Plasma Thymidine Kinase Activity as a Biomarker in Patients with Luminal Metastatic Breast Cancer Treated with Palbociclib within the TREnd Trial

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ABSTRACT

Purpose: Thymidine kinase 1 (TK1) is downstream to the CDK4/6 pathway, and TK activity (TKa) measured in blood is a dynamic marker of outcome in patients with advanced breast cancer (ABC). This study explores TK1 as a biomarker of palbociclib response, both *in vitro* and in patients with ABC.

Experimental Design: Modulation of TK1 levels and activity by palbociclib were studied in seven estrogen receptor–positive breast cancer cell lines: sensitive (PDS) and with palbociclib acquired resistance (PDR). TKa was assayed in plasma obtained at baseline (T0), after one cycle (T1), and at disease progression on palbociclib (T2) in patients enrolled in the "To Reverse ENDocrine Resistance" (TREnd) trial ($n = 46$).

Results: Among E2F-dependent genes, TK1 was significantly downregulated after short-term palbociclib. Early TKa reduction by palbociclib occurred in PDS but not in PDR cells. In patients, median TKa (mTKa) at T0 was 75 DiviTum units per liter (Du/L), with baseline TKa not proving prognostic. At T1, mTKa decreased to 35 Du/L, with a minority of patients ($n = 8$) showing an increase—correlating with a worse outcome than those with decreased/stable TKa ($n = 33$; mPFS 3.0 vs 9.0 months; $P = 0.002$). At T2, mTKa was 251 Du/L; patients with TKa above the median had worse outcomes on post-study treatment compared with those with lower TKa (2.9 vs 8.7 months; $P = 0.05$).

Conclusions: TK is a dynamic marker of resistance to palbociclib which may lead to early identification of patients in whom treatment escalation may be feasible. In addition, TKa may stratify prognosis in patients with acquired resistance to palbociclib.

Introduction

In the past decade, cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors represent arguably the most significant contribution to the management of advanced hormone receptor–positive, HER2-negative breast cancer. CDK4/6 inhibitors, administered in combination with endocrine therapy, have been adopted into widespread clinical practice following landmark studies that describe superior progression-free survival in the first- (1–3) and later-line setting (4–6). CDK4/6 inhibitors lead to a reduction in phosphorylation of the Rb protein, which in turn decreases E2F activity, eventually leading to cell-cycle arrest. Thymidine kinase-1 (TK1) plays a pivotal role in DNA replication (7), is a well-documented marker of cancer proliferation in breast cancer, and can be measured in plasma or serum samples as a marker of tumor proliferation (8–10). TK1 synthesis is regulated by the E2F pathway, and as such represents a potential marker of CDK4/6 inhibitor activity, due to the common convergence on this pathway. Circulating levels of TK1 activity (TKa) have previously been shown to be prognostic in patients with metastatic breast cancer treated with endocrine therapy, both when measured at baseline and during treatment (11, 12).

Despite positive clinical results, acquired resistance to CDK4/6 inhibitor agents over time is considered inevitable in all patients who initially derive a positive clinical response, with a smaller subset expressing *de novo* resistance. The mechanisms underpinning primary and acquired resistance to CDK4/6 inhibitors is not yet comprehensively understood, but are widely acknowledged to be multifactorial (13). It is therefore perhaps unsurprising that the identification of definitive prognostic and predictive biomarkers in this field has so far remained elusive (14–17). In a retrospective analysis of the single-arm phase II NeoPalAna trial (18), TKa has previously been shown to decrease in patients treated with neoadjuvant palbociclib plus endocrine therapy for 15 days, which was attributed as reflecting pharmacodynamic change on palbociclib treatment (19). The prognostic value of TKa changes during treatment with palbociclib, as well as the dynamics of TKa changes on palbociclib-containing treatments are not yet comprehensively defined.

On this basis, we conducted preclinical studies in palbociclib-sensitive (PDS) and palbociclib-resistant (PDR) breast cancer cell lines to examine TKa in the context of CDK4/6 inhibition. For its clinical validation of as a biomarker, we performed retrospective analyses of baseline and on-treatment levels of plasma TKa (pTKa)

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Translational Relevance

Despite their proven activity, *de novo* resistance to CDK4/6 inhibitors is seen in at least 10%–15% of patients with luminal metastatic breast cancer, posing a significant clinical problem. Even patients with initial response inevitably acquire adaptive resistance to CDK4/6 inhibitors, and therapeutic choices for these patients are currently empirical. There is a lack of biomarkers to either identify patients with *de novo* resistance or to stratify the prognosis of those with acquired resistance. This report shows that thymidine kinase activity (TKa) measured in plasma may be a biomarker of palbociclib response and also of prognosis at the point of acquired palbociclib resistance. This is supported by thymidine kinase being an E2F-dependent gene, which in preclinical models is negatively modulated by palbociclib in sensitive but not in resistant models. TKa can be monitored noninvasively throughout the treatment course, with a potential role as a dynamic biomarker for early identification of resistance and potentially adapting treatment strategies in clinical trials.

as correlated to clinical outcome in patients receiving palbociclib as a part of the phase II "To Reverse ENDocrine Resistance" (TREnd) trial (NCT02549430). The primary aims of these analyses were to test pTKa as an early biomarker of primary resistance to palbociclib, as well as investigating the potential clinical value of pTKa in predicting outcome on the next-line of therapy received after exiting TREnd.

