Cyclin E1 (*CCNE1*) Expression and Palbociclib Efficacy in Previously-Treated Hormone Receptor−Positive Metastatic Breast Cancer

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Abstract

PURPOSE: A large panel gene expression analysis was conducted to identify biomarkers associated with effectiveness of adding palbociclib to fulvestrant.

METHODS: PALOMA-3 randomized 521 endocrine-pretreated metastatic breast cancer patients to receive palbociclib+fulvestrant or placebo+fulvestrant. Primary analysis was first conducted on 10 genes based on pathway biology and evidence from previous studies, followed by a systematic panel-wide search among 2534 cancer-related genes. The association of gene expression with the effect of palboiclib on progression-free survival (PFS) was evaluated using Cox regression analysis, with gene expression as a continuous variable or dichotomized by median. An independent breast cancer cohort from the Preoperative Palbociclib (POP) study was used for validation, with 61 patients treated with 2 weeks of palbociclib.

RESULTS: In the PALOMA-3 trial, 302 patients had tumor tissue analyzed (palbociclib arm, 194; placebo arm, 108). Palbociclib efficacy was lower in patients with high versus low *CCNE1* mRNA expression (median PFS: palbociclib arm, 7.6 mo vs 14.1 mo; placebo arm, 4.0 mo vs 4.8 mo, respectively; interaction *P* value unadjusted *P*=0.00238; false discovery adjusted *P*=0.0238). *CCNE1* mRNA was more predictive in metastatic compared with archival primary biopsies. There was no significant interaction between treatment and expression levels of CDK4, CDK6, cyclin D1, and Rb1. Palbociclib was efficacious in both luminal A and B tumors. High *CCNE1* mRNA expression was associated with poor antiproliferative activity of palbociclib in the POP trial (*P*=0.005).

CONCLUSIONS: Addition of palbociclib to fulvestrant demonstrated efficacy in all biomarker groups, although high *CCNE1* mRNA expression was associated with relative resistance to palbociclib.

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Introduction

Palbociclib is an oral cyclin-dependent kinase (CDK) 4/6 inhibitor that decreases retinoblastoma protein (Rb) phosphorylation, blocking cell cycle progression from the G1 to S phase, and reduces proliferation of breast cancer cells.[1-3] Large randomized prospective clinical studies have demonstrated the efficacy and safety of palbociclib in combination with letrozole or fulvestrant,[4-7] supporting palbociclib plus an aromatase inhibitor or fulvestrant as a standard of care for treating hormone receptor–positive (HR+), human epidermal growth factor receptor 2–negative (HER2−) metastatic breast cancer (MBC) in premenopausal or postmenopausal women.[2,3,8] Extensive analyses have shown that clinical subgroups derive similar benefit from palbociclib combination treatment. [9-11] Identification of biomarkers would assist in distinguishing patient subgroups who derive the greatest efficacy from palbociclib, and elucidating resistance mechanisms, could lead to rationale selection of patients with CDK4/6 combination therapy.

Preclinical research has suggested potential mechanisms of resistance to CDK4/6 inhibitors, including bypass activation of *CDK2*,[12] with high cyclin E1 (*CCNE1*) expression correlated with palbociclib resistance in cell line models of breast and ovarian cancer.[13,14] Other studies have shown that *CDK6* amplification was associated with acquired resistance to CDK4/6 inhibitors[15] and that luminal subtype breast cancer cell lines were more responsive to CDK4/6 inhibitors than non-luminal subtypes.[16] In the small nonrandomized Neoadjuvant Palbociclib and Anastrozole (NeoPalAna) study, exploratory

analysis showed that high levels of *CCNE1* and *CDKN2D* mRNA may predict palbociclib resistance.[17]

No predictive biomarkers have been identified in randomized trials of CDK4/6 inhibitors. In PALOMA-1, neither *CCND1* amplification nor p16 loss were predictive for palbociclib efficacy.[5] In PALOMA-2, CDK4 and CDK6 expression were not predictive of efficacy for palbociclib plus letrozole.[18] In PALOMA-3, neither estrogen receptor 1 (*ESR1*) nor phosphatidylinositol-4,5-biphosphate 3-kinase (*PIK3CA)* mutations predicted palbociclib plus fulvestrant efficacy.[4,19] Additionally, data from the Preoperative Palbociclib (POP) study showed that *PIK3CA* mutations or *CCND1* amplication were not predictive for palbociclib efficacy.[20]

Herein, we describe an analysis of baseline tumor tissue from PALOMA-3 using a large gene expression panel to identify predictive biomarkers for the relative efficacy of adding palbociclib to fulvestrant.

Materials and Methods

Samples

PALOMA-3 randomized 521 patients with endocrine-pretreated MBC to receive palbociclib plus fulvestrant or placebo plus fulvestrant.[4] This study was approved by an institutional review board or independent ethics committee at each site; all patients provided informed consent before enrollment. Patients consented to the assessment of biomarkers associated

with sensitivity or resistance to palbociclib combination treatment per study protocol. Except for patients with bone-only disease or relapse while on adjuvant therapy and who had surgery within 3 years who could provide an archival primary sample, all patients provided formalin-fixed paraffin-embedded (FFPE) tissue taken from metastatic disease. One FFPE tissue sample (2 slides per patient) was stained with hematoxylin and eosin. A board-certified pathologist assessed tumor content and tissue necrosis (additional details in **Supplement**).

To independently validate the association between *CCNE1* mRNA expression and efficacy of palbociclib, we analyzed gene expression data from 61 patients in the POP window trial (additional details in **Supplement**).[21] This trial allocated women with untreated early stage breast cancer 3:1 to receive oral palbociclib for 14 days until the day before surgery, or no treatment.

