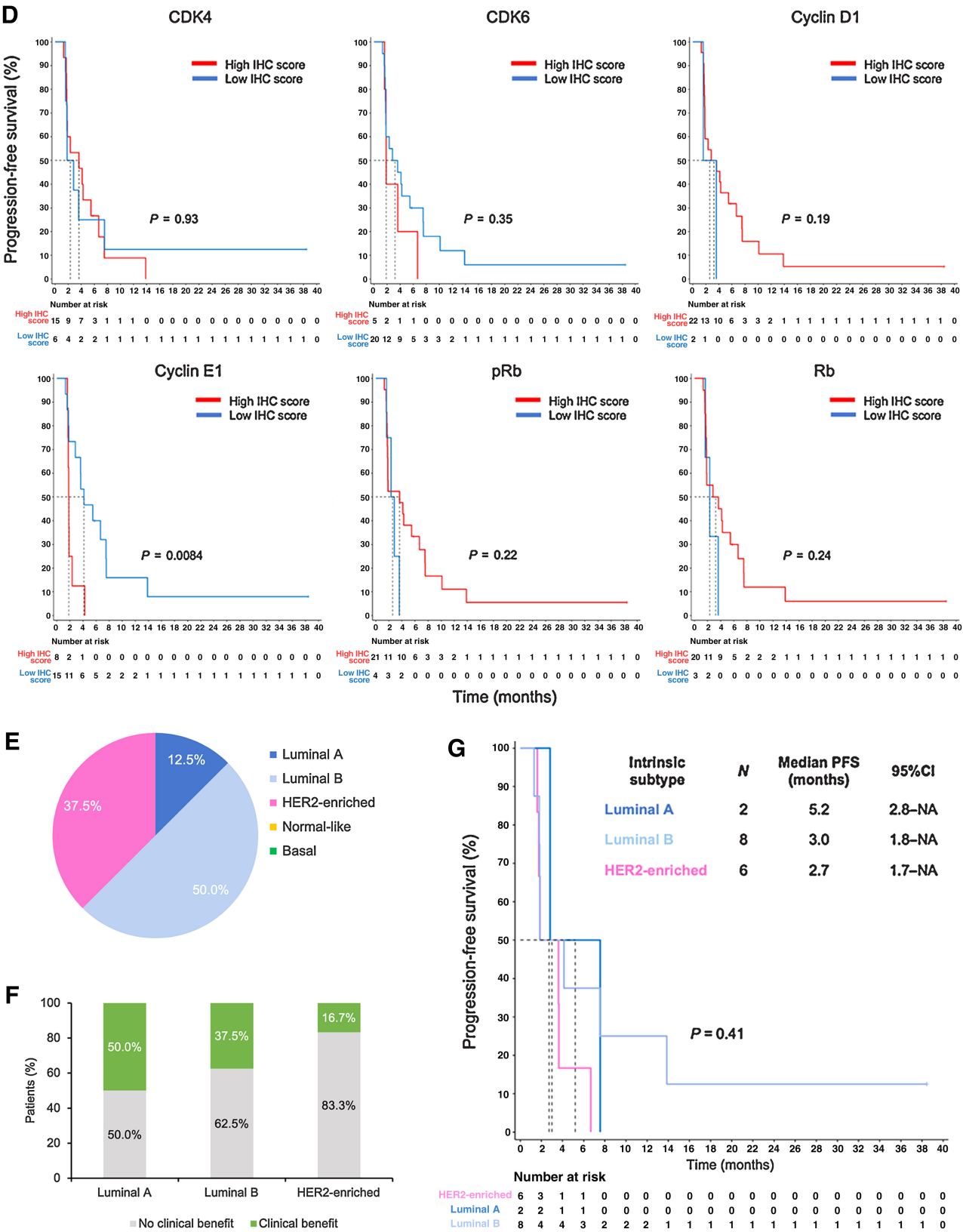


Figure 2. (Continued.) (D). Intrinsic molecular subtypes by PAM50 (E) and their correlation with clinical benefit (F) and progression-free survival (G). (Continued on the following page.)