Materials and Methods

Cell lines, cell culture, and reagents

Cells were cultured at 37°C and 5% CO_2. T47D, ZR75-1, MCF7, MDAMB361, and BT474 cells were grown in DMEM with 4.5 g/glucose and L-glutamine (Lonza) supplemented with 10% heat-inactivated FBS (HyClone) and 10,000 U penicillin and 10 mg streptomycin/mL solution (Sigma-Aldrich). MCF7 estrogen deprivation–resistant (EDR) and MCF7 tamoxifen-resistant (TamR) cells were grown in DMEM with 4.5 g/glucose and without L-glutamine and phenol red (Lonza) supplemented with 10% charcoal-stripped FBS (GIBCO), and penicillin/streptomycin. MCF7 TamR cell medium was supplemented with 100 nmol/L final concentration of (Z)-4-Hydroxytamoxifen (Sigma-Aldrich) dissolved in 100% ethanol.

Palbociclib-resistant derivatives were generated as described previously (20) and maintained in their original media with the addition of palbociclib 1 μmol/L. The starting treatment concentration (STC) was defined as the initial concentration used to induce drug resistance in the different cell lines (50 nmol/L for T47D, ZR75-1, MCF7 EDR, and MCF7 TamR cell lines and 350 nmol/L for MCF7, MDA MB 361, and BT474 cell lines; ref. 20). Palbociclib (provided by Pfizer) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

All cell lines and their PDR derivatives have been authenticated by short tandem repeat DNA profiling analysis. This analysis was performed by BMR Genomics in January 2016. Absence of *Mycoplasma* contamination was verified with the MycoAlert Mycoplasma Detection Kit (Lonza) in 2017. Cells used in these experiments had been thawed and maintained in culture for approximately 3 months. T47D and ZR75-1 cell lines were obtained from Dr Livia Malorni (CNR Avellino, Italy) in 2013. MCF7 parental cells, MCF7 EDR, and MCF7 TamR were described previously (21). MDAMB361 (ECACC catalog no. 92020423, RRID:CVCL_0620) were purchased from Sigma-Aldrich in 2015 and BT474 (ICLC catalog no. HTL00008, RRID: CVCL_0179) from Interlab Cell Line Collection in 2013.

Gene expression analysis

The Direct-zol RNA MiniPrep Kit (Zymo Research) was used to isolate RNA, according to the manufacturer's instructions. RNA was isolated from PDS cells treated with 0.01% DMSO as a control, or with palbociclib at the STC for 3 days, and from PDR cells grown in their individual media supplemented with palbociclib 1 μmol/L. Gene expression profiles were obtained as described previously (20). The top 15 most differentially expressed E2F target genes between PDR and PDS-treated cells were selected as most the variable (i.e., by SD, top 25%) genes included in the "Hallmark_E2F_/targets" gene list (http://software.broadinstitute.org/gsea/msigdb/cards/HALLMARK_E2F_TARGETS) that showed an absolute value of average normalized expression with respect to corresponding untreated PDS >0.2. Differential expression between $\log_2$-ratio expression values was tested by Wilcoxon–Mann-Whitney (WMW) test.

TKa in cell lysates

Cell lysates for TKa were prepared according to a standard protocol from Biovica International. Briefly, 150,000 cells/well were plated in 6-well plates in triplicate in their full medium. After 24 hours, treatments consisting of palbociclib 50 nmol/L, 350 nmol/L, and 1 μmol/L and 0.01% DMS0 (vehicle) were added. After 3 days of treatment, cell extracts were prepared by scraping cells into 1 mL of ice-cold RB Lysis Buffer (Biovica International). Cell extracts were transferred to Eppendorf tubes and snap frozen at −80°C until shipment from Italy to Biovica laboratories in Sweden, wherein the cell extracts were thawed, spun for 10 minutes, and supernatants collected. Cell extract samples were analyzed using the DiviTum assay (see below) without any knowledge of cell line or treatment identifiers. TKa was normalized to total protein concentration for each sample, assessed with BCA Protein Assay (Thermo Scientific Pierce).

Two biological replicates for each PDS or PDR cell line were performed. Values were normalized against TKa in the presence of DMSO and represented means of the two biological replicates ± SEM. Two-way ANOVA with Dunnett multiple comparisons test was performed with GraphPad Prism version 7.03 and $P < 0.05$ was considered significant.

Proliferation assays

A total of 3,000 cells/well of PDS or PDR cell lines were seeded in 96-well plates in triplicate in their full medium. After 24 hours, cell lines were treated with palbociclib 1 μmol/L, palbociclib 350 nmol/L, or 0.01% DMS0 (vehicle) for 9 days. Media were replaced every 72 hours. Cells were fixed with 4% glutaraldehyde (Sigma-Aldrich) and stained with 0.05% methylene blue (Sigma-Aldrich). The dye was subsequently extracted with 3% HCl (Carlo Erba) and absorbance measured at 655 nm. Three biological replicates for each PDS or PDR cell line were performed. Mean value absorbance of all experiments ± SEM was plotted. Two-way ANOVA with Dunnett multiple comparisons test was performed with GraphPad Prism version 7.03 and $P < 0.05$ was considered significant.

Clinical study design and patients

The conduct and results of TREnd have been reported in detail elsewhere (22). Briefly, this phase II, open-label, multicentre study randomized postmenopausal women with moderately pretreated estrogen receptor–positive, HER2-negative advanced breast cancer ($N = 115$) to receive either oral palbociclib monotherapy (125 mg daily

for 3 weeks, followed by 1 week off until disease progression or withdrawal from study) or palbociclib at the same dose and regimen, given in combination with the endocrine therapy upon which the patient had progressed in a previous line prior to trial enrollment (oral anastrozole 1 mg/day or letrozole 2.5 mg/day or exemestane 25 mg/day, or intramuscular fulvestrant 500 mg every 4 weeks). Patients were eligible if they had received one or two lines of prior endocrine therapy for metastatic disease, and were permitted to have received a maximum one line of chemotherapy for metastatic disease. A preplanned translational substudy, "c-TREnd," designed to identify potential biomarkers, was run in parallel with TREnd under approval from the institutional review board of each participating center, and conducted in accordance with the Declaration of Helsinki. Separate informed written consent was prospectively obtained from patients joining in this substudy.