Gene Expression Analysis

The EdgeSeq Oncology BM Panel (HTG Molecular Diagnostics; https://www.htgmolecular.com/assays/obp) was used for mRNA profiling, assessing 2534 cancer-related genes. Gene expression analysis was performed blinded to the clinical information. The EdgeSeq system used targeted capture sequencing to quantitate RNA expression levels of gene targets in FFPE tissues and was extensively validated **(Supplementary Fig 1)**. Sample preparation was conducted by following the laboratory process and manufacturer protocols. Sequencing was performed on the Illumina

NextSeq 500 Sequencer. Raw data is deposited in the Gene Expression Omnibus.

In the POP trial, *CCNE1* mRNA expression data were obtained from gene expression analysis on Affymetrix Human Gene ST2.1 arrays.

Gene Expression Hypothesis-Driven Statistical Analysis

Gene expression data were quantile normalized and log2 transformed (HTG Molecular Diagnostics). Hypothesis-driven analysis was conducted on 10 genes based on pathway biology and evidences from preclinical and the NeoPalAna studies.[17] Cox regression analysis was performed to investigate potential interaction between biomarker levels, as a continuous variable or dichotomized by median level, and treatment effect in terms of PFS. Interaction *P* values were adjusted using Benjamini-Hochberg false discovery rate (FDR) to account for multiplicity. *CCNE1* mRNA expression by treatment interaction was further evaluated using the nonparametric subpopulation treatment effect pattern plot (STEPP) method for varying levels of *CCNE1* mRNA expression[22] (additional details in **Supplement**). Data analyses were performed using R and Matlab. All tests were 2-sided, unless otherwise noted.

To investigate whether higher levels of *CCNE1* mRNA were associated with lower absolute antiproliferative response (ln Ki67 of <1% at day 15) in the POP trial, we performed a Cochran-Armitage test for trend using the three tertiles of *CCNE1* mRNA expression. We also performed an ANCOVA of the change from baseline in ln Ki67 in the palbocicib arm across the three *CCNE1*

mRNA tertiles. Analyses were carried out based on a 2-sided significance level of 0.05.

Molecular Subtype Classification

Only HR+ patients comprised the PALOMA-3 cohort, with no large diverse reference tumor sets profiled with EdgeSeq Oncology platform, limiting classification with PAM50, where the subtype is determined relative to a baseline of heterogeneous tumors.[23] The Absolute Intrinsic Molecular Subtyping (AIMS) single sample predictor algorithm was thus applied to assign subtypes through a set of binary rules that compare expression measurements for pairs of genes from a single patient (additional details in **Supplement**).[24]

Exploratory Unbiased Discovery Statistical Analysis

A data-driven exploratory unbiased discovery analysis was performed for gene expression biomarkers suggestive of greater efficacy from adding palbociclib to fulvestrant. Using Cox regression analysis, the search was initially narrowed down to genes whose expression as a continuous variable was significantly associated with treatment effect within the palbociclib arm (P<0.01); a cross-arm interaction analysis was then performed on these genes. To further investigate the underlying biological processes mediating palbociclib plus fulvestrant response, all genes were sorted by the coefficient values of the expression-treatment interaction from continuous analysis followed by Gene Set Enrichment Analyses (GSEA; additional details in **Supplement**).

Results

Gene Expression Analysis of PALOMA-3 Tumor Tissue

In total, 462 tumor samples from 521 patients were analyzed by HTG EdgeSeq for gene expression (**Fig 1**), with 302 tumor samples evaluable for analysis; 159 archival primary samples (53%) and 142 metastatic biopsies (47%). Of the evaluable samples, 194 (64%) were from the palbociclib arm (102 primary, 92 metastatic samples) and 108 (36%) were from the placebo arm (57 primary, 50 metastatic samples). Baseline clinical and pathologic characteristics **(Table 1)** and progression free survival **(Supplementary Fig 2)** were similar between the biomarker and overall PALOMA-3 populations. Gene expression of *ESR1* mRNA and progesterone receptor (PR) mRNA showed high correlation with protein expression of the estrogen receptor (ER; Spearman R=0.54; *P*<0.0001) and PR (Spearman R=0.77; *P*<0.0001) assessed centrally by immunohistochemistry H score at the same time point (**Supplementary Fig 3**). ER and PR H-scores were most correlated with their own transcript across all genes in the EdgeSeq Oncology panel.

Our primary hypothesis was that the expression of CDK4/6-RB1 axis genes would have an impact on the addition of palbociclib to fulvestrant. Expression of *CDK4*, *CDK6,* and *CCND1* mRNA were not predictive of palbociclib efficacy (**Fig 2 and Supplementary Fig 4A**). Similarly, although *ESR1* mRNA expression was prognostic with low expression associated with shorter PFS in both treatment arms, the efficacy of palbociclib did not differ significantly by *ESR1* mRNA expression level (**Supplementary Fig 4B**).