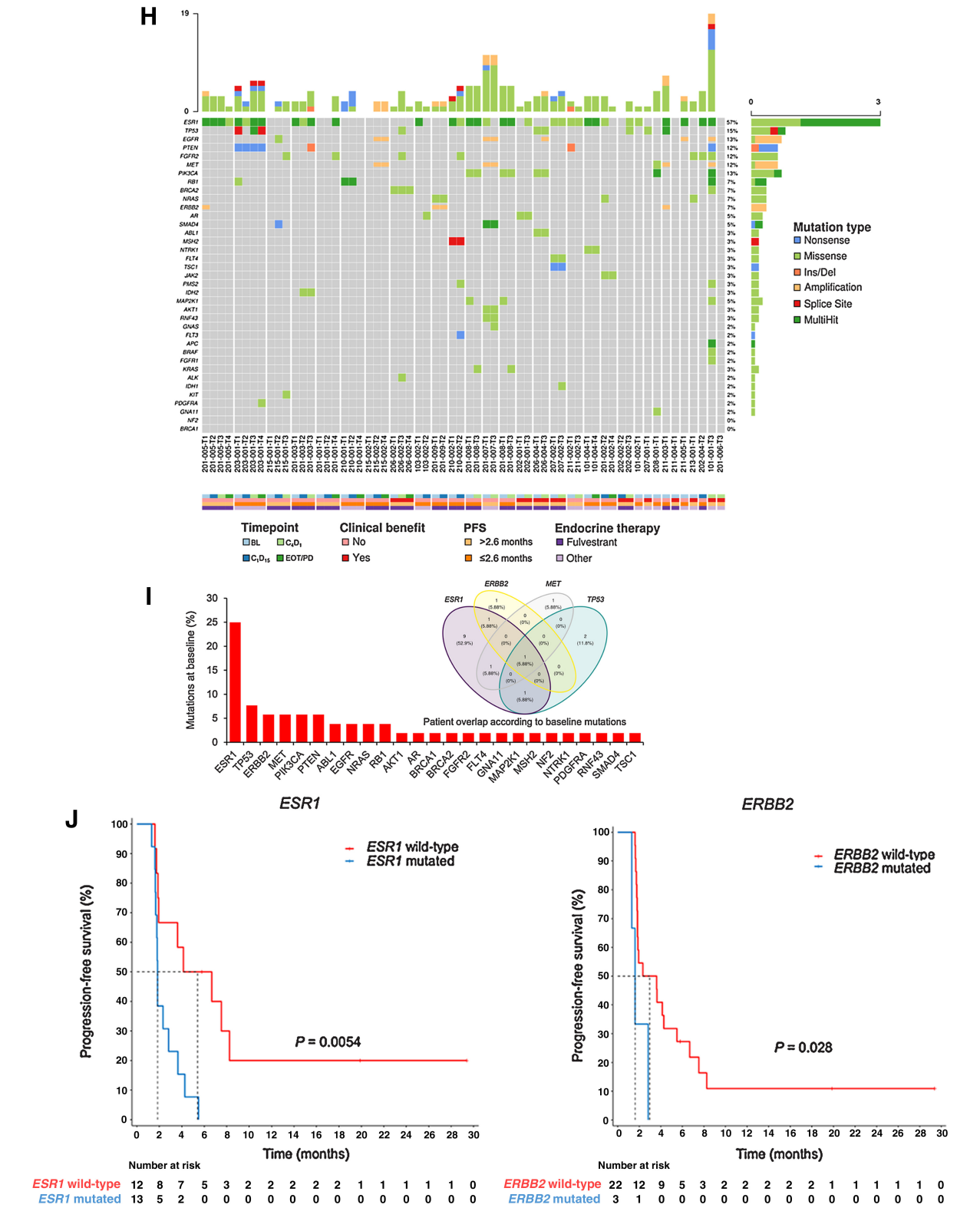


Figure 2. (Continued.) **H**, Distribution and number of gene mutations by patient in circulating tumor DNA. **I**, Prevalence of gene mutations and Venn diagram with the four most frequent mutated genes observed in circulating tumor DNA at baseline. **J**, Correlation of baseline gene mutations with progression-free survival. BL, baseline; C₁D₁₅, day 15 of cycle 4; C₄D₁, day 1 of cycle 4; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; ctDNA, circulating tumor DNA; EOT/PD, end of treatment or progressive disease; ET, endocrine therapy; Ins/Del, insertions or deletions; NA, not achieved; PFS, progression-free survival; pRb, phosphorylated retinoblastoma; Rb, retinoblastoma.

Table 3. Trial participants included in the efficacy population according to endocrine therapy received, clinical benefit, PAM50 intrinsic subtype, protein expressions of cyclin E1 and Rb, and *ESR1* mutation.