Primary endpoints of this analysis were median progression-free survival (mPFS) according to baseline pTKa, and mPFS according to dynamic change of pTKa after completion of one cycle of trial treatment (approximately 4 weeks from baseline). TREnd defined mPFS as the median time from randomization to radiological disease progression or death on study. In addition, pTKa was measured in a number of patients at the point of disease progression on trial, and correlated to median time to treatment failure (mTTF) on subsequent poststudy treatment. For this objective, TTF was defined as the time from disease progression on TREnd, to the point of treatment cessation for any cause on the line of therapy received directly after exiting TREnd. Post-TREnd management strategies were directed by individual physician choice, with subsequent clinical outcomes having been separately published previously (23).

Plasma collection in c-TREnd

From 46 consenting patients enrolled in TREnd, blood samples were collected at baseline prior to starting trial treatment (time point T0; sample $N = 44$), between C1D24 and C2D3 on trial (T1; $N = 43$), and after disease progression on trial, prior to starting a new line of therapy (T2; $N = 35$). Overall, 32 patients had available, valid samples collected at all three time points. Blood samples were processed within 1 hour from withdrawal by centrifugation at 1,600 × *g* for 10 minutes at 4°C, followed by a second centrifugation at 14,000 rpm for 10 minutes at 4°C. Plasma was aliquoted and stored at −80°C until shipment with dry ice to Biovica laboratories. A CONSORT diagram describing plasma collection at designated time points and selection for analysis is presented in **Fig. 1**.

Analysis of pTKa using DiviTum assay

TKa was determined by the DiviTum assay, a refined ELISA-based method, at Biovica laboratories. Analysis was performed with no access to, nor any knowledge of, patient or tumor characteristics. Each sample was diluted 1/10 in a dilution buffer and then incubated with a reaction mixture on the assay microtiter plate. Bromodeoxyuridine (BrdU), a thymidine analogue, is phosphorylated to BrdU monophosphate by the TK present in the sample, then further phosphorylated and incorporated in a DNA strand bound to the bottom of the wells. BrdU incorporation is detected by ELISA technique using an anti-BrdU mAb conjugated to alkaline phosphatase and a chromogenic substrate, producing a yellow reaction product. Absorbance was measured at 405 nm with the reference wavelength of 630 nm after 30 and 60 minutes of incubation. The measured optical density was proportional to the enzymatic TKa of each sample that was expressed as DiviTum units per liter (Du/L), calculated from a standard curve based on calibrators of known activity. The working range of the assay was 20—4,000 Du/L; at 100 Du/L, the coefficient of variation (CV) was <20%. In samples analyzed from c-TREnd, the median CV was 6.1% (mean, 7.4%).

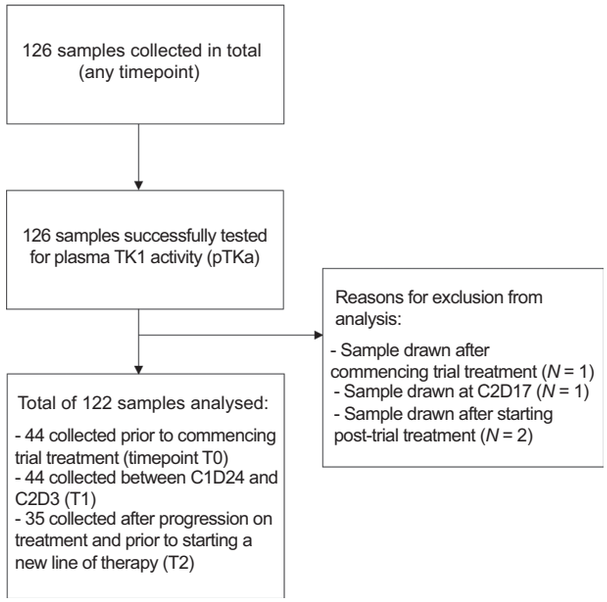

Figure 1.
CONSORT diagram of the study.

Statistical analysis (c-TREnd)

The distributions of PFS and TTF were estimated using the Kaplan–Meier method and compared with the log-rank test. HRs with 95% confidence intervals (CI) were calculated with the Cox proportional hazards model.

DiviTum does not have a predetermined, uniform, absolute cut-off value to define "high" and "low" levels in a single reading at baseline for all patients. In its current use, a unique cutoff is established for each cohort studied with the assay, with previous published analyses (11, 12, 24) employing the cohort's median baseline value as the cut-off point. In measuring dynamic changes in TKa on treatment, the individual baseline reading of each patient serves as the point from which changes are estimated. When comparing matched baseline (T0) and on-treatment (T1) levels, a difference in pTKa value between the two time points was considered meaningful if it was more than 10% of either T1 or T0 (whichever was the greatest). In accordance with previous work by our group (11, 12), we accounted for a coefficient of variation of 10% in estimating pTKa measure uncertainty, this was predetermined ahead of statistical analysis.

Ethics statement

This work was conducted in accordance with Good Clinical Practice standards and the Declaration of Helsinki.

