Biomarker Analyses of Response to Cyclin-Dependent Kinase 4/6 Inhibition and Endocrine Therapy in Women with Treatment-Naïve Metastatic Breast Cancer

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ABSTRACT

Purpose: Preclinical data identified the cyclin-dependent kinase 4/6 (CDK4/6) inhibitor palbociclib as synergistic with antiestrogens in inhibiting growth of hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2−) human breast cancer models. This observation was validated clinically in the randomized, placebo-controlled, phase III PALOMA-2 study.

Experimental Design: To determine markers of sensitivity and resistance to palbociclib plus letrozole, we performed comprehensive biomarker analyses, investigating the correlation with progression-free survival (PFS), on baseline tumor tissues from PALOMA-2.

Results: Despite a broad biomarker search, palbociclib plus letrozole demonstrated consistent PFS gains versus placebo plus letrozole, with no single biomarker or cassette of markers associated with lack of benefit from combination treatment. Palbociclib plus letrozole confers efficacy on both luminal A and B patients. Higher *CDK4* levels were associated with endocrine resistance which was mitigated by the addition of palbociclib, whereas lower PD-1 levels were associated with greater palbociclib plus letrozole benefit. Tumors with more active growth factor signaling, as exemplified by increased expression of *FGFR2* and *ERBB3* mRNA, appeared to be associated with greater PFS gain from the addition of palbociclib.

Conclusions: These data underscore the importance of CDK4/6 signaling in HR+/HER2− breast cancer and suggest that the interplay between steroid hormone and peptide growth factor signaling could drive dependence on CDK4/6 signaling.

See related commentary by Anurag et al., p. 3

Introduction

The molecular heterogeneity of breast cancer is well established (1), but breast cancer is still approached clinically as three large therapeutic subgroups: hormone receptor-positive (HR+), human epidermal growth factor receptor 2-amplified (HER2+), and the so-called triple-negative breast cancer (TNBC), defined by the lack of hormone receptors and HER2 amplification/overexpression. Approximately 65% of breast cancers fall into the HR+/HER2− category (2, 3). The standard treatment for these patients has been hormonal blockade–based therapies directed at inhibiting the estrogen/estrogen receptor (ER) signaling pathway by various modalities (4). However, it is well understood that a subset of women with HR+/HER2− disease will present with either *de novo* or acquired resistance to endocrine-based therapies (5). Numerous signaling pathways have been implicated as possible mechanisms for resistance, including aberrant peptide growth factor signaling pathways mediated by the epidermal growth factor, insulin growth factor, fibroblast growth factor, and other pathways (6). In addition, alterations in intracellular signaling pathways such as the PI3K, MAPK, Src, and others have been implicated in endocrine resistance in laboratory studies, and many of these alterations are correlated with poor clinical outcomes (7).

Therapeutic targeting of most of these signaling pathways in the frontline setting of advanced HR+/HER2− breast cancer has not yielded significant improvements in outcomes. In 2015, the approval of palbociclib, a small-molecule inhibitor of CCND1/CDK4 kinase activity and CDK6 (a homolog of CCND1/CDK4 kinase activity; ref. 8), in combination with letrozole represented a breakthrough in the management of women with HR+/HER2− advanced breast cancer (ABC). This was the first molecularly targeted agent that demonstrated a significant improvement in outcomes for this group of women (9, 10). These data were initially met with some skepticism given the lack of specific biomarkers, other than ER-positivity (ER+), as a way to identify patients who would receive benefit from treatment. However, since then, multiple phase III studies have validated the clinical benefit of cyclin-dependent kinase 4/6 (CDK4/6) inhibitors in HR+/HER2− breast cancers (11–13).

Cyclin-dependent kinases 4 and 6, together with the protein cyclin D1 (CCND1), are critical regulators of cell-cycle progression (14). Preclinical studies have shown that CDK4/6 inhibition prevents DNA

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors have demonstrated significant activity in estrogen receptor–positive/human epidermal growth factor receptor 2–negative (ER+/HER2–) advanced breast cancer. Further biomarkers to refine the population beyond ER+/HER2– that benefits remain to be identified. We performed a comprehensive biomarker analysis using patient tissues from PALOMA-2, a randomized phase III study of palbociclib-letrozole versus placebo-letrozole. These data were correlated with progression-free survival (PFS) to determine markers of sensitivity and resistance to the combination. Palbociclib-letrozole demonstrated consistent PFS gains versus placebo-letrozole. No single biomarker or cassette of markers was associated with lack of benefit from combination treatment. Our findings support the mechanism that the interplay between two growth-promoting pathways (steroid hormone and peptide hormone signaling pathways) drives CDK4/6 dependence, resulting in clinical benefit from palbociclib-letrozole. These results may guide additional therapeutic options in patients with ER+/HER2– advanced breast cancer and contribute to our understanding of appropriate patient selection.

replication by arresting progression from the G_1 to the S phase during cell division, thereby preventing tumor cell proliferation (8, 15). The CDK4/6–CCND1 complex phosphorylates the retinoblastoma (RB) protein, a tumor suppressor, releasing the E2F and DP transcription factors that regulate expression of genes required for entry into S phase of the cell cycle (16, 17). CDK4/6 activity and progression to the G_1 phase are negatively regulated by the CDK4/6-interacting protein-kinase inhibitory protein (Cip-Kip) and by the inhibitor of the cyclin-dependent kinase 4 (INK4) families, typified by p16, the protein product of *CDKN2A* (16, 18).

Using a panel of 47 human breast cancer and immortalized cell lines, we found that ER+ models were most sensitive to growth inhibition by palbociclib (15). This included data from a cell line that was antiestrogen-resistant (15). The PALOMA-1 study was an open-label, randomized phase II trial designed to validate this observation (9). The study prospectively enrolled women with advanced frontline ER+/HER2− breast cancer and included two distinct cohorts of patients; cohort 1 enrolled patients based solely on ER+/HER2− status, whereas cohort 2 further restricted eligibility to enroll ER+/HER2− patients whose tumor also contained *CCND1* amplification and/or *CDKN2A* (p16) loss (9). Findings from this study were consistent with the preclinical studies and were the first to clinically demonstrate a role for CDK4/6 inhibitors in ER+ breast cancer. The biomarker analysis of the PALOMA-1 study failed to demonstrate a relationship between *CCND1* amplification or p16 loss and efficacy of palbociclib in combination with letrozole (9). Consequently, the pivotal phase III PALOMA-2 study enrolled ABC patients who had not received prior treatment for advanced disease using only ER+/HER2− status, without further biomarker enrichment (11). A total of 666 postmenopausal patients were enrolled to receive palbociclib plus letrozole or placebo plus letrozole (11); the addition of palbociclib to letrozole resulted in a statistically significant, robust, and clinically meaningful 13-month improvement in median progression-free survival (PFS) compared with placebo plus letrozole [27.6 months vs. 14.5 months; HR, 0.56; 95% confidence interval (CI), 0.46-0.69; $P < 0.0001$; data cutoff date: May 31, 2017; ref. 19]. This benefit was consistent across patients with various clinical parameters, including disease-free interval >5 years, non-measurable or measurable disease, visceral or nonvisceral disease, and bone-only disease, as well as those who had received prior endocrine therapy (19).