CCNE1 mRNA Expression Is Predictive of Palbociclib Efficacy When Assessed in Metastatic Tissues

In line with prior preclinical evidence, lower *CCNE1* mRNA (cyclin E1 mRNA) expression was associated with improved efficacy from palbociclib (**Fig 3A**). Dividing samples by median *CCNE1* mRNA expression value, the median PFS of patients with high CCNE1 mRNA levels was 7.6 months with palbociclib plus fulvestrant and 4.0 months with placebo plus fulvestrant (hazard ratio [HR], 0.85; 95% CI, 0.58−1.26), while the median PFS of those with lower *CCNE1* mRNA levels was 14.1 months with palbociclib plus fulvestrant and 4.8 months with placebo plus fulvestrant (HR, 0.32; 95% CI, 0.20−0.50), with a significant interaction between treatment effect and *CCNE1* mRNA expression (unadjusted *P*=0.00238; false discovery adjusted *P*=0.0238; **Fig 2 and Fig 3A**). The interaction with *CCNE1* mRNA remained significant after accounting for baseline clinicopathological characteristics, including recurrence type, tumor tissue collection site, baseline Eastern Cooperative Oncology Group performance status, visceral metastases, prior chemotherapy, prior aromatase inhibitor, and prior tamoxifen (*P*=0.00167).

The STEPP analysis further supported a significant interaction between *CCNE1* mRNA expression and the relative treatment effect based on the HR across *CCNE1* mRNA expression levels (supremum HR *P*=0.0008; **Fig 3B**). STEPP analysis of absolute treatment effect based on 6-month PFS across *CCNE1* mRNA expression levels consistently provided evidence of

heterogeneous treatment effects related to *CCNE1* mRNA expression (*P*=0.016; **Fig 3C**).

The source of tumor biopsy had an impact on the association between *CCNE1* mRNA expression and palbociclib efficacy. *CCNE1* mRNA was highly predictive in metastatic biopsies (N=142; interaction *P*=0.00047), but marginal in primary biopsies (N=159; interaction *P*=0.09). Interestingly, primary and metastatic biopsies expressed similar levels of *CCNE1* mRNA at baseline (**Supplementary Table 1**; *P*=0.57), suggesting that a more contemporaneous assessment of gene expression may explain the improved prediction power of assessment in metastatic biopsies. Tumors with documented sensitivity to prior hormone therapy tended to have lower *CCNE1* mRNA expression levels (**Supplementary Table 1**; *P*=0.0032). Overall, 54% of primary and 52% of metastatic tumor samples were supplied by slides, while 45% of primary and 47% of metastatic samples were supplied by blocks. As anticipated, both ER and PR immunohistochemistry levels were significantly higher in blocks than slides (*P*=0.00016 and *P*=0.004, respectively). *CCNE1* mRNA levels were not affected by block versus slide tissue type analyzed (slide versus block *P*=0.085).

Independent Validation of high CCNE1 mRNA as a Marker of Palbociclib Resistance in the POP Trial

In the POP trial, high *CCNE1* mRNA expression was associated with lower absolute anti-proliferative response to palbociclib (high *CCNE1* mRNA, 36% versus, intermediate *CCNE1* mRNA, 79%, and low *CCNE1* mRNA, 80%;

P=0.005; **Figure 4A**). High *CCNE1* mRNA expression was also associated with a reduced geometric mean change in Ki67 with palbociclib treatment (high *CCNE1* mRNA, –49% versus, intermediate *CCNE1* mRNA, –82%, and low *CCNE1* mRNA, –82%; *P*=0.015; **Figure 4B**)

Intrinsic Subtypes and Efficacy of Palbociclib

Of tumors with gene expression data, 133 (44%) were luminal A, 93 (31%) luminal B, and 76 (25%) non-luminal (5 basal-like, 63 HER2-enriched, 8 normal-like; **Fig 5A**). In patients with luminal A tumors, median PFS was 16.6 months with palbociclib plus fulvestrant and 4.8 months with placebo plus fulvestrant (HR, 0.41; 95% CI, 0.25−0.66), while in patients with luminal B tumors, median PFS was 9.2 months with palbociclib plus fulvestrant and 3.5 months with placebo plus fulvestrant (HR, 0.64; 95% CI, 0.38−1.09; **Fig 5B**). There was no significant interaction between luminal A versus luminal B and treatment effect of palbociclib (*P*=0.20). Patients with non-luminal HR+ tumors had a median PFS of 9.5 months with palbociclib plus fulvestrant and 5.5 months with placebo plus fulvestrant (HR, 0.58; 95% CI, 0.34−0.99; **Supplementary Fig 5)**.

CCNE1 mRNA expression appeared highest in the few basal-like subtype tumors, followed by luminal B (across all subtypes, *P*<0.0001; **Fig 5C; Supplementary Table 1)**. The *CCNE1* mRNA expression level of luminal A tumors was significantly lower than luminal B (*P*<0.0001, Mann-Whitney test). In an exploratory subtype-specific analysis, the effect of *CCNE1* mRNA as a continuous variable on improvement in PFS from adding palbociclib to

fulvestrant was apparent in both luminal B and non-luminal subtypes (interaction *P*=0.03 and 0.007 respectively), but not luminal A (interaction *P*=0.49).

Discovery Analysis of Genes and Pathways Associated With Efficacy of Palbociclib

After correcting for multiple hypothesis testing, 20 candidate genes were identified with FDR<0.1, including 11 relative resistance markers and 9 relative sensitivity markers (**Supplementary Table 2**). The unbiased search independently identified high *CCNE1* mRNA expression as the second most significant gene panel-wide linked to lack of efficacy from the addition of palbociclib to fulvestrant. The only more significant gene was Neuromedin U, which had previously been implicated in drug resistant HER2+ breast cancer by driving increased levels of TGFβ1[25] and expanding the cancer stem cell phenotype.[26] We noted that higher *CDKN2D* mRNA (p19) expression level was also associated with reduced efficacy with palbociclib combination, as well as possibly *CDKN2C* mRNA (p18) expression (**Supplementary Fig 6**). Both genes belong to the INK4 family, which regulates kinase activities of CDK4/6.