Results

TK1 mRNA levels are modulated by treatment with palbociclib in PDS, but not PDR cells

TK1 synthesis is regulated by the E2F pathway, the target of CDK4/6 inhibitors. To test whether palbociclib affects TK1 expression, in

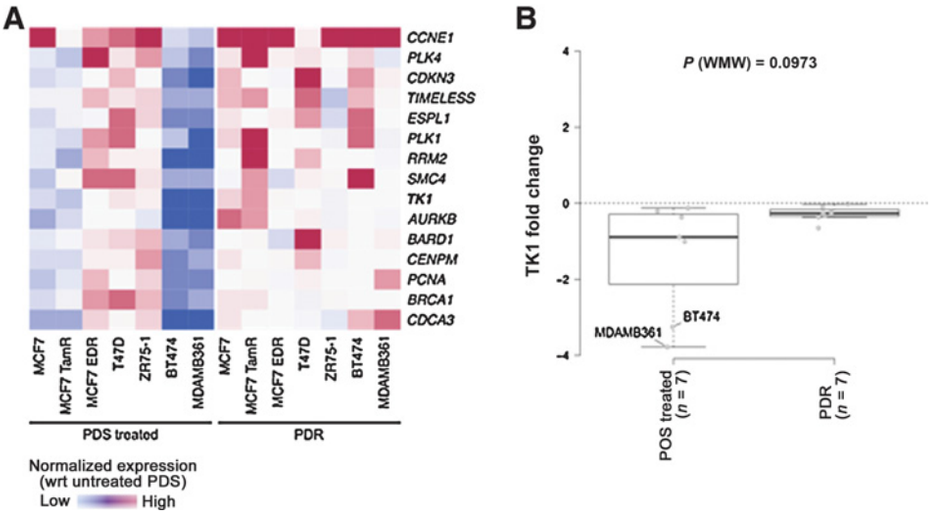

Figure 2.

A, Heatmap showing the top 15 most differentially expressed E2F target genes between PDR- and PDS-treated cells (columns). Values are the $\log_2$ of fold change (FC) of normalized expression in each cell lines with respect to the corresponding untreated PDS. **B,** Box plot showing the distribution of fold change of TK1 expression in PDS-treated and PDR cells. *P* value by WMW test.

hormone receptor–positive cell lines, we analyzed gene expression profiles of PDS cells treated with drug vehicle (untreated PDS) or with palbociclib STC for 3 days (PDS treated) and PDR cells continuously receiving palbociclib 1 μmol/L. Data demonstrated that, among *E2F* target genes, TK1 was one of the most differentially expressed genes between PDR and PDS-treated cells (**Fig. 2A**). In PDS-treated cells compared with control, palbociclib induced a general TK1 under expression, with the HER2-positive models (BT474 and MDAMB361) showing the highest reduction. Interestingly, in PDR models TK1 expression returned to levels comparable with untreated PDS cells despite the presence of the drug (**Fig. 2B**).

TKa may be an early marker of growth inhibition in response to palbociclib in sensitive cells

We investigated the effects of palbociclib on TK1 enzymatic activity both in PDS and PDR cells. We selected a hormone receptor–positive/HER2-negative and a hormone receptor–positive/HER2-positive cell line, MCF7 and BT474, respectively, and analyzed TKa in cell lysates extracted from PDS and PDR cells treated for 3 days with different doses of palbociclib. TKa was significantly reduced in PDS cells treated with palbociclib compared with vehicle ($P < 0.05$), even at the lower dose (50 nmol/L; **Fig. 3A**). In accordance with the expression data, TKa response to palbociclib was more dramatic in the HER2-positive model BT474 as compared with MCF7. As expected, cell proliferation of PDS models was inhibited by treatment with palbociclib (**Fig. 3C**), with a significant reduction of proliferation rate being observed only after 6 days of exposure to the drug. Conversely, no significant alterations in TKa (**Fig. 3B**) or proliferation rate (**Fig. 3D**) were observed in PDR cells, at any dose of palbociclib.

Patient characteristics in c-TREnd

The baseline characteristics of the patients analyzed in c-TREnd were representative of the overall cohort of the original TREnd study (Supplementary Table 1), and were well-balanced across randomized treatment arms. At trial entry, the majority had visceral metastatic disease, had completed one prior line of endocrine therapy in the advanced setting, to which most had a durable response in excess of 6 months. Cumulatively, 65% of patients had received only one line of therapy (endocrine and/or chemotherapy) prior to trial enrollment in the monotherapy arm, compared with 46% in the combination arm. Characteristics according to baseline pTKa levels (high vs. low), and pTKa after one cycle of treatment (rise or no rise) are presented in **Table 1**.

Correlation between baseline pTKa and clinical outcome

The overall baseline median pTKa level prior to commencing treatment on TREnd (T0) was 75 Du/L (range, 20–4,302). When the median value was employed as the defined cutoff between "high" and "low" levels of baseline pTKa, no notable difference in the mPFS was seen between groups ($P = 0.35$). When the top quartile was used as the cut-off point (318 Du/L), those with high pTKa at baseline ($N = 11$) had mPFS of 5.8 months (95% CI, 4.6–NA), versus 8.5 months (95% CI, 3.7–14.2) in the group with low baseline pTKa ($N = 33$), a difference which did not reach statistical significance ($P = 0.12$; **Fig. 4A**). Similarly, when an "optimal cut-off point" (44.8 Du/L) was determined via an outcome-orientated method that provided the value corresponding to the most significant relationship between pTKa and time to progression, baseline pTKa remained statistically insignificant as a prognostic marker ($P = 0.082$). In addition, there was no significant difference between pTKa levels at baseline and CBR on treatment (**Table 1**).