N	Patient ID	ET agent	Clinical benefit ^a	PAM50 subtype	Cyclin E1 (%)	Rb (%)	<i>ESR1</i> ^b
1	101-002	AI	No	NE	NE	0.5	Mutated
2	101-004	AI	No	NE	NE	NE	Mutated
3	101-005	Fulvestrant	No	NE	0	70	Wild-type
4	103-002	AI	No	Luminal B	13	75	Mutated
5	201-001	Fulvestrant	No	Luminal B	1	21	Wild-type
6	201-002	AI	No	NE	16	40	Wild-type
7	201-003	Fulvestrant	No	Luminal B	14	97	Mutated
8	201-005	Fulvestrant	No	Luminal A	0	27	Mutated
9	201-007	AI	No	NE	42	57	Mutated
10	201-008	Fulvestrant	No	NE	NE	1	Mutated
11	201-009	Fulvestrant	No	HER2-E	65	99	Wild-type
12	202-004	Fulvestrant	No	NE	NE	NE	Wild-type
13	203-001	AI	No	NE	12	0.5	Mutated
14	208-001	AI	No	HER2-E	24	46	Wild-type
15	210-001	AI	No	NE	NE	NE	Wild-type
16	210-002	Fulvestrant	No	NE	8	NE	Mutated
17	211-002	AI	No	HER2-E	0	48	Mutated
18	211-003	Fulvestrant	No	Luminal B	10	90	Mutated
19	211-005	AI	No	Luminal B	6	42	Mutated
20	215-001	Fulvestrant	No	HER2-E	5	0.5	Wild-type
21	215-002	Fulvestrant	No	HER2-E	17	80	Wild-type
22	101-001	AI	Yes	NE	NE	NE	Wild-type
23	201-006	AI	Yes	Luminal B	7	65	Wild-type
24	202-001	Fulvestrant	Yes	NE	NE	NE	Wild-type
25	202-002	Fulvestrant	Yes	Luminal B	5	38	Wild-type
26	206-002	Fulvestrant	Yes	NE	3	70	Wild-type
27	206-004	Fulvestrant	Yes	HER2-E	5	37	Wild-type
28	207-001	AI	Yes	NE	NE	NE	Wild-type
29	207-002	Fulvestrant	Yes	NE	4	90	Mutated
30	211-004	Fulvestrant	Yes	NE	NE	NE	Wild-type
31	213-001	AI	Yes	Luminal B	3	80	Wild-type
32	213-002	Fulvestrant	Yes	Luminal A	2	38	Wild-type

Abbreviations: AI, aromatase inhibitor; ET, endocrine therapy; HER2-E, HER2-enriched molecular subtype; NE, not evaluable; Rb, retinoblastoma; RECIST, Response Evaluation Criteria in Solid Tumors.

^aClinical benefit was evaluated in patients with measurable disease at baseline as per RECIST version 1.1 who received at least one cycle of study treatment and experienced complete or partial response, or stable disease lasting at least 24 weeks. Clinical responses were confirmed at the subsequent tumor assessment.

^b*ESR1* mutation status evaluated on ctDNA.

sponsor. All data provided are anonymized to respect the privacy of patients who have participated in the trial in line with applicable laws and regulations.

Results

Patient characteristics

Between June 2017 and April 2019, 33 patients were included. One patient who did not achieve clinical benefit on prior palbociclib-based regimen was excluded from the efficacy analysis ($n = 32$). All the patients received at least one dose of study treatment and were included in the safety analysis ($n = 33$). Data cut off was October 20, 2020, 18 months after last patient's first treatment in the study. **Fig. 1A** shows patient disposition.

The median age was 59.5 years (range, 42–80) and all patients were postmenopausal. A total of 25 (78.1%) patients had visceral disease (72.0% of whom with liver metastases), 19 (59.4%) presented ≥ 3 metastatic sites, and 14 (43.8%) had ECOG performance status 0. Overall, 24 (75.0%) patients received study therapy as second-line and four (12.5%) were treated with a prior line of chemotherapy for ABC.