Among the 50 hallmark gene sets from the Molecular Signature Database (MSigDB),[27] E2F targets (regulon) demonstrated the most significant association with lack of improvement in PFS from palbociclib combination (NES=−2.36; FDR<0.001), followed by other cell cycle-related pathways, including Myc regulon, mechanistic target of rapamycin complex 1 (mTORC1)

signaling, G2/M checkpoint, DNA repair, and mitotic spindle (**Supplementary Fig 7**). These results from the unbiased search support and confirm the critical role of E2F transcriptional activity, and *CCNE1* mRNA in particular, in determining the relative clinical efficacy from the addition of palbociclib to fulvestrant.

Discussion

We present a gene expression analysis of breast cancer tissues in the PALOMA-3 trial and identified the first predictive marker of efficacy from CDK4/6 inhibition, with low expression of *CCNE1* mRNA associated with greater efficacy of palbociclib. Low E2F transcriptional activity was associated with relative improved efficacy, with *CCNE1* mRNA appearing to be the most significant predictive biomarker gene within the regulon. In contrast, we found no evidence that either ER expression or luminal subtype associated with efficacy of palbociclib.

Previous studies have shown discordance between primary and metastatic biopsies in genetic profiles [28]and *HER2*[29] and ER and PR status.[30] PALOMA-3 was one of the first phase 3 trials to mandate the provision of recurrent disease biopsies, unless patients had bone-only disease or relapsed in the first 3 years of adjuvant endocrine therapy and could provide tissue from the primary tumor. Our results demonstrate that the collection of tissue temporally closer to the time of trial entry greatly facilitates the identification of predictive markers and could be considered a norm in all phase 3 advanced breast cancer trials.

Cyclin E1 canonically activates CDK2,[31] and our findings build on a wealth of preclinical and early clinical evidence that cyclin E1 expression is a marker of resistance to CDK4/6 inhibition.[13,14,17,32] High *CCNE1* mRNA expression correlates with resistance to therapy in cell line models of breast and ovarian cancer.[13,14] In triple negative cancer cell lines with resistance to CDK4/6 inhibition, *CCNE1* mRNA expression remains high directly after mitosis, bypassing the restriction point at which CDK4/6 has traditionally been viewed as being required for G1 transition by activating CDK2.[33] High expression of low molecular weight cyclin E1 assessed by immunohistochemistry was associated with poor outcomes, including in a cohort of patients treated with palbociclib in routine clinical practice,[34 35] It will be interesting in future research to explore the relative importance of assessing *CCNE1* mRNA *versus* cyclin E1 protein, and the post-translational modification of cyclin E1.

We independently validated high *CCNE1* mRNA as a resistance biomarker in the POP trial. The POP trial assessed gene expression with Affymetrix arrays, demonstrating the potential of *CCNE1* mRNA expression to be predictive across different platforms.Limited biomarker work from other preoperative palbociclib trials also supports high *CCNE1* mRNA as a biomarker identifying ER+ and HER2− cancers that are resistant to CDK4/6 inhibition.[17] These data suggest that *CCNE1* mRNA expression may be associated with the benefit from palbociclib in early stage breast cancer. Future research will be required to assess to the impact endocrine therapy resistance on *CCNE1* mRNA expression, and to identify the cellular processes that allow *CCNE1* mRNA

expression to become decoupled from the requirement for prior CDK4/6 activation.

Overall, both luminal A and B breast cancer subtypes derived benefit from adding palbociclib to fulvestrant. Our data add to recent data that the small subset of non-luminal ER+ breast cancers may be a distinct and separate entity characterized by disparate treatment responsiveness.[16,36] Non-luminal ER+ breast cancers may derive less benefit from endocrine therapy compared with luminal breast cancers.[37] Our data suggest that CDK4/6 inhibition combination therapy may improve PFS in non-luminal cancers, which were dominated by the HER2-enriched phenotype (**Supplementary Fig 5**). In unsupervised exploratory analysis of genes and pathways associated with palbociclib efficacy, we identified a potential association between high expression levels of p19-*CDKN2D* mRNA, as well as p18-*CDKN2C* mRNA, and reduced efficacy from palbociclib. This observation is exploratory and requires validation, but possibly identifies that high levels of an intrinsic CDK4/6 inhibitor may predict lower response to palbociclib. mTORC1 signaling was associated with reduced response to palbociclib addition, consistent with previous pre-clincial studies.[38]

Our study has important limitations. The PALOMA-3 backbone endocrine therapy was fulvestrant,[4,7] and it is unknown if the biomarkers identified in this study are relevant to aromatase inhibitor-CDK4/6 combinations. Our analysis was not conducted with a clinical assay and should not be used to select patients for therapy without further validation of the results and validation of

clinical grade diagnostics. In addition, it is not clear from this analysis if measuring *CCNE1* mRNA would be useful to make decisions for individual patients, as the subgroup with high *CCNE1 mRNA* expression potentially derived some PFS improvement with the addition of palbociclib to fulvestrant, albeit to a substantially less degree than cancers of low expression.

In this correlative analysis of the PALOMA-3 trial, we have identified the first potential biomarker that was predictive of the efficacy of palbociclib. Our findings reinforce CDK2 as a key bypass kinase of CDK4/6 inhibition, identifying potential therapeutic approaches to prevent early progression on CDK4/6 inhibitors. The effect of *CCNE1* mRNA expression was most evident in metastatic biopsies, which are more contemporaneous to treatment than archival primary biopsies, demonstrating the importance of collecting metastatic/recurrent tissue biopsies in clinical studies. Further methodologic and clinical validations are warranted to elucidate the role of *CCNE1* mRNA expression as a biomarker pf CDK4/6 inhibitor therapy.