Prognostic role of on-treatment pTKa

The overall median pTKa at timepoint T1 was 35 Du/L (range, 20–2,780). When comparing matched T0 and T1 levels, a difference in pTKa value between the two time points was predetermined as meaningful if it was more than 10% of either T1 or T0 (whichever was the greatest). The majority of patients exhibited either a decrease or no significant change in pTKa at T1 compared with baseline. In this group ($N = 33$), median pTKa at T1 was 21 Du/L (range, 20–2,780) and the mPFS was 9.0 months (95% CI, 5.8–12.0). Interestingly, a small group of patients ($n = 8$; 20%) showed a rise in pTKa at T1 (**Fig. 5**). In this group, median pTKa at T1 was 131 Du/L (range, 30–2,286) and mPFS was significantly shorter when compared with the group with decreased or stable reading (mPFS 3.0 months; 95% CI, 2.7–NA vs. 9 months 95% CI, 5.8–12.0; $P = 0.002$; **Fig. 4B**). In addition, within the group of patients with increasing pTKa during treatment, only two patients (12%) achieved clinical benefit on study, as compared with 73% of patients in the other group ($P = 0.035$; **Table 1**). A greater proportion of patients randomized to the palbociclib monotherapy arm demonstrated a rise in pTKa on treatment (75%) compared with

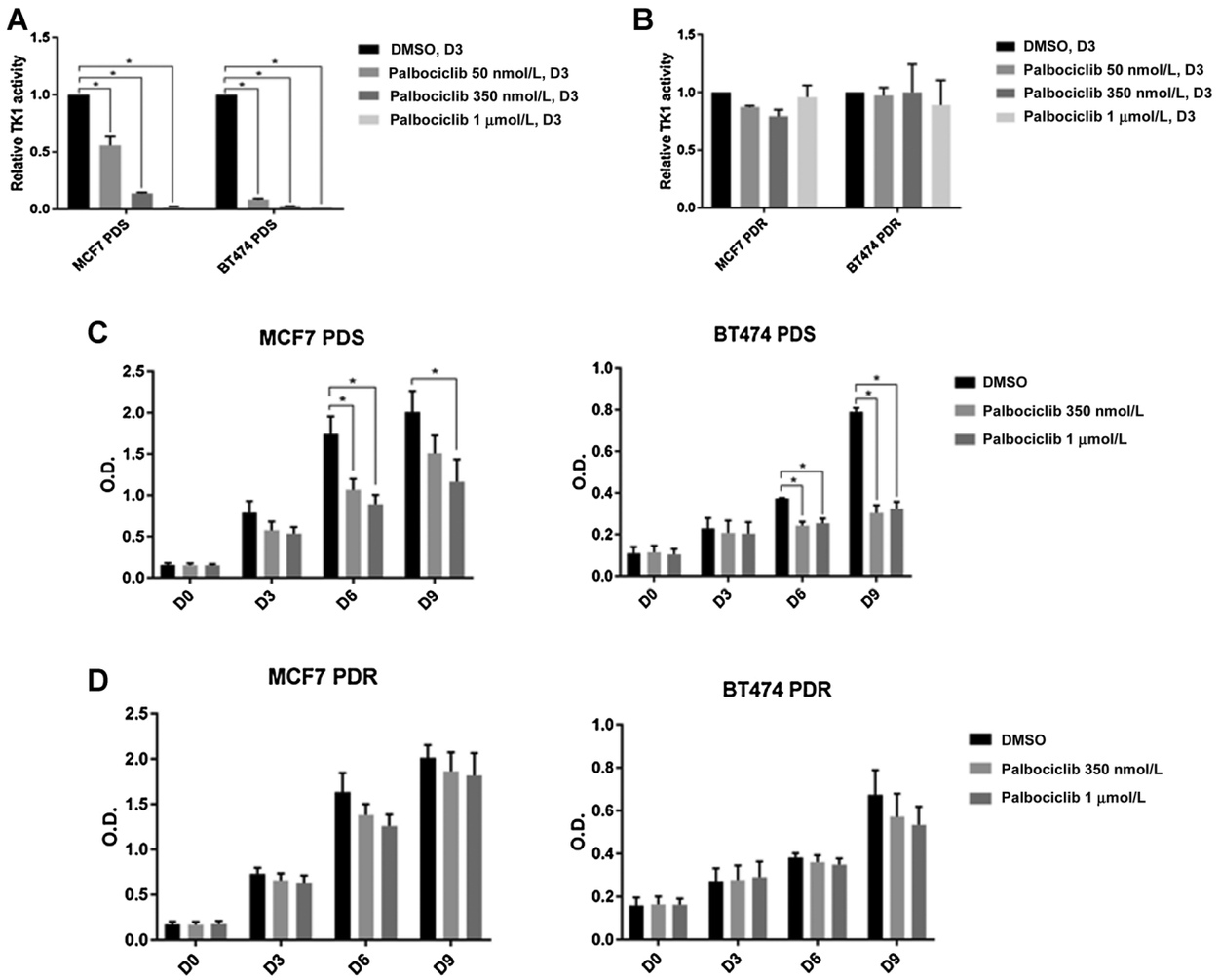

Figure 3.

A, TKa in cell lysates of MCF7 and BT474 PDS and **B**, PDR cells treated with DMSO (vehicle), palbociclib 50 nmol/L, 350 nmol/L, and 1 μmol/L was assessed at day 3 (D3) by DiviTum assay. Values are normalized against TKa in the presence of DMSO and represent means of two biological replicates ± SEM (*, $P < 0.05$, two-way ANOVA with Dunnett multiple comparisons test). **C,** Proliferation rate of MCF7 and BT474 PDS and **D**) PDR cells treated with DMSO (vehicle), palbociclib 350 nmol/L, and 1 μmol/L was assessed at day 0 (D0), 3(D3), 6 (D6), and 9 (D9) by methylene blue assay. Values represent means of three independent experiments ± SEM (*, $P <$ 0.05, two-way ANOVA with Dunnett multiple comparisons test).

those assigned to receive palbociclib plus endocrine therapy (25%; **Table 1**). **Figure 5** illustrates pTKa dynamics according to allocated trial treatment arm.