In an attempt to garner further information on a population of patients who received benefit from the addition of a CDK4/6 inhibitor in ER+/HER2− breast cancer, baseline tumor tissues were required for enrollment in the PALOMA-2 study. Using these tissues, we performed a comprehensive preplanned assessment evaluating additional DNA, mRNA, and protein biomarkers and correlated them with the clinical outcomes observed in the PALOMA-2 study.

Materials and Methods

PALOMA-2 study design

The details of the PALOMA-2 study have been previously published (11). Briefly, it was a double-blind, phase III placebo-controlled study that randomized patients 2:1 to receive palbociclib 125 mg daily (3 weeks of treatment, then 1 week off) plus letrozole (2.5 mg daily) or placebo plus letrozole (2.5 mg daily) in postmenopausal women who had not received prior systemic therapy for ER+/HER2− ABC. This study was approved by an Institutional Review Board or equivalent ethics committee at each study site. The primary endpoint was investigator-assessed PFS. The study was conducted in accordance with the International Conference on Harmonization Good Clinical Practice guidelines and the provisions of the Declaration of Helsinki, and all patients provided written informed consent before enrollment.

Tissue samples

The submission of formalin-fixed paraffin-embedded (FFPE) tumor samples was mandatory in PALOMA-2. Patients consented to the assessment of biomarkers associated with sensitivity and/or resistance to palbociclib plus letrozole per study protocol. If an FFPE tissue block could not be provided, a minimum of 12 glass slides, each containing an unstained 5-μm FFPE tissue section, were required for analysis. Whenever possible, recently biopsied tissue samples from a metastatic or recurrent tumor lesion were provided. If archival tissue was unavailable, a new biopsy was required.

Gene expression analysis

Analyses of gene expression (RNA) were performed by using the EdgeSeq Oncology Biomarker Panel (HTG Molecular Diagnostics, Inc). This system uses targeted capture sequencing to quantitate RNA expression levels of gene targets in FFPE tissues. The first section of breast cancer FFPE tissue was stained with hematoxylin and eosin (H&E). A board-certified pathologist assessed the tumor cell content and tissue necrosis. Tumor content was estimated based on the number of malignant cells as a percentage of all cells (i.e., malignant plus normal cells in the tissue section). The acceptance criterion for analysis was set at >70% of tumor content. Necrosis was assessed based on the percentage of necrotic tissue within the total tissue area. The acceptance criterion for analysis was set at <20% necrosis. Macrodissection was performed on the tissue sections if the tumor content was <70% or if necrosis was ≥20%. Sample preparation was conducted following laboratory processes and manufactory protocols. Sequencing was performed on an Illumina NextSeq 500 Sequencer (Illumina, Inc.).

To normalize the sequencing data, probe counts were transformed into $\log_2$ counts per million. Expression values were quantile normalized. HTG Molecular Diagnostics, Inc., was blinded to patient information and clinical outcomes.

Molecular subtype classification

Given the lack of large diverse reference tumor sets profiled with the EdgeSeq Oncology platform and the fact that only ER+ patients were included in the PALOMA-2 study, we could not use the PAM50 classification scheme, which determines subtype relative to a baseline of heterogeneous tumors from the same cohort or from a comparable reference database (20). Therefore, the single sample predictor algorithm Absolute Intrinsic Molecular Subtyping (AIMS) was adopted to classify subtypes through a set of binary rules that compare expression measurements for pairs of genes from each patient independently (21). As only 42 of the 100 binary rules could be applied based on genes in the EdgeSeq Oncology BM panel, classification performance was assessed by downsampling TCGA data from genome-wide to the EdgeSeq Oncology panel subset. Using all genes versus EdgeSeq Oncology panel genes only, the agreement with PAM50 classification was 77% versus 76%, respectively.

Exploratory unbiased discovery statistical analysis

A data-driven exploratory unbiased discovery analysis was performed for gene expression biomarkers predictive of the benefit from palbociclib plus letrozole. Using Cox regression analysis, the search first identified genes whose expression was significantly associated with PFS as a continuous variable within the palbociclib plus letrozole or placebo plus letrozole arms (FDR < 0.1), followed by a cross-arm interaction analysis both before and after accounting for known clinical–pathological factors (i.e., site of disease, prior hormone therapy, disease-free interval, age, race, region, baseline Eastern Cooperative Oncology Group status; Supplementary Table S1). To further evaluate underlying biological processes mediating palbociclib plus letrozole response, we transformed data from gene to pathway activity using MSigDB curated (c2) gene sets and a Gene Set Variation Analysis (GSVA) algorithm (22). Cox regression analysis as described for the gene-level search indicated above and then carried out with pathway activity scores.

Protein expression analysis by IHC

Expressions of ER (Dako 1D5 anti-ER antibody), RB (BD Biosciences G3-245 anti-RB antibody), CCND1 (Biocare Medical SP4 anti-Cyclin D1 antibody), p16 (Ventana E6H4 anti-p16 antibody), and Ki67 (Ventoan 30-9 anti-Ki67 antibody) were analyzed retrospectively at a Clinical Laboratory Improvement Amendments–certified laboratory using validated IHC assays. The results were quantified using H-score methodology (3 × percentage of strongly staining nuclei + 2 × percentage of moderately staining nuclei + percentage of weakly staining nuclei, giving a range of 0–300). For ER, CCND1, and p16, positive expression was defined as an H-score of $\geq$1.

RB was reported as positive if $\geq$5% of tumor cells exhibited a positive staining reaction, in a nuclear pattern (Vision Biosystems). The staining intensity of tumor cells was reported based on the following guidelines: 0 = nuclear staining not detectable, 1+ = nuclear staining translucent, 2+ = nuclear staining opaque, and 3+ = nuclear staining solid.

FISH analysis

FISH was performed using a 4-color FISH test to detect both *CCND1* amplification and *CDKN2A* (p16) deletion simultaneously from a single tissue section. A minimum of two 5-μm FFPE tissue sections were used for this assay. The first section was stained with H&E to visualize the tumor compartment within the tissue. The second and subsequent sections were reserved for FISH analysis. A 4-color FISH probe set was hybridized to the section. Using the H&E as a guide, the invasive component of the tumor was analyzed for *CCND1* amplification and *CDKN2A* deletion. A minimum of 20 cells were scored in each case.

The DNA probes [*CCND1* (Spectrum Red), *CEP11* (Spectrum Green), *CDKN2A* (Spectrum Gold), and *CEP9* (Spectrum Qua)] were purchased from Abbott Molecular. Manual visualization and interpretation of fluorescence signals were performed using an Olympus BX51 Fluorescence microscope. *CCND1* amplification was quantified by *CCND1/CEP11* ratio, with a cutoff of >1.5 considered positive. The *CDKN2A* deletion was quantified by *CDKN2A/CEP9* ratio, with a cutoff of <0.8 considered positive. Neogenomics Laboratories, Inc., was blinded to patient information and clinical outcomes.