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Table 1. Baseline Clinical and Pathological Characteristics of the Biomarker Subset and Overall Intent-to-Treat Population From PALOMA-3

	Intention-to-Treat Population, n (%)		Biomarker Subset, n (%)	
	FUL+PAL (n=347)	FUL+PBO (n=174)	FUL+PAL (n=194)	FUL+PBO (n=108)
Recurrence type				
Recurrent	278 (80)	146 (84)	160 (82)	90 (83)
Newly diagnosed	67 (19)	25 (14)	32 (16)	15 (14)
Visceral metastasis				
Yes	200 (58)	104 (60)	108 (56)	60 (56)
No	147 (42)	70 (40)	86 (44)	48 (44)
Prior chemotherapy				
Yes	253 (73)	138 (79)	144 (74)	87 (81)
No	94 (27)	36 (21)	50 (26)	21 (19)
Prior AI				
Yes	296 (85)	151 (87)	168 (87)	91 (84)
No	51 (15)	23 (13)	26 (13)	17 (16)
Prior tamoxifen				
Yes	210 (61)	104 (60)	111 (57)	66 (61)
No	137 (39)	70 (40)	83 (43)	42 (39)
Baseline ECOG performance status				
0	206 (59)	116 (67)	110 (57)	70 (65)
1	141 (41)	58 (33)	84 (43)	38 (35)
ER/PgR status				
ER-/PgR+	1 (0)	2 (1)	0 (0)	1 (1)
ER+/PgR-	92 (27)	50 (29)	48 (25)	24 (22)
ER+/PgR+	240 (69)	111 (64)	137 (71)	76 (70)
Menopausal status				
Pre/perimenopausal	72 (21)	36 (21)	32 (16)	21 (19)
Postmenopausal	275 (79)	138 (79)	162 (84)	87 (81)
Documented sensitivity to prior hormone therapy				
Yes	273 (79)	133 (76)	150 (77)	82 (76)
No	74 (21)	41 (24)	44 (23)	26 (24)
Measurable disease Present at baseline				
Yes	267 (77)	138 (79)	143 (74)	83 (77)
No	80 (23)	36 (21)	51 (26)	25 (23)

AI=aromatase inhibitor; ECOG=Eastern Cooperative Oncology Group; ER=estrogen receptor; FUL=fulvestrant; PAL=palbociclib; PBO=placebo; PgR=progesterone receptor.

Figures

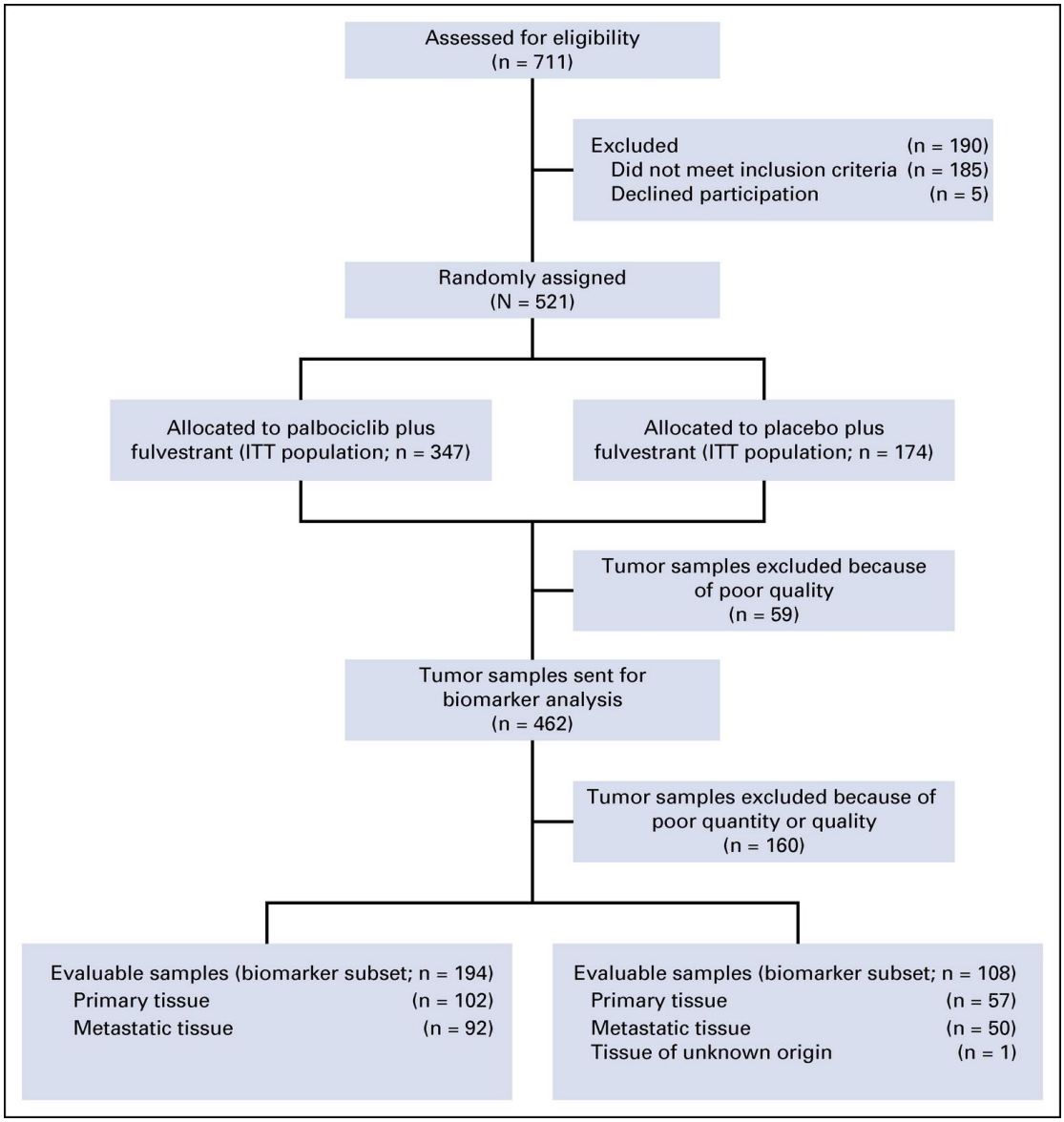

Fig 1. CONSORT diagram of breast cancer tissues analyzed for gene expression. ITT=intent-to-treat.