Correlation between pTKa at time of progression on trial and mTTF on next-line therapy

At the point of disease progression on TREnd (T2), the overall median pTKa was 251 Du/L (range, 20–4,504). Using this median value as a cut-off point, we correlated pTKa values (high vs. low) with outcome on the treatment received immediately after exiting TREnd. One patient had an available T2 sample which was included in the calculation of the median TKa value for this cohort, but due to an absence of outcome data following trial exit, is not represented in the TTF analysis. On next-line treatment, the mTTF of patients with low pTKa at T2 was found to be significantly longer than those patients with a high pTKa at the time of disease progression. The mTTF was 8.7 months (95% CI, 3.3–13.4) in patients with pTKa less than 251 Du/L at T2, versus 2.9 months (95% CI, 2.2–NA) in patients with values in excess of 251 Du/L ($P = 0.05$; **Fig. 4C**).

Discussion

The link between tumor cell proliferation and serum TKa (evaluated by the DiviTum assay) has previously been reported within the neoadjuvant NeoPalAna trial (19). Patients with clinical stage II/III ER-positive, HER2-negative breast cancer received an initial 28 days of anastrozole monotherapy, followed by the addition of palbociclib for four cycles, followed by 3–5 weeks of palbociclib washout ahead of surgery. A small subset of patients continued on palbociclib and anastrozole until surgery, with no washout. Investigators showed that 2 weeks following the initiation of palbociclib, overall median serum TKa significantly reduced from the median baseline. Furthermore, TKa levels rose significantly after palbociclib was withdrawn prior to surgery, but remained suppressed in the small group of patients who

Table 1. Illustrating baseline levels of pTKa and on-treatment changes according to baseline characteristics of studied patient.

Baseline characteristic	Baseline pTKa LOW ($N = 33$)	Baseline pTKa HIGH ($N = 11$)	*P*	On-treatment pTKa NO INCREASE ($N = 33$)	On-treatment pTKa INCREASE ($N = 8$)	*P*
Age (range)	66 (45–80)	59 (39–82)	0.14	64 (44–82)	66 (39–80)	0.91
ECOG performance status						
0	26 (79%)	9 (82%)	0.30	26 (79%)	6 (75%)	0.72
1	7 (21%)	1 (9%)		6 (18%)	2 (25%)	
2	0 (0%)	1 (9%)		1 (3%)	0 (0%)	
Sites of metastases						
Visceral	24 (73%)	8 (73%)	1.00	24 (73%)	6 (75%)	0.47
Bone-only	4 (12%)	1 (9%)		5 (15%)	0 (0%)	
Other nonvisceral	5 (15%)	2 (18%)		4 (12%)	2 (25%)	
Number of prior lines of endocrine therapy received for advanced disease						
One line	22 (67%)	7 (64%)	1.00	20 (61%)	6 (75%)	0.69
Two lines	11 (33%)	4 (36%)		13 (39%)	2 (25%)	
Duration of most recent endocrine therapy received						
≤6 months	4 (12%)	3 (27%)	0.34	6 (18%)	1 (12%)	1.00
>6 months	29 (88%)	8 (73%)		27 (82%)	7 (88%)	
Endocrine therapy most recently received prior to enrolment						
Aromatase inhibitor	21 (64%)	6 (55%)	0.72	19 (58%)	5 (62%)	1.00
Fulvestrant	12 (36%)	5 (45%)		14 (42%)	3 (38%)	
Receipt of prior chemotherapy for advanced disease?						
Yes	6 (18%)	2 (18%)	1.00	5 (15%)	1 (12%)	1.00
No	27 (82%)	9 (82%)		28 (85%)	7 (88%)	
Total number of prior lines of systemic therapy received for advanced disease						
One line	19 (58%)	6 (55%)	1.00	19 (58%)	5 (62%)	0.86
Two lines	11 (33%)	4 (36%)		10 (30%)	3 (38%)	
Three lines	3 (9%)	1 (9%)		4 (12%)	0 (0%)	
Clinical benefit observed on trial?						
Yes	21 (64%)	7 (64%)	1.00	24 (73%)	2 (12%)	0.035
No	12 (36%)	4 (36%)		9 (27%)	6 (88%)	
Trial arm assignment						
Palbociclib alone	17 (52%)	3 (27%)	0.29	12 (36%)	6 (75%)	0.11
Palbociclib plus ET	16 (48%)	8 (73%)		21 (64%)	2 (25%)	

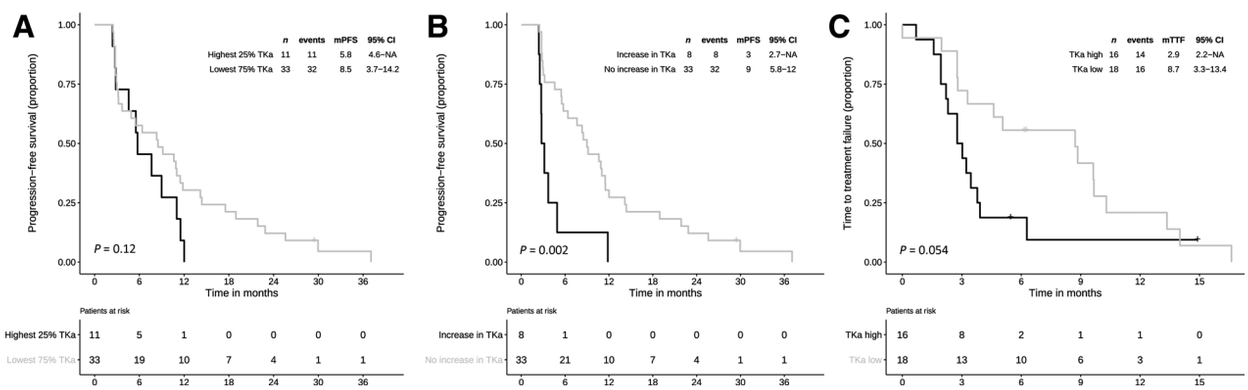

Figure 4.