Statistical analysis

Baseline characteristics and demographics in the biomarker analysis set were summarized by treatment arms. Analyses of investigator-assessed PFS were performed in the subgroups, defined by corresponding baseline biomarker tests using the Kaplan–Meier method and Cox proportional hazard models to investigate potential association between biomarker levels and PFS from palbociclib plus letrozole or placebo plus letrozole treatments. Biomarker analyses were performed using both hypothesis-driven (supervised) and data-driven (unsupervised) approaches. Results were visualized using Kaplan–Meier plots by median or quantile dichotomization. A log-rank test was used to compare PFS differences, with multiple testing corrections made and FDRs based on the Benjamini–Hochberg procedure.

Results

Consistent PFS benefit in the biomarker and intent-to-treat populations

A total of 568 of 666 (85%) baseline tumor tissues from the 666 patients randomized were collected: 379 of 444 patients (85%) in the palbociclib plus letrozole arm and 189 of 222 patients (85%) in the placebo plus letrozole arm. A total of 455 of 568 (80%) patient tissues were evaluated using gene expression analyses, 520 (92%) were evaluated by FISH, and 568 (100%) were evaluated using IHC (Supplementary Fig. S1). Baseline patient demographics and clinical characteristics were similar between the gene expression, IHC, and FISH biomarker subsets and the overall PALOMA-2 intent-to-treat (ITT) population (**Table 1**). Of note, the PFS benefit associated with palbociclib plus letrozole was similar between the various biomarker subsets defined below, as well as in the overall ITT population (Supplementary Fig. S2).

CDK6, cyclin D, cyclin E, RB, and p16 expression levels do not predict the benefit of palbociclib in combination with letrozole by IHC, FISH, or gene expression analysis

Analyzing genes involved in the cyclin D-CDK4/6-Rb pathway showed that irrespective of the baseline gene expression levels (higher

Table 1. Demographics of the overall ITT population and gene expression, IHC, and FISH biomarker subsets.

	Palbociclib plus letrozole				Placebo plus letrozole			
	ITT ($n = 444$)	Gene Expression ($n = 303$)	IHC ($n = 379$)	FISH ($n = 344$)	ITT ($n = 222$)	Gene Expression ($n = 152$)	IHC ($n = 189$)	FISH ($n = 176$)
Race, *n* (%)								
White	344 (77)	243 (80)	295 (78)	264 (77)	172 (77)	116 (76)	142 (75)	133 (76)
Non-white	86 (19)	50 (17)	71 (19)	68 (20)	38 (17)	28 (18)	37 (20)	32 (18)
Missing data	14 (3)	10 (3)	13 (3)	12 (3)	12 (5)	8 (5)	10 (5)	11 (6)
ECOG performance status, *n* (%)								
0	257 (58)	176 (58)	218 (58)	199 (58)	102 (46)	75 (49)	87 (46)	82 (47)
1	178 (40)	122 (40)	153 (40)	137 (40)	117 (53)	75 (49)	100 (53)	93 (53)
2	9 (2)	5 (2)	8 (2)	8 (2)	3 (1)	2 (1)	2 (1)	1 (1)
Disease site,[a] *n* (%)								
Visceral	214 (48)	148 (49)	189 (50)	170 (49)	110 (50)	78 (51)	97 (51)	92 (52)
Nonvisceral	230 (52)	155 (51)	190 (50)	174 (51)	112 (50)	74 (49)	92 (49)	84 (48)
Disease-free interval,[a,b] *n* (%)								
>12 mo	179 (40)	112 (37)	147 (39)	125 (36)	93 (42)	65 (43)	79 (42)	72 (41)
≤12 mo	98 (22)	73 (24)	86 (23)	79 (23)	48 (22)	38 (25)	42 (22)	39 (22)
De novo metastatic	167 (38)	118 (39)	146 (39)	140 (41)	81 (36)	49 (32)	68 (36)	65 (37)
Prior hormonal therapy,[a] *n* (%)								
No	194 (44)	132 (44)	168 (44)	161 (47)	96 (43)	61 (40)	81 (43)	77 (44)
Yes	250 (56)	171 (56)	211 (56)	183 (53)	126 (57)	91 (60)	108 (57)	99 (56)

Abbreviation: ECOG, Eastern Cooperative Oncology Group.
[a]Stratification factors based on randomization.
[b]Time between the end of prior neoadjuvant or adjuvant treatment and the onset of metastatic disease.

or lower based on dichotomization by median) of *CCND1, CCNE1/2, CDK2/6, RB1, CDKN2A*, or *ESR1*, the addition of palbociclib to letrozole demonstrated a benefit versus placebo plus letrozole, in line with the overall ITT population (**Fig. 1A**). Similar results were obtained when gene expression levels were stratified by upper and lower quartiles (Supplementary Fig. S3). Similar results were observed for the protein levels of ER, RB, CCND1, and p16 when expression was assessed by quartiles using an H-score analysis (**Fig. 1B**). As seen in PALOMA-1 and based on copy-number (FISH) analysis from the current study, patients benefited from the addition of palbociclib to letrozole regardless of whether their tumor contained *CCND1* amplification and/or *CDKN2A* (p16) loss (**Fig. 1C**).

Concordance analysis showed a reasonable agreement between protein and mRNA expression for ER, CCND1, and Ki67 (Spearman $R = 0.50$, 0.39, and 0.48, respectively), but weaker correlation for RB (Spearman $R = 0.22$) and p16 ($R = 0.14$; Supplementary Table S2). In addition, the protein levels of ER, CCND1, and RB were most correlated with their own transcript among all genes across the EdgeSeq Oncology panel, whereas the cis-correlation for Ki67 was ranked fifth panel wide, demonstrating the high specificity of the relationship. It is also worth noting that the concordance between the expression by IHC and amplification by FISH analyses of CCND1 (coefficient $R = 0.19$) and p16 ($R = 0.09$) was also very low (Supplementary Table S3).

The incidence of RB loss in ER+ breast cancer is low (<5%; ref. 23). In our cohort, total RB expression as assessed by IHC was positive in 90.9% (512/563) of patients and negative in 9.1% (51/563) of patients. In patients with RB-positive (RB+) tumors, the observed HR from the unstratified analysis was 0.543 (95% CI, 0.433-0.681; log-rank $P < 0.0001$ in favor of palbociclib plus letrozole). The RB-"negative" (RB−) subset had a weaker HR of 0.868 (95% CI, 0.424-1.777, log-rank $P = 0.698$), but this is clearly limited by the small numbers of patients in this cohort. Similarly, we analyzed RB gene expression based on a cutoff of 8 from negative control probes (**Fig. 2A**) as well as dichotomized by the median (**Fig. 2B**) and by quartile (**Fig. 2C**) to define RB higher versus lower expression between the two treatment groups. Exploratory assessments of PFS by *RB1* gene using this approach revealed a consistent benefit with palbociclib plus letrozole. Of note, there was no evidence of palbociclib benefit in truly *RB*-null tumors (**Fig. 2**; Supplementary Fig. S4).