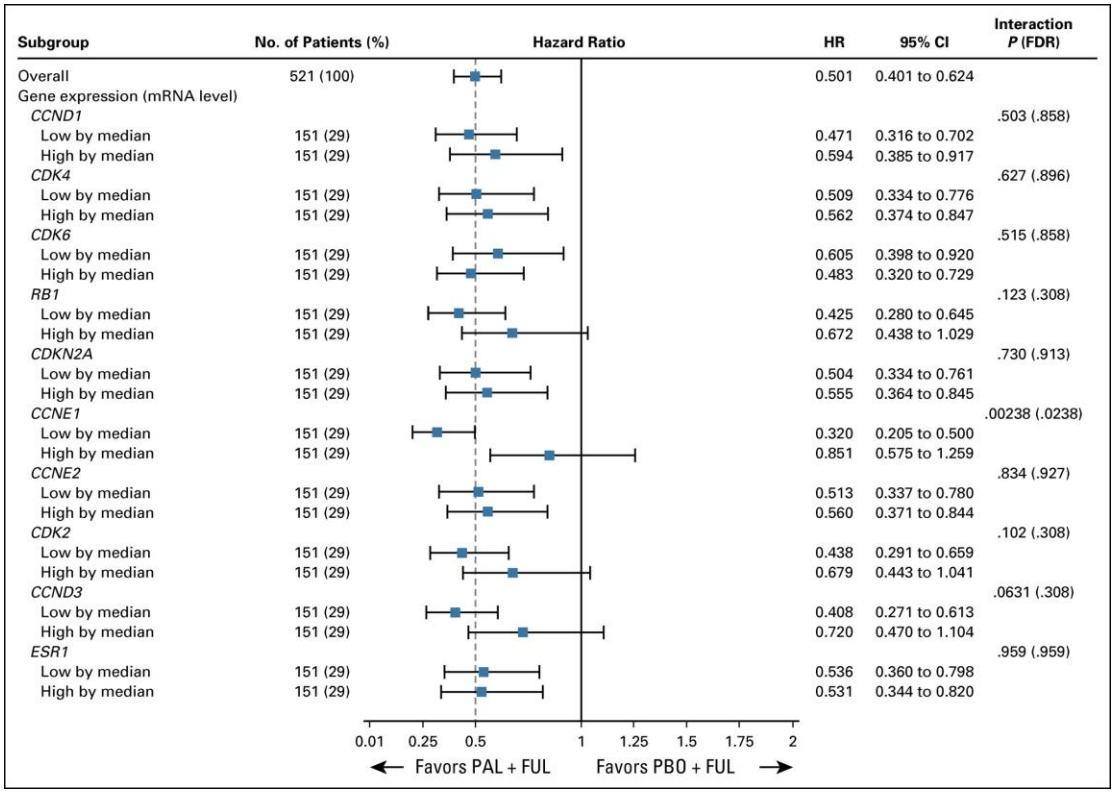

Fig 2. Association of cell cycle pathway gene expression and the efficacy of palbociclib in combination with fulvestrant.

Expression of cell cycle pathway genes, dichotomized by median expression, with HRs for PFS of palbociclib plus fulvestrant versus placebo plus fulvestrant. HRs were derived with a Cox regression model. Interaction *P* value for statistical interaction between gene expression and treatment. *CCND1*=cyclin-D1; *CCND3*=cyclin-D3; *CCNE1*=cyclin-E1; *CCNE2*=cyclin-E2; *CDK2*=cyclin-dependent kinase 2; *CDK4*=cyclin-dependent kinase 4; *CDK6*=cyclin-dependent kinase 6; *CDKN2A*=cyclin-dependent kinase inhibitor 2A; CI=confidence interval; *ESR1*=estrogen receptor 1; FDR=false discovery rate; FUL=fulvestrant; HR=hazard ratio; mRNA=messenger ribonucleic acid; PAL=palbociclib; PBO=placebo; PFS=progression-free survival; *RB1*=retinoblastoma 1.