Outcome of patients in the TREnd study according to pTKa. **A,** Baseline (T0) pTKa divided according to the top quartile, correlated to mPFS. **B,** On-treatment pTKa changes after one cycle of palbociclib therapy (T1), correlated to mPFS. **C,** mTTF on the line of treatment received immediately after exiting TREnd according to pTKa at the point of progression on trial (T2) using the median value as threshold.

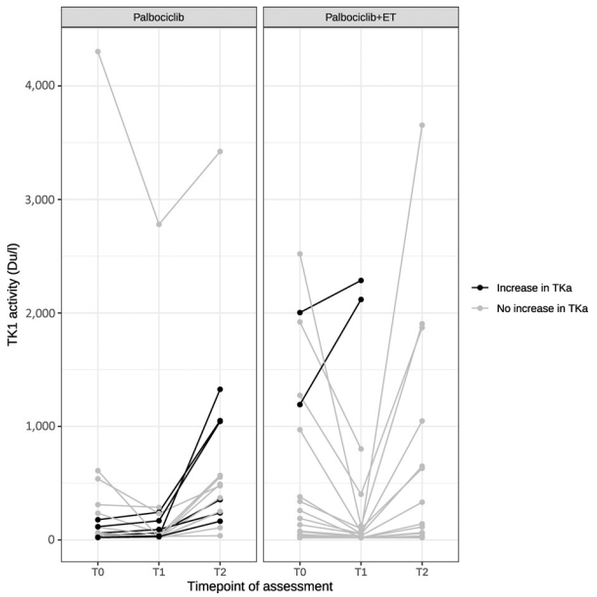

Figure 5.

Spaghetti plot illustrating TKa values across available time points (T0–T1) or (T0–T1–T2) for individual patients divided by study treatment arm. Not shown are patients who did not have appropriately matched samples, but who are included in the prognostic analysis.

continued palbociclib until surgery. Overall, there was a high concordance rate (89.9%) between the direction of palbociclib-induced TKa changes and that of tumor Ki67 levels, leading the investigators to conclude that serum TKa may serve as a pharmacodynamic marker of the antiproliferative effect of CDK4/6 inhibition. A small phase I study in Chinese women receiving palbociclib and letrozole as first-line treatment for advanced ER-positive, HER2-negative breast cancer (NCT02499146) demonstrated a reduction in phosphorylated Rb protein levels and Ki67 quantified in skin biopsies in response to treatment, remaining low at steady-state levels of palbociclib, with a similar, corresponding pattern noted in TKa levels measured in contemporaneous serum collections (25). Collectively, this suggests TKa has a dominant function as a marker of tumor proliferation, and therefore ongoing proliferation observed on treatment may be an early manifestation of treatment resistance. This hypothesis has been further supported in previous retrospective analyses conducted by our group in patients receiving endocrine monotherapy for advanced disease (11, 12).

We and others have previously shown that E2F target genes and proteins are generally downregulated by palbociclib in sensitive breast cancer models, and that recurrent alterations in key mediators of the E2F pathways (such as *CCNE1* and *RB1*) may mediate a general return of E2F target genes expression to the baseline levels observed in untreated cells (20, 26). This data suggest that, among E2F targets, TK1 levels may serve a sensitive indicator or surrogate marker of pathway inhibition in response to palbociclib. Our preclinical data show that indeed, TK1 was among the top differentially expressed E2F dependent–genes between PDS cells treated with short-term palbociclib and PDR cells, with an observed general downregulation across models upon treatment. We acknowledge that changes in E2F-dependent gene expression between sensitive and resistant cell lines are heterogeneous across the presented models, a phenomenon which we have reported in a previous study (20). Nonetheless, concordantly with TK1 downregulation, a reduction in TKa in response to palbociclib was observed exclusively in PDS cell lines and not in the drug-resistant derivatives, suggesting TKa modulation as a biomarker of response to palbociclib. Interestingly, TKa reduction in sensitive cells was observed within 3 days of drug exposure, whereas it took 6 days to detect any significant concomitant reduction in the rate of cellular proliferation. The fact that changes in TKa may precede discernible alternations in cellular turnover support its theoretical utility as a marker of early response to therapy, as was borne out in our clinical validation.