CDK4 expression levels predict resistance to placebo plus letrozole but not to palbociclib plus letrozole

Contrary to results with the other cell-cycle genes and proteins analyzed above, higher *CDK4* gene expression level was associated with an important clinical parameter measured in the study. These patients demonstrated shorter PFS with placebo plus letrozole ($P = 0.000972$ and $P = 0.000779$ when gene expression levels were treated as a continuous variable or dichotomized by median, respectively; **Fig. 3A**; Supplementary Fig. S5). This relationship was not significant in the palbociclib plus letrozole arm ($P = 0.91$ and $P = 0.13$ by continuous and median analysis, respectively). As noted above, the expression of *CDK6* did not have a similar effect (lower expression, HR = 0.596; higher expression, HR = 0.592; **Fig. 1A**).

ESR1 expression levels are associated with longer PFS in both placebo plus letrozole and palbociclib plus letrozole arms

Higher *ESR1* gene expression was found to be associated with greater benefit in both the placebo plus letrozole and the palbociclib plus letrozole arms (**Fig. 3B**), underscoring the importance of ER in predicting response to both letrozole and its combination with palbociclib. Importantly, in both the *ESR1* high- and low-expression groups, the addition of palbociclib increased PFS by approximately 10 months (11.2 to 21.5 months for the lower *ESR1* expression level and 18.2 to 27.7 months for the higher *ESR1* expression level). This is consistent with protein data showing that regardless of the expression level of ER

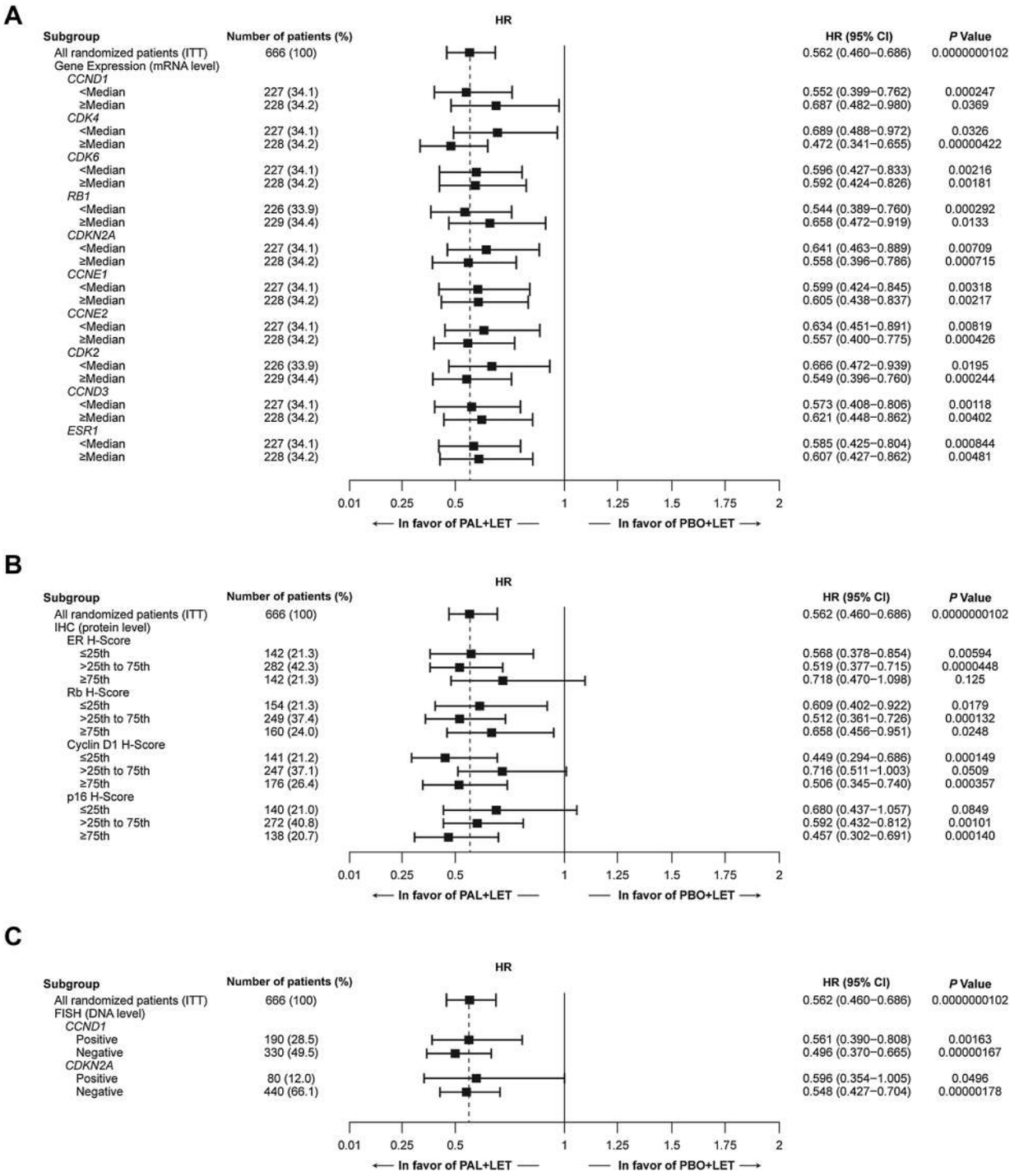

Figure 1.

Forest plot analysis of PFS benefit from the addition of palbociclib to letrozole based on target genes in the Cyclin D-CDK4/6-RB pathway by (**A**) mRNA expression (HTG), (**B**) protein expression (IHC), and (**C**) DNA copy number (FISH). *CCND1*, cyclin D1; *CCND3*, cyclin D3; *CCNE1*, cyclin E1; *CCNE2*, cyclin E2; *CDK2,* cyclin-dependent kinase 2; *CDK6*, cyclin-dependent kinase 6; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *ESR1*, estrogen receptor 1; HR, hazard ratio; LET, letrozole; PAL, palbociclib; PBO, placebo; *RB1*, retinoblastoma 1.