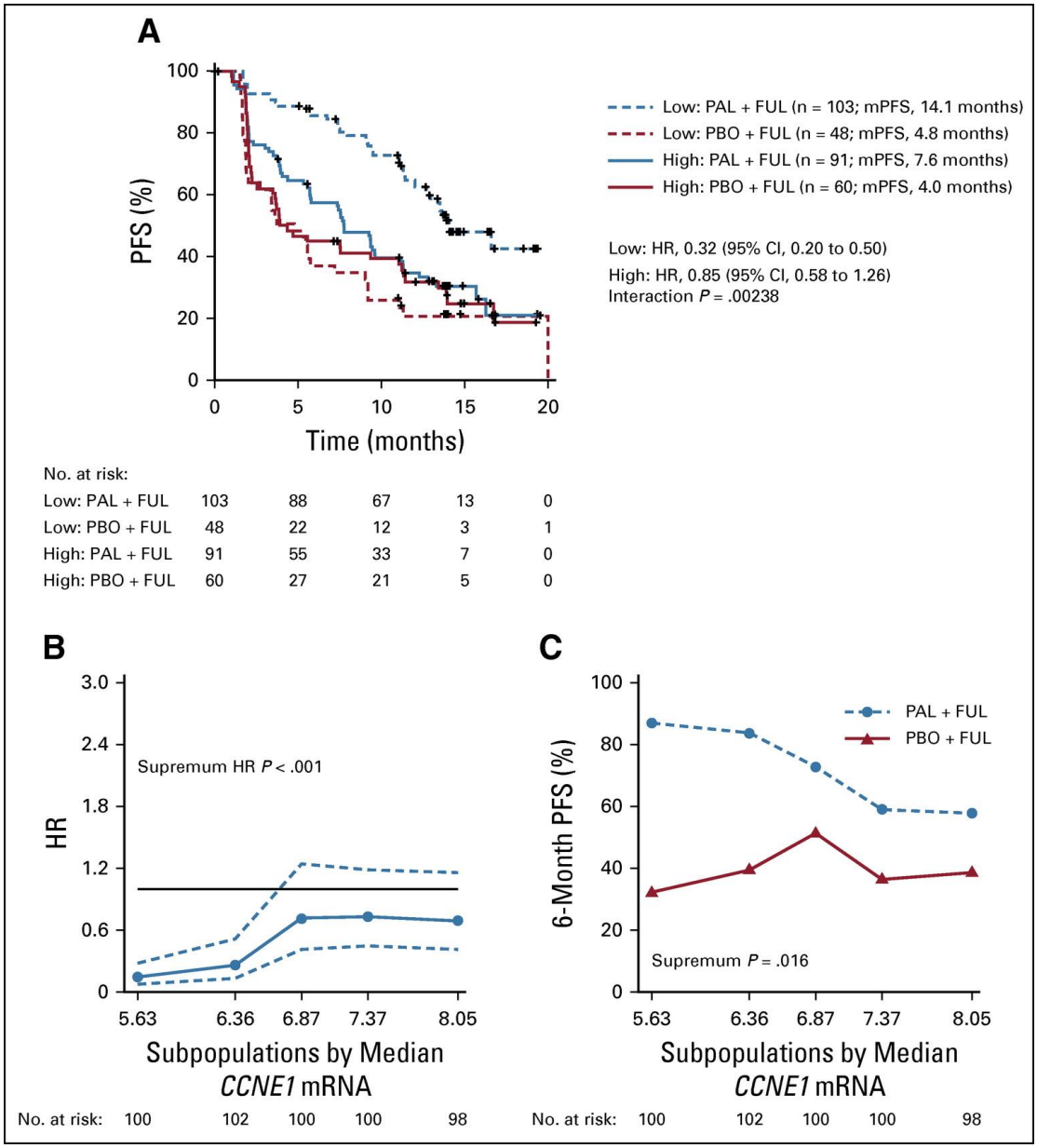

Fig 3. Association between *CCNE1* mRNA expression and palbociclib efficacy.

(A) PFS in tumors with low or high *CCNE1* mRNA expression by median. HRs were derived with a Cox regression model. *P* value from the interaction test between gene expression and treatment. **(B)** STEPP analysis of *CCNE1* mRNA expression as measured by HR (palbociclib plus fulvestrant versus placebo plus fulvestrant). The x-axis represents the median *CCNE1* mRNA expression for patients in each of the overlapping subpopulations. The

dashed lines represent the corresponding 95% pointwise confidence intervals. The horizontal solid black line indicates a reference HR of 1 with HR<1 favoring palbociclib plus fulvestrant combination. **(C)** STEPP analysis of *CCNE1* mRNA expression as measured by 6-month PFS rates.

CCNE1=cyclin E1; CI=confidence interval; FUL=fulvestrant; HR=hazard ratio; mPFS=median PFS; PAL=palbociclib; PBO=placebo; PFS=progression-free survival; STEPP=subpopulation treatment effect pattern plot.