In-line with previous data established in studies of patients receiving endocrine therapy alone (11, 12), on-treatment pTKa observed in patients receiving TREnd-mandated palbociclib was prognostic, with those who recorded an increase in pTKa after one cycle of palbociclib ultimately demonstrating a poor PFS while on study. This provides the first evidence that TKa may be a feasible marker of early resistance to CDK4/6 inhibition, thus potentially allowing clinicians to recognize patients who are unlikely to derive benefit at a timepoint that long precedes the point at which routine radiological assessments and/or clinical signs of progression usually occur. Given emerging substantiation of overall survival benefit associated with CDK4/6 inhibitors received in first-line treatment for advanced disease (27), biomarkers that allow early identification of the subset of patients known to have primary resistance to these agents is key. This subset is not insignificant, representing at least one patient in every 10 who receives CDK4/6 inhibition plus endocrine therapy in the first-line setting for advanced breast cancer. PFS analyses of patients randomized to receive palbociclib and letrozole as a part of PALOMA-2 revealed 49 censored observations made by investigators within the first 3 months of the trial, increasing to 60 at central assessment (1). This equates to an incidence of primary resistance to palbociclib of 11% and 13.5%, respectively. Similarly, 12% of patients assigned to ribociclib plus letrozole in MONALEESA-2 recorded censored events within the first 2 months of trial entry (2), and of those on the abemaciclib arm of MONARCH-3, 17% incurred censored observations within the first 4 months (3). Timely identification of patients with primary resistance to CDK4/6 inhibitors has potential to lead to an early switch to alternative regimens, such as triplet therapy combinations which combine another agent that inhibits an alternative target (e.g., an inhibitor of PI3K, mTOR, or MEK) with the preexisting endocrine therapy plus CDK4/6 inhibitor doublet.

Unlike our previous studies conducted within the context of endocrine therapy (11, 12), baseline pTKa in the c-TREnd study did not prove prognostic in the setting of CDK4/6 inhibition, regardless of chosen cut-off value between "high" and "low" levels. This may be attributable to the small sample size, but as cumulative experience in employing TK1 assays in the context of CDK4/6 inhibitors is still limited, this should be explored further in a larger cohort. However, in-line with our results, previous studies have shown that tumor cell proliferation measured by Ki67 on archival or baseline tumor samples is not predictive of benefit from CDK4/6 inhibitors (28). Similarly, other studies suggest there is not a significant interaction between intrinsic breast cancer subtype (luminal A vs. the more proliferative luminal B type) and treatment effect of palbociclib (17). Cumulatively, these data seem to suggest that the dynamic measurement of tumor cell proliferation during treatment with CDK4/6 inhibitors may be more informative than static baseline testing. These observations require further research. Data from the NeoPalAna trial suggest that TK activity may increase after washout from palbociclib, reflecting a recovery in tumor cell proliferation (19). In our study, the T1 sample was obtained approximately 4 weeks after the baseline timepoint T0.

As palbociclib is administered on a 3-weeks-on, 1-week-off schedule, the T1 sample was obtained while "off-palbociclib-treatment" in all patients, and as such it cannot be excluded that some of the increase in pTKa observed at T1 may represent an early measure of escape from treatment with palbociclib with or without endocrine therapy during the week-off treatment. More detailed studies on the kinetics of TKa changes during treatment are needed to understand the best timing for early TKa testing.

Limitations of this study include the lack of an independent validation cohort, the small sample size studied, and consequent inability to perform multivariate analyses to distinguish whether pTKa functioned as a prognostic marker independent of other significant variables such as tumor burden, visceral involvement, and previous lines of treatment received prior to trial enrollment. However, multivariate analyses adjusting for previous sensitivity to aromatase inhibitors, number of metastatic sites, presence of visceral involvement, and study treatment received as a part of the landmark EFECT trial established serum TKa as an independent marker of prognosis in women receiving fulvestrant or exemestane (12). Correspondingly, in the analysis of c-TREnd, there was no statistical difference observed between patients according to site of metastases, number of prior lines of therapy received, prior response to endocrine therapy, and Eastern Cooperative Oncology Group (ECOG) status, and corresponding TKa levels at baseline (T0) and on-treatment dynamics (T1; **Table 1**).

One strength to this study was the finding that TKa levels measured at the point of progression on palbociclib (T2) correlated with clinical outcome on the next-line of systemic treatment received for metastatic disease. We have previously reported that responses to subsequent-line treatment in TREnd were generally short-lived, with an overall median time to posttrial treatment failure of 3.8 months (23). This phenomenon was consistent, irrespective of previous lines of treatment prior to trial enrollment, randomized arm allocation, evidence of response on trial, or type of posttreatment received. Similarly, exploratory analysis of the duration of immediate subsequent line of postprogression therapy in patients enrolled in PALOMA-3 revealed a median of 4.9 months in those allocated to the palbociclib/fulvestrant arm, versus 6.0 months in those who received fulvestrant alone. However, cumulatively, no significant difference in overall survival was observed in the entire trial group (29). There are a number of currently ongoing trials exploring optimum treatment choices following progression on CDK4/6 inhibitors, including potentially continuing or rechallenging with these agents beyond progression. There is limited real-world (30) evidence to suggest that this approach may be feasible, although no biomarkers currently exist to select patients best suited for rechallenge. In view of the prognostic nature of point-of-progression pTKa levels shown in this study, it might be hypothesized that patients demonstrating a relatively low level of TK activity at progression, perhaps suggestive of residual sensitivity to therapy, may represent an eligible population in whom CDK4/6 and endocrine therapy may be appropriately revisited. Conversely, patients progressing on CDK4/6 inhibitors with high TK activity may represent a population with a comparatively more aggressive disease where escalation of therapy may be an option within dedicated clinical trials.

Disclosure of Potential Conflicts of Interest

G. Curigliano reports receiving speakers bureau honoraria from Seattle Genetics, Daiichi Sankyo, Roche, AstraZeneca, and Pfizer. M. Bergqvist is an employee of and holds ownership interest (including patents) in Biovica. M. Benelli is a paid consultant for Novartis Farma SPA. E. Risi reports receiving other remuneration in the form of registration and accommodation for oncological congress from Pfizer. L. Biganzoli reports receiving speakers bureau honoraria from Pfizer. A. Di Leo is a paid consultant for Novartis, Pfizer, and Lilly. L. Malorni reports receiving commercial research grants and speakers bureau honoraria from Pfizer and Novartis. No potential conflicts of interest were disclosed by the other authors.

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