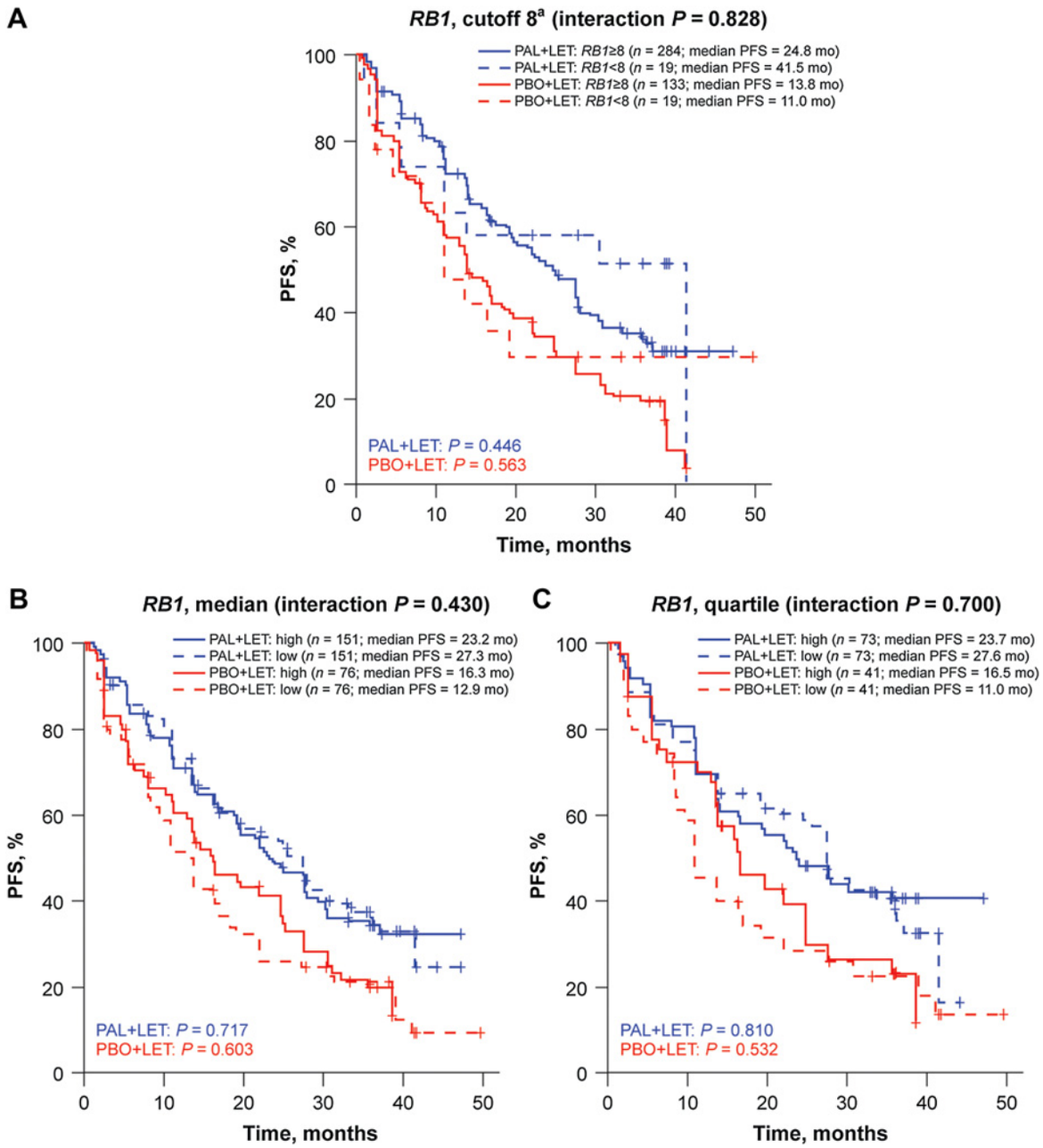

Figure 2.

PFS by *RB1* expression (**A**) based on a cutoff of 8, (**B**) dichotomized by the median, and (**C**) by quartile. LET, letrozole; PAL, palbociclib; PBO, placebo; *RB1*, retinoblastoma 1. [a]A cutoff of 8 is based on the expression pattern of negative control probes on the EdgeSeq Oncology Biomarker panel.

protein in the tumor as measured by IHC, patients with ER+ disease benefited from palbociclib plus letrozole over placebo plus letrozole (**Fig. 1B**).

Both luminal A and B subtypes determined by either Ki67 or gene expression–based profiles benefit from the addition of palbociclib to letrozole

We explored whether the benefit of palbociclib plus letrozole may differ by intrinsic breast cancer subtype. Based on published literature, Ki67 protein cutoffs of >20% (24, 25) or >15% (26, 27) were used to subdivide tumors into luminal B versus A subtypes. In total, 58% or 46% of patients with Ki67 IHC data available had luminal A (Ki67 ≤20% or ≤15%), and 42% or 54% of patients had luminal B tumors (Ki67 >20% or >15%), respectively. Analyses using either cutoff led to the observation that patients with either luminal A or B subtype benefited from treatment with palbociclib plus letrozole compared with placebo plus letrozole (Supplementary Fig. S6).

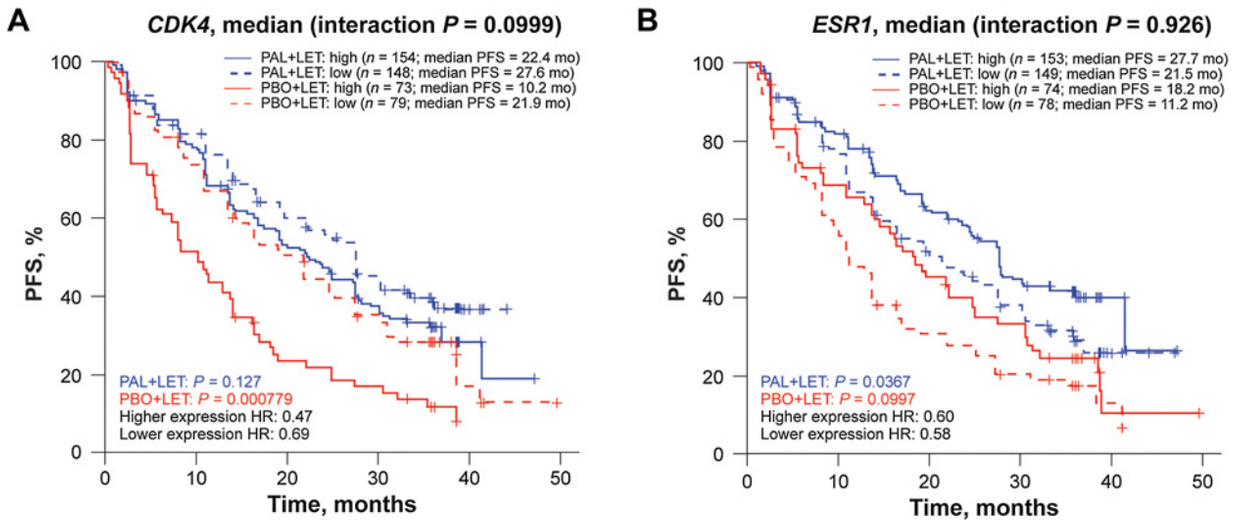

Figure 3.
PFS by (**A**) *CDK4* and (**B**) *ESR1* expression. *ESR1*, estrogen receptor 1; HR, hazard ratio; LET, letrozole; PAL, palbociclib; PBO, placebo.

Tumors were also classified according to their mRNA profiles using the AIMS algorithm (21). This analysis demonstrated that 50% of study patients had luminal A ($n = 229$), and 30% had luminal B tumors ($n = 135$; **Fig. 4A**). Gene expression–based analysis also showed that patients of either luminal subtype benefited from the addition of palbociclib to letrozole (luminal A: HR, 0.55; 95% CI, 0.39-0.77; $P = 0.000547$ and luminal B: HR, 0.51; 95% CI, 0.34-0.77; $P = 0.00109$; **Fig. 4B** and **C**). The number of patients with nonluminal molecular subtypes were smaller, with the largest group being the HER2−like subtype (19%, $n = 85$), and the study only had very small numbers of patients with basal-like ($n = 2$) and normal-like ($n = 4$) subtypes (**Fig. 4A**); these groups were not large enough for a formal analysis. The increase in median PFS with the addition of palbociclib in the HER2−like group was less than in the luminal subgroups, but again, these observations are limited by the small group size.