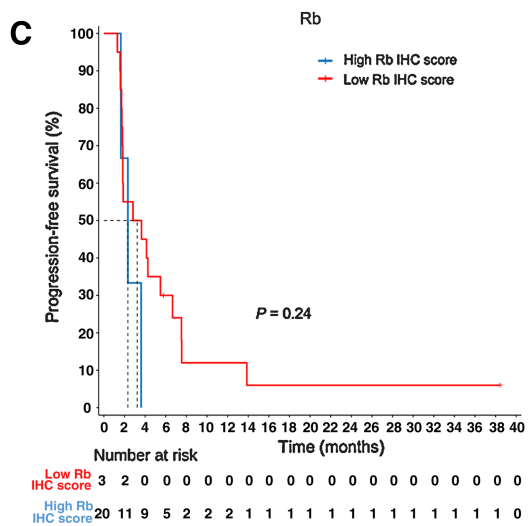
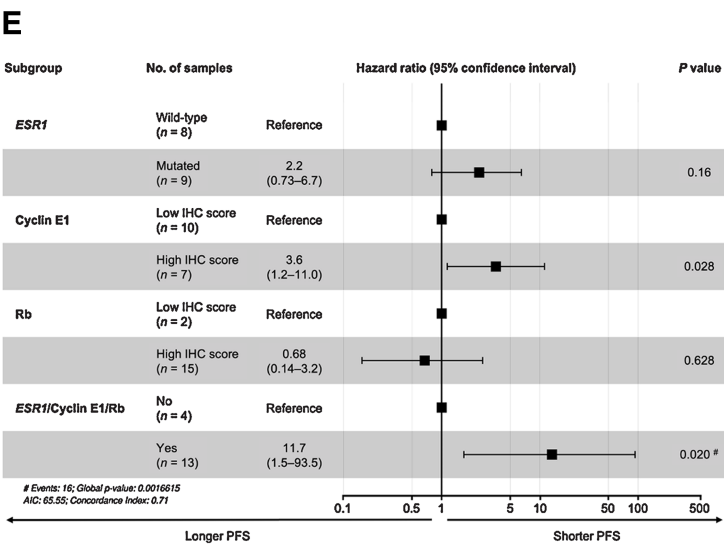
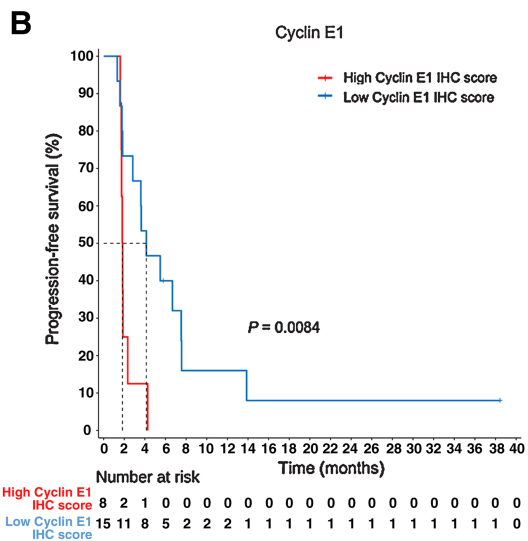
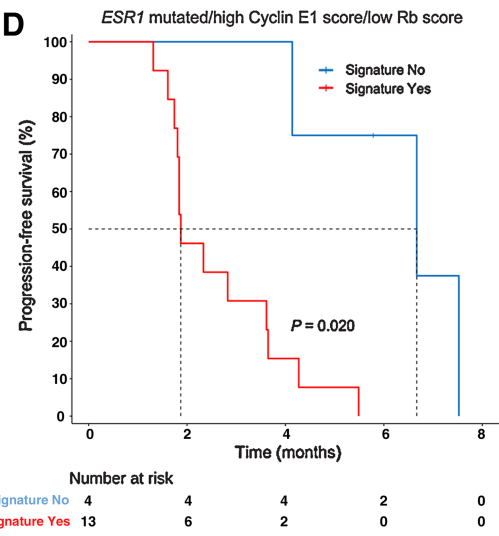
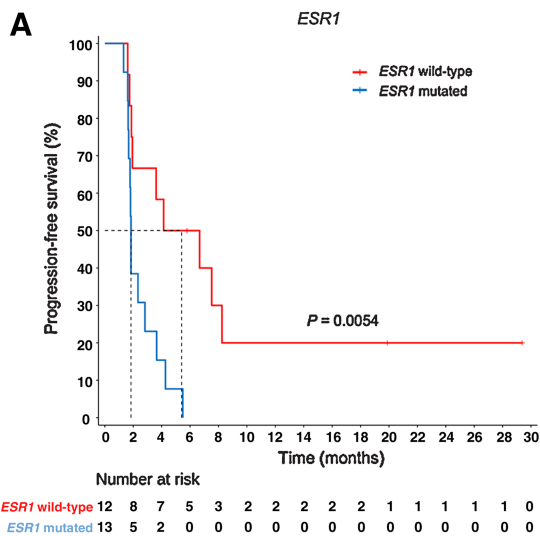
Of 32 patients, 15 (46.9%) received letrozole, 14 (43.8%) fulvestrant, and 3 (9.4%) exemestane in the prior palbociclib-based regimen. Median PFS for the prior palbociclib-based regimen was 13.8 months (range, 5.5–47.1). Baseline characteristic of the efficacy population are listed in **Table 1**. Representativeness of studied patients is shown in Supplementary Table S7.