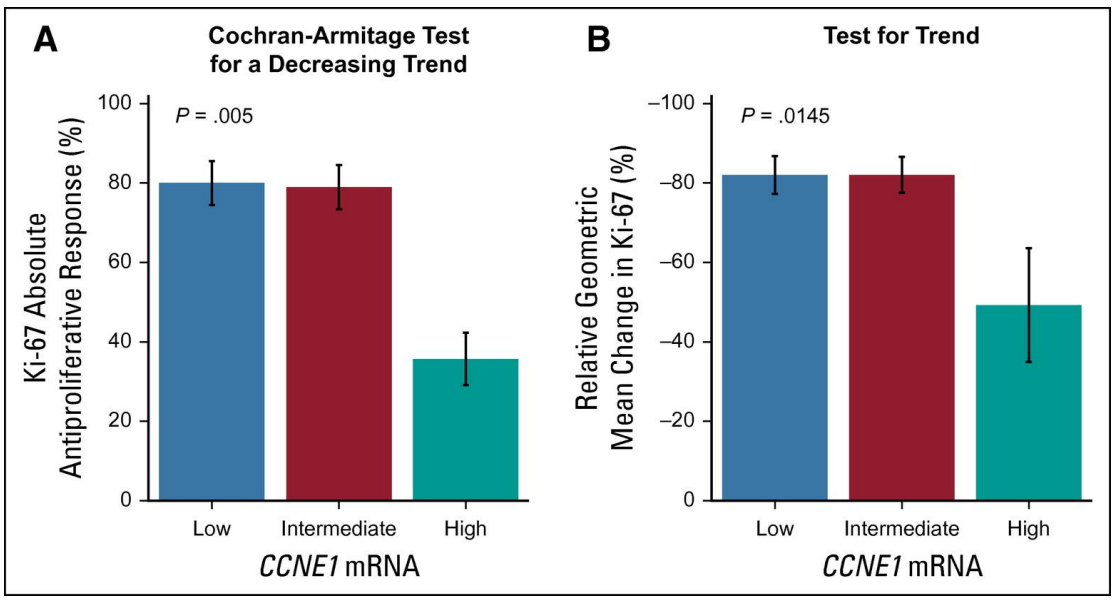

Fig 4. Independent validation of high *CCNE1* mRNA as a marker of palbociclib resistance in the POP trial. **(A)** Anti-proliferative response by *CCNE1* expression tertile. **(B)** Geometric mean change in Ki67 expression with palbociclib treatment by *CCNE1* expression tertile.*CCNE1*=cyclin E1.

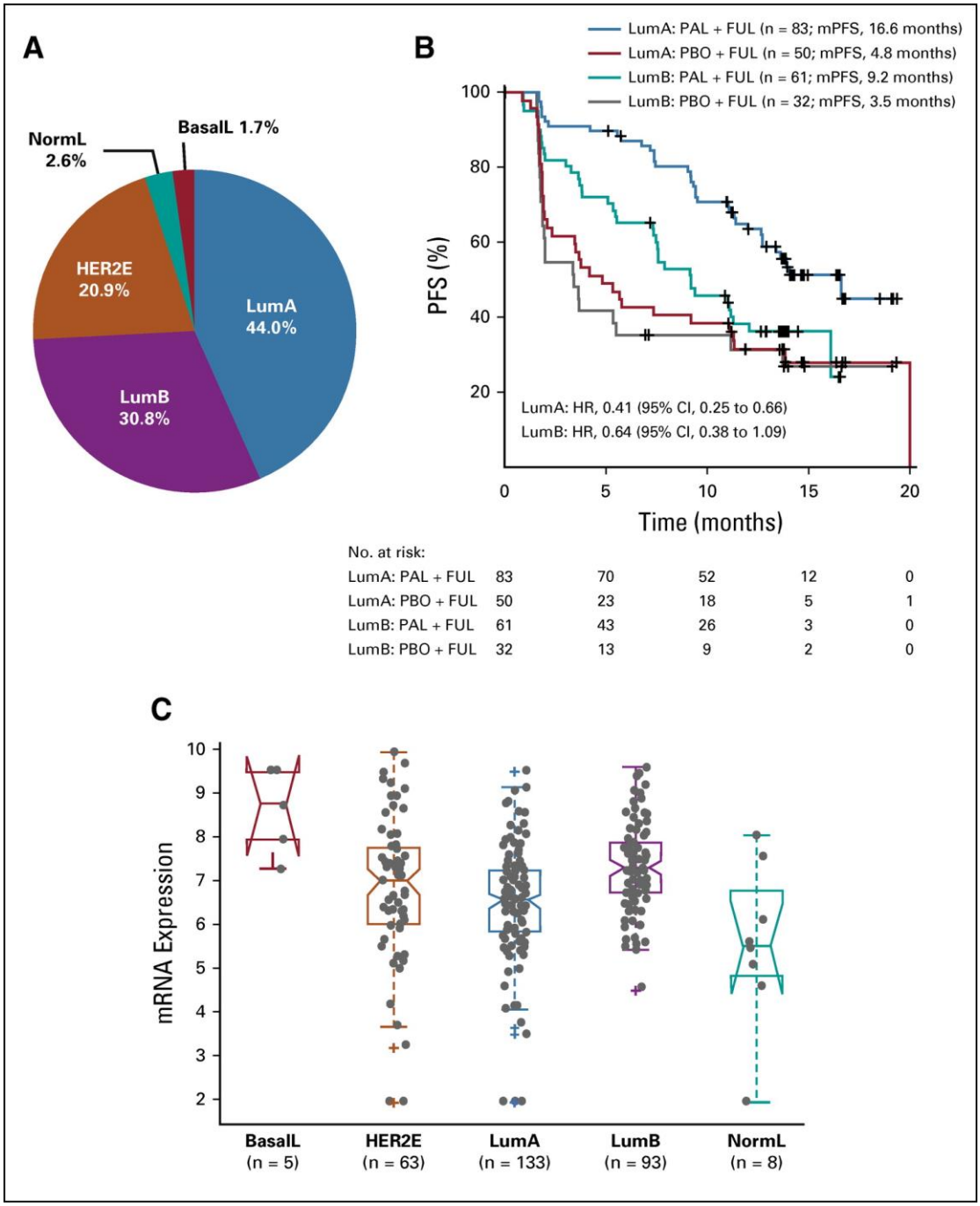

Fig 5. Intrinsic molecular subtype and efficacy of palbociclib. **(A)** Intrinsic subtype distribution of tumors in PALOMA-3. **(B)** Progression-free survival in luminal A and B tumors. **(C)** *CCNE1* mRNA expression by intrinsic molecular subtype.

BasalL=basal-like; *CCNE1*=cyclin E1; FUL=fulvestrant; Her2E=human epidermal growth factor receptor 2-enriched; HR=hazard ratio; LumA=luminal

A; LumB=luminal B; mPFS=median progression-free survival; mRNA=messenger ribonucleic acid; NormL=normal-like; PAL=palbociclib; PBO=placebo; PFS=progression-free survival.