PALOMA-2 enrolled patients with ER+/HER2− tumors based on the local site assessments. Although centralized FISH analysis was not performed, we confirmed the lack of HER2 amplification by examining the mRNA expression of the *ERBB2* gene itself and found that it did not show a bimodal distribution, with exceptionally high outliers typically associated with amplified tumors (28).

High level of fibroblast growth factor receptor 2 and Erb-B2 receptor tyrosine kinase 3 predicts greater degree of benefit from palbociclib plus letrozole

In addition, we performed a panel-wide unsupervised analysis to identify potential predictive biomarkers of benefit in each arm of the study. After correcting for multiple hypothesis testing, 9 and 16 candidate genes were identified from the palbociclib plus letrozole and placebo plus letrozole arms, respectively, using an FDR of <0.1 for within-arm PFS association and a cross-arm gene expression treatment interaction of $P < 0.1$ (Supplementary Table S1). This was performed both before and after accounting for known clinical–pathological factors, resulting in a total of 12 relative resistance markers (*CHI3L1, PDCD1, PHGDH, PORCN, LPL, PIK3R5, STEAP4, NUMB, ATF6, TNFRSF25, HHAT,* and *BMPR1B*) and 13 relative sensitivity markers (*SMAD2, TIRAP, SETBP1, KRT19, GSTM3, SORD, CYR61, CDK4, EZH2, MDK, THBS2, NNMT,* and *TMSB10*). Importantly, as noted in the supervised approach, *CDK4* expression was significantly associated with an enhanced benefit from palbociclib plus letrozole. Interestingly, tumors with higher levels of pretreatment programmed cell death protein 1 (*PDCD1*) mRNA expression tended to receive less benefit from addition of palbociclib to letrozole than those with lower levels (FDR = 0.099, interaction $P = 0.020$ from continuous analysis; **Fig. 5A**).

In addition to individual genes, we also assessed whether any biologically defined gene signatures might predict benefit with palbociclib plus letrozole by summarizing the data in thousands of MSigDB gene sets using the GSVA algorithm (22). Analogous Cox regression analysis highlighted the PD-1 signaling pathway as being associated with reduced PFS benefit from the addition of palbociclib to letrozole (palbociclib plus letrozole, FDR = 0.039 and interaction $P = 0.0893$). Meanwhile, tumors with more active growth factor signaling appeared to be associated with greater PFS gain from the combination of palbociclib plus letrozole (FDR = 0.027 and interaction $P = 0.0888$), as exemplified by increased expression of *FGFR2* and Erb-B2 receptor tyrosine kinase 3 (*ERBB3*; **Fig. 5B** and **C**).

Discussion

The development of CDK4/6 inhibitors in breast cancer has followed a rational clinical development plan based on compelling preclinical observations indicating that having an ER+ luminal phenotype was the most predictive for response to this class of targeted therapy. The clinical benefit of the CDK4/6 inhibitor palbociclib in combination with endocrine therapy initially shown in the PALOMA-1 study (9) has now been confirmed in multiple randomized phase III clinical studies in HR+/HER2− breast cancer using three different CDK4/6 inhibitors (11–13, 29–31). As a result, CDK4/6 inhibitors are now established as a standard-of-care option for both endocrine-sensitive and endocrine-resistant HR+/HER2− metastatic breast cancer (32, 33). Despite this observed clinical benefit, there remains interest in further defining the responsive population for

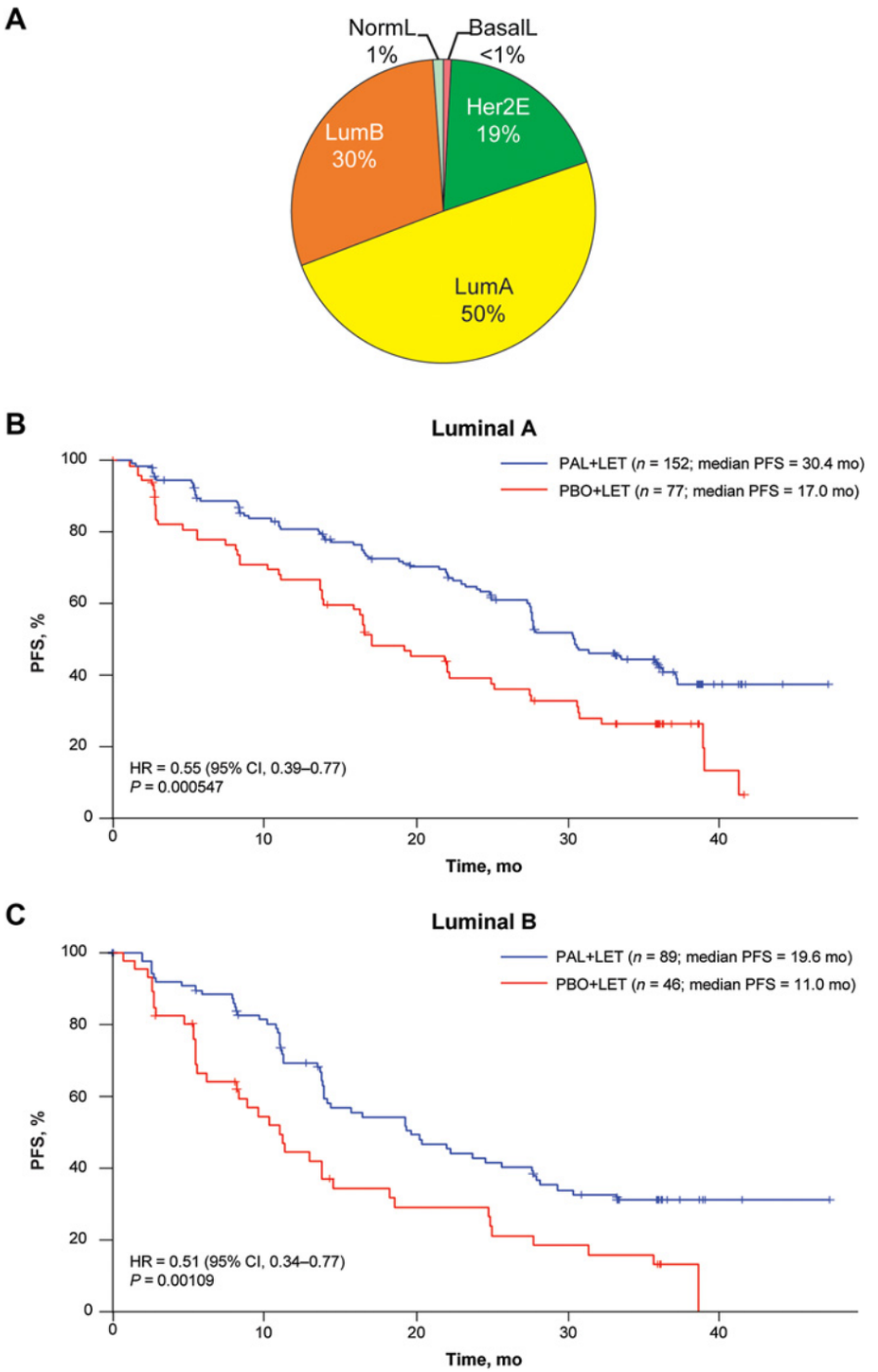

Figure 4.