Efficacy

The median follow-up was 18.4 months (range, 1.8–40.2). Clinical benefit was achieved by 34.4% of patients (11 of 32; 95% CI, 18.6–53.2; $P < 0.001$; **Table 2**) and the percentage of tumors with loss of Rb protein expression was 13.0% (3 of 23; 95% CI, 5.2–27.5), meeting both study coprimary endpoints (**Fig. 2**).

The ORR was 6.3% (2 of 32 patients; 95% CI, 0.1–20.8). Median PFS was 2.6 months (95% CI, 1.8–6.7) with a 6-month PFS rate of 31.2% (95% CI, 18.7–52.2; Supplementary Fig. S1). Median OS was 23.9 months (95% CI, 16.4–not estimable), with a total of 13 deaths (40.6%) at the time of data cut off (Supplementary Fig. S1).

The analysis of clinical and pathologic characteristics revealed that only patients with an ECOG performance status 0 at baseline were



more likely to achieve clinical benefit ($P = 0.016$). A nonsignificant trend towards better clinical benefit was observed with higher palbociclib doses ($P = 0.065$; Supplementary Table S8). No major differences in median PFS were seen in efficacy population according to certain clinical characteristics, such as number of metastatic sites, palbociclib dose, or pattern of metastatic spread (data not shown).

Among 24 patients who received study treatment as second-line regimen, CBR was 33.3% (95% CI, 15.6–55.3), median PFS was 3.2 months (95% CI, 1.8–7.5), and 6-month PFS rate was 33.3% (95% CI, 18.7–58.7; data not shown).

Safety

Out of 33 patients included in the safety analysis, 3 (9.1%) were still receiving study treatment at the date of cut-off analysis. The main reason for treatment discontinuation was PD, which occurred in 29 patients (87.9%; Supplementary Table S9).

The median relative dose intensity was 100% [interquartile range (IQR), 89.1–100] for endocrine therapy [99.4% (IQR, 96.9–100) for letrozole, 100% (IQR, 89.1–100) for fulvestrant, and 100% (IQR, 98.2–100) for exemestane] and 100% (IQR, 96.1–100) for palbociclib. The dose of palbociclib was reduced according to the protocol in 1 of the 33 patients (3.0%; Supplementary Table S9).

Among all patients included in the safety analysis, the most common adverse events of any grade reported were neutropenia (57.2%), fatigue (30.3%), leukopenia (18.2%), anemia (18.2%), asthenia (15.2%), and diarrhea (12.1%). Except for neutropenia [13 of 19 (39.4%) of grade 3; 1 of 19 (3.0%) of grade 4], most adverse events were of grade 1 or 2 severity. No cases of febrile neutropenia were reported (Supplementary Table S10). Nearly all the most frequent adverse events were deemed possibly related to study treatment.

The incidence of grade 3–4 toxicities and serious adverse events was 51.5% and 6.1%, respectively. No discontinuations due to adverse events, new safety signals, and treatment-related deaths were observed (Supplementary Fig. S2). A treatment-related grade 3 pulmonary embolism occurred in 1 patient (3.0%), who was continuing treatment with low molecular weight heparin at the time of data cut off (Supplementary Table S11).

Biomarkers

IHC

The protein expression of CDK4, CDK6, cyclin D1, cyclin E1, pRb, and Rb was evaluated by IHC in tumor biopsies obtained at baseline from metastatic lesions (Figs. 1B and C and 2A). Evaluable tissue samples were available in 100% of patients included in the efficacy analysis (32 of 32; Fig. 1C). However, some of them were too small or lacking in sufficient preanalytic quality to analyze all biomarkers. Therefore, 23 patients were included in the analysis of Rb protein expression (Rb subset) and 17 patients with all biomarkers evaluated were included in the signature analysis (signature subset; Fig. 1C). Baseline characteristics (Supplementary Table S12) and best response (Supplementary Table S13) were similar between participants included in the Rb and signature subsets, and overall population.

Representative staining of each biomarker is showed in Fig. 2A. Overall, 65.2%, 20.0%, 91.7%, 34.8%, 84.0%, and 87.0% of tumors presented a high IHC score of CDK4, CDK6, cyclin D1, cyclin E1, pRb, and Rb, respectively (Fig. 2B).

Patients whose tumors presented a loss of Rb protein expression at baseline did not achieve clinical benefit and the median PFS was 2.3 months (95% CI, 1.6–not estimable).

High expression of cyclin E1 was statistically associated with lack of clinical benefit ($P = 0.029$) and shorter PFS ($P = 0.0084$) than patients with low expression. However, CDK4, CDK6, cyclin D1, pRb, and Rb did not show statistically significant association with clinical outcomes. Figure 2C and D shows the relationship of each biomarker with clinical benefit and PFS.

PAM50 intrinsic subtyping

Out of 32 tumor samples, 16 (50.0%) had sufficient tissue for PAM50 intrinsic subtyping because sufficient tissue for RNA analysis after using the specimens for IHC was relatively uncommon in the small metastatic biopsies (Fig. 1C). Only 12.5% of tumors had luminal A subtype (Fig. 2E). Intrinsic subtypes were not statistically associated with clinical benefit ($P = 0.73$; Fig. 2F) nor PFS ($P = 0.41$; Fig. 2G). HER2-enriched subtype was numerically associated to absence of clinical benefit (83.3% vs. 50.0% vs. 62.5% in HER2-enriched, luminal A, and luminal B, respectively; Fig. 2F) and shorter PFS (2.7 months vs. 5.2 vs. 3.0 months in HER2-enriched, luminal A, and luminal B, respectively; Fig. 2G).

Liquid biopsy

Matched plasma samples collected at baseline, C₁D₁₅, C₄D₁, and EOT/PD were available for 25, 13, 14, and 8 patients, respectively (Fig. 1C). At baseline, cfcDNA (corresponding to mutant and wild-type DNA copies) was detected in all 25 patients, whereas ctDNA (corresponding to mutant DNA copies) was detected in 21 of 25 (84.0%) patients (Supplementary Fig. S3).

At C₁D₁₅, the median level of ctDNA copies presented a numerical decrease compared with baseline. At the time of radiological progression, all patients had detectable ctDNA levels, that were statistically significantly higher than median copies at baseline ($P = 0.016$) and C₄D₁ ($P = 0.028$). Undetectable ctDNA at C₁D₁₅ was associated with a longer PFS than detectable ctDNA [4.1 months [95% CI, 4.1–not achieved (NA)] vs. 1.8 months (95% CI, 1.6–2.3); $P = 0.021$]. However, detection of ctDNA at C₄D₁ was not associated with PFS (Supplementary Fig. S4).

Exploring 77 driver breast cancer genes by next-generation sequencing, mutations in 25 genes were identified at baseline and the 6 most frequent altered genes were *ESR1*, *TP53*, *ERBB2*, *MET*, *PIK3CA*, and *PTEN* (Fig. 2I). Patients who experienced clinical benefit had a statistically significant lower baseline number of mutations compared with patients without clinical benefit ($P = 0.033$; Supplementary Table S14). Fifteen mutations were detected in C₁D₁₅ plasma samples and 4 of them were not detected at baseline. (Fig. 2H).

ESR1 gene mutations were detected in the baseline plasma of 52.0% of patients (13 of 25; Fig. 2H). Patients with baseline *ESR1* mutated had lack of clinical benefit ($P = 0.015$; Supplementary Table S14) and shorter PFS than patients with wild-type *ESR1* (1.8 vs. 5.4 months, respectively; $P = 0.0054$; Fig. 2J) irrespective of the endocrine therapy administered.

Among the 6 most frequent mutated genes in the baseline plasma, *ERBB2* gene mutations were detected in 12.0% of patients (3 of 25; Fig. 2H), who had shorter PFS than patients with wild-type *ERBB2* (1.8 vs. 5.4 months, respectively; $P = 0.0054$; Fig. 2J). *PIK3CA*

Figure 3.