(**A**) Intrinsic subtype distribution of tumors and PFS in (**B**) luminal A and (**C**) luminal B tumors. BasalL, basal-like; Her2E, human epidermal growth factor receptor 2-enriched; HR, hazard ratio; LET, letrozole; LumA, luminal A; LumB, luminal B; NormL, normal-like.

efficacy with CDK4/6 inhibitors, given the side effects and costs associated with addition of a CDK4/6 inhibitor to endocrine therapy. Using tissues from a prospective phase III study (PALOMA-2), we performed a comprehensive molecular analysis of patient tissues at the DNA, mRNA, and protein levels in an attempt to identify biomarker(s) associated with benefit from the addition of palbociclib to letrozole for HR+/HER2− breast cancer. Ultimately, our analysis underscores the importance of selecting patients that are ER+; this subgroup appears to be dependent on signaling through the CDK4/6:cyclin D:RB pathway, given a dependence on estrogen signaling, and we have observed convergent actions on the CDK4/6:cyclin D:RB pathway in some patients with increased expression of receptor

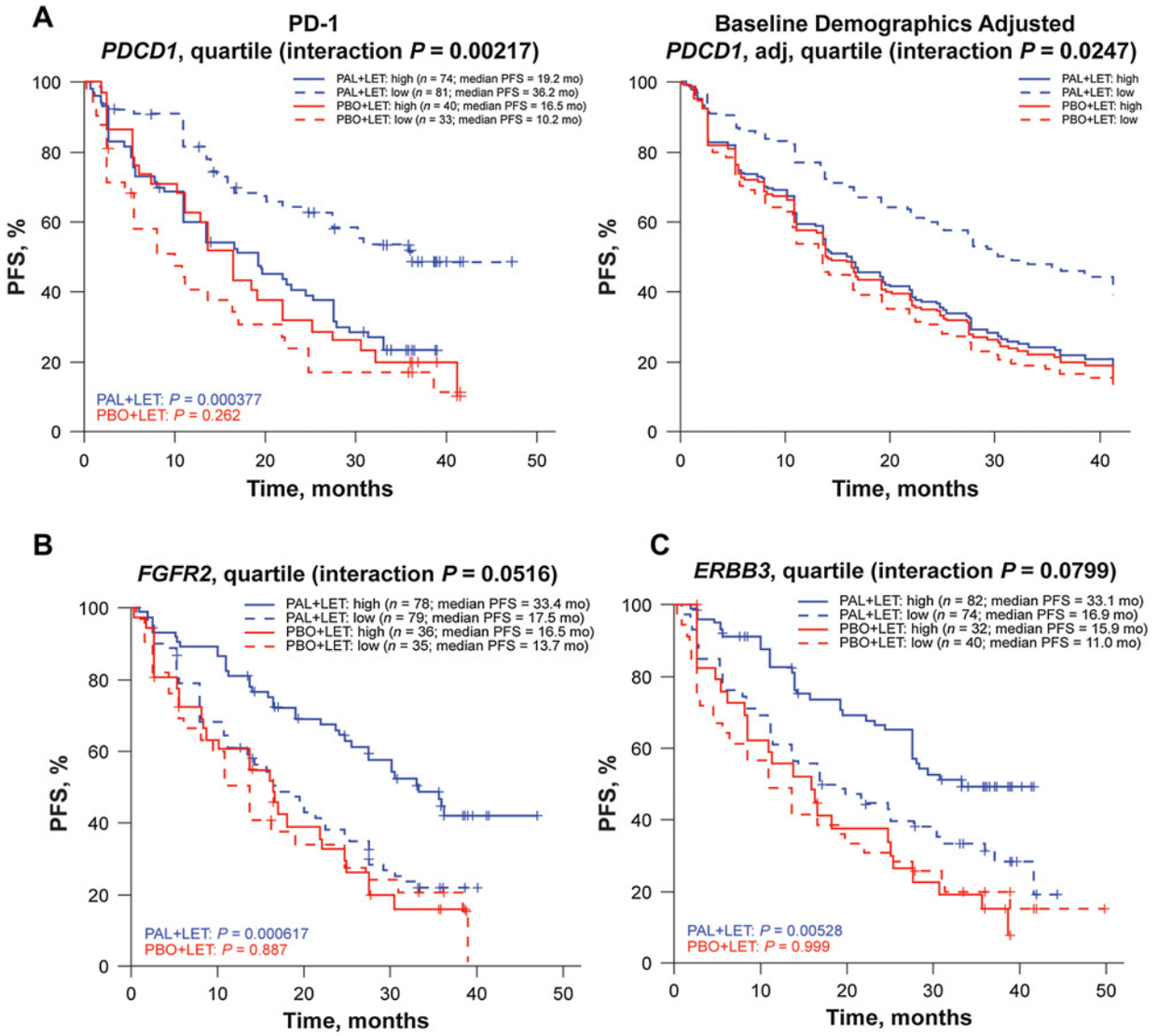

Figure 5.

PFS by expression levels of (**A**) *PDCD1*, (**B**) *FGFR2*, and (**C**) *ERBB3*. *ERBB3*, Erb-B2 receptor tyrosine kinase 3; *FGFR2*, fibroblast growth factor receptor 2; LET, letrozole; PAL, palbociclib; PBO, placebo; *PDCD1*, programmed cell death protein 1; PFS, progression-free survival.

tyrosine kinases. In addition, proposed alterations that are likely associated with resistance to a CDK4/6 inhibitor (i.e., *CCNE1* amplification or RB loss) appear to be rare events in this breast cancer subtype (23).

We initially focused our approach on potential biomarkers within the cyclin D:CDK4/6:RB pathway, including *CDK4, CDK6*, cyclin D, cyclin E, p16, and RB. Expression of these markers, as determined by IHC or mRNA levels, was not predictive of palbociclib benefit in combination with letrozole in this population, i.e., patients who had not received prior systemic therapy for ER+/HER2− ABC. When considering these data, it should be noted that an ER+ status already classifies these tumors into a higher RB expression level group (15), indicating that we are comparing relative RB levels within a "high RB expression" group. It has been hypothesized that amplification of *CCNE1* or RB loss could be associated with resistance to CDK4/6 inhibitor effects (34). However, the incidence of these alterations in ER+ luminal breast cancer is rare and (23), therefore, the numbers of patients in PALOMA-2 with such alterations were not sufficient to validate this hypothesis. Similarly, when evaluating RB expression by IHC, the number of patients with truly RB "negative" status was small. Furthermore, the validation of this assay in tissues collected from multiple centers without controlled processing and fixation is limited in our analysis. There was minimal concordance between *RB1* gene expression and RB protein level, indicating potential methodological limitations in the detection of RB in HR+ breast cancer. Based on these data, we could not conclude that palbociclib has activity in truly RB-null or *CCNE1*-amplified tumors.