Kaplan-Meier analyses of investigator-assessed progression-free survival according to *ESR1*-mutated gene (A), protein expression of cyclin E1 (B), Rb (C), and composite signature of *ESR1*-mutated gene/high cyclin E1 score/low Rb score (any of three biomarkers versus none of them; D). E, Forest plot showing the association of *ESR1* gene, cyclin E1, Rb, and the composite signature (*ESR1*-mutated/high cyclin E1 score/low Rb score) with progression-free survival. AIC, Akaike Information Criteria; PFS, progression-free survival; Rb, retinoblastoma.

mutations [3 of 25 of patients (12.0%); Fig. 2H] were not associated with clinical benefit ($P = 0.83$; Supplementary Table S14) or PFS (data not shown).

Biomarker signature predictive of outcome

In an exploratory analysis of biomarkers related to therapeutic resistance, almost all patients who did not experience clinical benefit had at least one baseline biomarker of worsened outcome (low Rb score, high cyclin E1 score, and *ESR1* mutation; Table 3).

Hypothesizing that a composite signature might be superior to each biomarker alone in identifying potential predictors of prolonged benefit to palbociclib rechallenge, we first performed a univariate analysis of each of these biomarkers for clinical benefit (Supplementary Table S14), followed by a multivariate analysis (Fig. 3). A signature that included mediators of therapeutic resistance at baseline (low Rb score, high cyclin E1 score, and *ESR1* mutation) was independently associated with shorter median PFS (HR, 11.7; 95% CI, 1.5–93.5; $P = 0.020$; Fig. 3). Median PFS was 1.9 months (95% CI, 1.7–3.6) in patients who showed this composite biomarker signature compared with 6.7 months (95% CI, 4.1–NA) in those in whom any of these biomarkers were detected ($P = 0.020$; Fig. 3).

Discussion

To our knowledge, BioPER is the first prospective published trial to evaluate the antitumor activity, safety, and predictive biomarkers of palbociclib rechallenge in patients with HR⁺/HER2[−] ABC. Prior retrospective studies had suggested a continued benefit from this clinical approach, but they must be interpreted with caution due to potential biases including patient selection, timing since prior CDK4/6 inhibitor-containing regimen, and the use of a different CDK4/6 inhibitor, mainly abemaciclib after PD on prior palbociclib/ribociclib-based regimens (21).

The randomized phase II MAINTAIN study has recently showed a significant PFS benefit (5.29 vs. 2.76 months; HR, 0.57; $P = 0.004$) for patients with HR⁺/HER2[−] ABC to switch endocrine therapy and receive ribociclib after progression on CDK 4/6 inhibitor. Of note, 84% of the patients had previously received palbociclib, making it difficult to extrapolate these results to other therapeutic strategies, such as changing the endocrine therapy and continuing the same CDK4/6 inhibitor (25).

The BioPER study achieved the prespecified clinical and biological endpoints among patients with HR⁺/HER2[−] ABC. However, although the CBR and 6-month PFS rate demonstrate good clinical efficacy, median PFS was modest [2.6 months (95% CI, 1.8–6.7)] in the efficacy analysis population despite the enrichment of the trial population by selecting patients with confirmed PD after having achieved clinical benefit (response or stable disease ≥ 24 weeks) on immediately prior palbociclib plus endocrine therapy-based regimen. Interestingly, two patients achieved a prolonged partial response that lasted 9.2 and 36.7 months. Among 24 patients who received study treatment as second-line regimen, median PFS increased to 3.2 months with a 6-month PFS rate of 33.3%.

The safety profile was in line with that described in previous studies, with no unexpected safety signals reported.

Antitumor activity of classical endocrine drugs as single agents is limited after progression on a CDK4/6 inhibitor. In the phase III EMERALD trial, elacestrant significantly improved PFS compared with standard endocrine therapy (HR, 0.697; 95% CI, 0.55–0.88; $P = 0.0018$) among CDK4/6 inhibitor-pretreated patients with

HR-positive/HER2-negative ABC in the second- and third-line settings. The 6-month PFS rates were 34.3% and 20.4% for elacestrant and control arm, respectively. Despite the clinically meaningful magnitude of the benefit, there was only a 1-month absolute benefit in PFS with elacestrant (2.79 vs. 1.97 months; ref. 14). Consequently, PFS from the BioPER trial appears similar to that achieved in the elacestrant and fulvestrant arms of the EMERALD study among unselected patients (14), but inferior to PFS reported in studies investigating the combination of endocrine therapy with alpelisib or everolimus beyond CDK4/6 inhibition (16–18, 20).