Previous studies have reported *CCND1* amplification in 29% of luminal A and 58% of luminal B tumors, and *CDK4* gains in 14%

and 25% of luminal A and luminal B tumors, respectively (35); low expression of p16 in luminal A tumors has also been reported (36). The PALOMA-1 study independently assessed *CCND1* amplification and/or p16 loss in an open-label randomized study (9). In the cohort of patients with *CCND1* amplification or p16 loss ($n = 99$), the benefit of palbociclib plus letrozole was similar to patients without those genomic changes (9). These observations are now confirmed in the current placebo-controlled PALOMA-2 study. The current results showed that *CDK6*, cyclin D, cyclin E, p16, and RB expression levels as measured here were not predictive of palbociclib benefit in combination with letrozole in this population of patients who had not received prior systemic therapy for ER+/HER2− ABC.

Large randomized studies have consistently shown that CDK4/6 inhibitors add to endocrine therapy activity in HR+/HER2– ABC, as evidenced by improvements in median PFS (9, 11–13). We also investigated endocrine resistance and sensitivity as potential markers of palbociclib activity. Our study demonstrated high concordance between quantitative IHC by H-score and *ESR1* mRNA expression using data from contemporary care settings. These data suggest that high *ESR1* expression may predict a potentially greater degree of benefit from letrozole in metastatic breast cancer and are consistent with previous literature supporting ER expression and its relationship to HER2 signaling (37). Preclinical data indicate that endocrine-resistant cell lines demonstrate high levels of *CDK4* expression (38, 39). Consistent with these observations, our findings showed that patients treated with placebo plus letrozole who have tumors expressing high levels of *CDK4* expression exhibited shorter median PFS than patients with lower levels. These findings suggest that elevated levels of *CDK4* may contribute to an endocrine resistance phenotype that can be circumvented with the addition of palbociclib. Thus, levels of *ESR1* expression appeared to be prognostic, and *CDK4* may be predictive of letrozole resistance (interaction $P = 0.016$). These findings suggest that the effect of CDK4/6 inhibition may be independent of those exerted by the endocrine-mediated pathway.

Multiple growth steroid peptides signal and prompt tumor growth via the ER (40). Cyclin D is a direct downstream effector of ER signaling (17, 41). From an unbiased panel-wide analysis, we demonstrated that more active growth factor signaling, including high levels of *ERBB3* and *FGFR2* expression, was associated with a larger PFS gain from the combination of palbociclib and letrozole. These data provide evidence that the interplay between steroid hormone and peptide growth factor signaling in ER+ breast cancer could drive dependence on CDK4/6, which leads to the greater clinical benefit seen with CDK4/6 inhibition through the addition of palbociclib to letrozole.

Genomic assays classify luminal disease into luminal A and luminal B subtypes. Published results in early breast cancer indicate that patients with luminal A status may derive more benefit from endocrine therapy than patients with luminal B disease (24, 25, 42). In the present study, Ki67 cutoffs (24, 25, 43) and the gene expression–based AIMS algorithm (21) were used to stratify luminal subtypes. Patients with both luminal A and B disease benefited to a similar degree from the addition of palbociclib to letrozole. There was less of a benefit associated with palbociclib plus letrozole in the HER2−like group; however, this was a relatively small subgroup, and further evaluation will be required to address this issue.

There is increasing interest in the development of immune-oncology agents in breast cancer, although to date, most efforts have focused on triple-negative disease (44). Preclinical studies have evaluated palbociclib combined with checkpoint inhibitors and shown potential efficacy suggesting effects of CDK4/6 inhibition in immune effector cells (15). Of note, an unsupervised analysis of the current study identified not only a higher level of PD-1 itself, but also that the pathway is associated with less benefit from the addition of palbociclib to letrozole versus lower expressors. PD-1 expression is a potential indication of lymphocyte infiltration, and emerging data suggest that low PD-1 expression levels may be prognostic of poor outcomes. This may represent a potential avenue to improve outcomes with intrinsic resistance to endocrine-CDK4/6 inhibitor–based therapy and warrant further clinical investigation. Ongoing studies are now evaluating immune checkpoint inhibitors targeting PD-1 in combination with CDK4/6 inhibitors, including palbociclib (NCT02778685).

One major limitation of the current analysis is that tissue samples could have been from either primary or metastatic biopsies. The origin of tissue samples was not recorded, and biomarkers may differ in samples from metastatic and primary biopsies. The impact of this was not assessed in the current study. In addition, host factors (e.g., pharmacogenomics) were not assessed in the present study, only tumor tissue factors. Despite these limitations, the current findings provide important biological insights into the interplay between the ER signaling, peptide growth factor signaling, and cell-cycle pathways that may ultimately help guide additional therapeutic opportunities in patients with ER+/HER2– ABC. These findings may contribute to understanding appropriate patient selection, particularly in early breast cancer, where greater clinical benefit with this class of agents may occur. Large adjuvant studies of palbociclib in early stage breast cancer are ongoing (clinicaltrials.gov: NCT02513394, NCT03609047) and will provide additional tissues for biomarker correlates.

Disclosure of Potential Conflicts of Interest

R.S. Finn is an employee/paid consultant for AstraZeneca, Bayer, Bristol-Myers Squibb, Eisai, Eli Lilly, Pfizer, Merck, and Roche/Genentech, and reports receiving other remuneration from Novartis. Y. Liu is an employee/paid consultant for and holds ownership interest (including patents) in Pfizer. Z. Zhu is an employee/paid consultant for and holds ownership interest (including patents) in Pfizer. M. Martin reports receiving commercial research grants from Roche and Novartis, and reports receiving other remuneration from Lilly, Roche, Novartis, PUMA, AstraZeneca, Pharmamar, Taiho Oncology, Pfizer, and Daiichi Sankyo. H.S. Rugo reports receiving other commercial research support from Pfizer. V. Diéras is an employee/paid consultant for and reports receiving speakers bureau honoraria from Pfizer, Novartis, and Lilly. S.-A. Im reports receiving commercial research grants from Pfizer; reports receiving other commercial research support from AstraZeneca; and is an unpaid consultant/advisory board member for AstraZeneca, Amgen, Hanmi, Novartis, Eisai, Roche, Pfizer, MediPacto. N. Harbeck is an employee/paid consultant for Lilly, Novartis, and Pfizer, and reports receiving speakers bureau honoraria from Novartis and Pfizer. D.R. Lu is an employee/paid consultant for, has immediate family members who are employees/paid consultants for, and holds ownership interest (including patents) in Pfizer. E. Gauthier is an employee/paid consultant for and holds ownership interest (including patents) in Pfizer. C. Huang Bartlett is an employee/paid consultant for and holds ownership interest (including patents) in Pfizer. D.J. Slamon holds ownership interest (including patents) in Pfizer. No potential conflicts of interest were disclosed by the other author.

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