The BioPER study confirms that palbociclib rechallenge does not work in all patients, so exploring predictors of response to CDK4/6 inhibitors is critical to identify those patients that are more likely to benefit from this strategy. The chronic loss of Rb has been specifically associated with evolution to a CDK4/6-independent state and, ultimately, resistance to palbociclib in breast cancer cell lines (29). Moreover, in the PALOMA-3 trial, high *CCNE1* mRNA expression was also associated with relative resistance to palbociclib (median PFS in patients with high versus low *CCNE1* mRNA was 7.6 vs. 14.1 months, respectively; ref. 30). Finally, *ESR1* gene mutations and the HER2-enriched subtype have been correlated with endocrine resistance (31–33). In BioPER, the analysis of biomarkers as mediators of therapeutic resistance at baseline revealed that low Rb score, high cyclin E1 score, and *ESR1* mutations were associated with worse outcome to palbociclib rechallenge, findings that are consistent with those observed in prior studies (33). Compared with patients with HR⁺/HER2[−] early ABC, the rate of HER2-enriched intrinsic subtype significantly increases in the advanced scenario (34). In this way, the HER2-enriched subtype represented 37.5% of all tumors in the BioPER study. Unfortunately, although HER2-enriched subtype was numerically associated to less clinical benefit and shorter PFS to palbociclib rechallenge, the small number of evaluable samples does not allow to draw definitive conclusions. Finally, undetected ctDNA at C₁D₁₅ seems to be associated with better PFS. However, this finding is also limited for the low number of matched samples available.

A signature that included mediators of therapeutic resistance at baseline (low Rb score, high cyclin E1 score, and *ESR1* mutation) seems to predict worse outcome. This approach of combining different biomarkers of resistance to predict efficacy of CDK4/6 inhibitor rechallenge has been also recently described by others (35). Of note, in the subset of patients in whom any of these biomarkers were detected the median PFS was superior to 7 months. Despite the encouraging results, BioPER is still far from data obtained in the PADA-1 trial in which patients who presented rising *ESR1* in ctDNA, without evidence of PD following treatment with palbociclib plus an aromatase inhibitor, were randomly assigned to continue with the same treatment or to switch to fulvestrant plus palbociclib. Median PFS was 11.9 months in the fulvestrant arm and 5.7 months in the standard therapy arm (HR, 0.61; 95% CI, 0.43–0.86; $P = 0.007$; ref. 35). Although these data reflect a higher efficacy of an early treatment change before radiographic disease progression, they could also suggest that resistance mechanisms to endocrine therapy could precede palbociclib resistance.

Main limitations of this study are the small sample size, a heterogeneous population including patients with different number of prior lines of therapy and endocrine partners, and descriptive nature of the molecular correlative associations. To overcome these limitations, the randomized, open-label, phase II PALMIRA trial (NCT03809988) is currently testing the value of continuing palbociclib in combination with second-line endocrine therapy in 198 patients with HR⁺/HER2[−] ABC who have achieved clinical benefit during first-line palbociclib-

based treatment. Patients are randomly assigned (ratio 2:1) to receive endocrine therapy alone (letrozole or fulvestrant) or in combination with palbociclib (36). It is worth noting that PALMIRA has important design strengths that help mitigate limitations of the MAINTAIN study (25), such as continuing palbociclib after progression on the same CDK4/6 inhibitor, excluding patients who received systemic treatment in the metastatic setting and did not achieve clinical benefit to the palbociclib-based endocrine regimen, and randomizing a larger number of subjects (198 vs. 120 patients, respectively).

In conclusion, BioPER achieved the prespecified clinical and biological endpoints among patients with HR⁺/HER2⁻ ABC. Although the single-arm study design of BioPER does not allow a definitive understanding of whether continuing CDK4/6 blockade might be a reasonable approach for these patients, the preliminary efficacy along with the favorable safety profile provide a support for further investigation. In addition, ancillary biomarker-driven studies are needed to confirm these preliminary findings. Larger randomized controlled trials will elucidate the antitumor activity of prolonging CDK4/6 blockade beyond progression on prior CDK4/6 inhibitor-based treatment and further confirm the role of our biomarker signature to identify patients that may not derive particular benefit from this strategy.

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Authors' Contributions

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Note

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