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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Translational relevance box (120-150 words)

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 initially responding patients inevitably acquire adaptive resistance to CDK4/6 inhibitors and therapeutic choices for these patients are currently empirical. Biomarkers to either identify patients with de-novo resistance or to stratify the prognosis of those with acquired resistance are lacking. We show in this report 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, negatively modulated by palbociclib in sensitive but not in resistant models. TKa can be monitored non-invasively throughout the treatment course, supporting its potential role as a dynamic biomarker for early identification of resistance and potentially adapting treatment strategies in clinical trials.

Abstract (250 words)

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 oestrogen 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 TREnd trial (n=46).

Results: Among E2F-dependent genes, *TK1* was significantly down-regulated 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 Du/L, with baseline TKa not proving prognostic. At T1, mTKa decreased to 36 Du/L, with a minority of patients (n=9) showing an increase – correlating with a worse outcome than those with decreased/stable TKa (n= 33) (mPFS 3.2 vs 9 months, p= 0.027). At T2, mTKa was 249 Du/L; patients with TKa above the median had worse outcomes on post-study treatment compared to those with lower TKa (3 vs 8.8 months, p= 0.039).

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. Additionally, TKa may stratify prognosis in patients with acquired resistance to palbociclib.

Introduction

In the past decade, the advent of cyclin dependent kinase 4 and 6 (CDK4/6) inhibitors represents arguably the most significant contribution to the management of advanced hormone receptor (HR) positive, HER2-negative breast cancer (BC). CDK4/6 inhibitors, administered in tandem with endocrine therapy, have been adopted into widespread clinical practice following landmark studies that describe superior progression-free survival in the first- (Finn 2016, Hortobagyi 2016, Goetz 2017) and later-line setting (Cristofanilli 2016, Slamon 2018, Sledge 2017). 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 (Schwartz 2003), is a well-documented marker of cancer proliferation in BC, and can be measured in plasma or serum samples as a marker of tumour proliferation (Robertson 1990, Romain 2000, Broet 2001). 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 BC treated with endocrine therapy, both when measured at baseline and during treatment (Bonechi 2018, McCartney 2019).

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 multi-factorial (Portman 2019). It is therefore perhaps unsurprising that the identification of definitive prognostic and predictive biomarkers in this field has so far remained elusive (Finn 2015, Hortobagyi 2018, Neven 2019, Turner 2019). In a retrospective analysis of the single-arm phase II NeoPalAna trial (Ma 2017), 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 (Bagegni 2017). The predictive 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 pre-clinical studies in palbociclib-sensitive (PDS) and palbociclib-resistant (PDR) BC cell lines to examine TKa in the context of CDK4/6 inhibition. For clinical validation of its utility as a biomarker, we performed retrospective analyses of baseline and on-treatment levels of plasma TKa (pTKa) as correlated to clinical outcome in patients receiving palbociclib as a part of the phase II "**T**o **R**everse **End**ocrine 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% CO2. T47D, ZR75-1, MCF7, MDAMB361 and BT474 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/glucose and L-glutamine (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and 10,000 U penicillin and 10 mg steptomycin/mL solution (P/S) (Sigma-Aldrich). MCF7 oestrogen deprivation resistant (EDR) and MCF7 tamoxifen resistant (TamR) cells were grown in DMEM with 4.5g/glucose and without L-glutamine and phenol red (Lonza) supplemented with 10% charcoal-stripped FBS (CS-FBS) (GIBCO), and P/S. MCF7 TamR cell medium was supplemented with 100 nM final concentration of (Z)-4-Hydroxytamoxifen (Sigma-Aldrich) dissolved in 100% ethanol.

Palbociclib resistant derivatives were generated as previously described (Guarducci 2018) and maintained in their original media with the addition of palbociclib 1 μM. The starting treatment concentration (STC) was defined as the initial concentration used to induce drug resistance in the different cell lines (Guarducci 2018). 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, Padova, Italy in January 2016. Absence of mycoplasma contamination was verified with the MycoAlert™ mycoplasma detection kit (Lonza). Cells used in these experiments had been thawed and maintained in culture for approximately three 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 previously described (Fu 2016). MDAMB361 (ECACC Cat# 92020423,

RRID:CVCL_0620) were purchased from Sigma-Aldrich in 2015 and BT474 (ICLC Cat# HTL00008, RRID:CVCL_0179) from Interlab Cell Line Collection, Genova, Italy 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 µM. Gene expression profiles were obtained as previously described (Guarducci 2018). The top fifteen most differentially expressed E2F target genes between PDR and PDS-treated cells are illustrated in Figure 2. These were selected as most the variable (i.e., by standard deviation, top 25%) genes included in the "Hallmark_E2F_/targets" HALLMARK_E2F_TARGETS gene list (http://software.broadinstitute.org/gsea/msigdb/cards/HALLMARK_E2F_TARGETS), that showed an absolute value of average normalised expression with respect to corresponding untreated PDS > 0.2. Differential expression between log2-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, Uppsala, Sweden. 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 nM, 350 nM and 1 µM and 0.01% DMS0 (vehicle) were added. After three days of treatment, cell extracts were prepared by scraping cells into 1mL of ice-cold RB lysis buffer (Biovica). 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 analysed using the DiviTum® assay (see below) without any knowledge of cell-line or treatment identifiers. TKa was normalised 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 normalised against TKa in the presence of DMSO and represented means of the two biological replicates +/- SEM. Two–way ANOVA with Dunnett's multiple comparisons test was performed with GraphPad Prism version 7.03 and p-values <0.05 were considered significant.

Proliferation assays

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 µM, palbociclib 350 nM or 0.01% DMS0 (vehicle) for nine days. Media were replaced every 72 h. 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 +/- standard error of the mean (SEM) was plotted. Two–way ANOVA with Dunnett's multiple comparisons test was performed with GraphPad Prism version 7.03 and p-values <0.05 were considered significant.

Clinical study design and patients

The conduct and results of TREnd have been reported in detail elsewhere (Malorni 2018). Briefly, this phase II, open-label, multicentre study randomised post-menopausal women with moderately pre-treated ER-positive, HER2-negative advanced BC (N=115) to receive either oral palbociclib monotherapy (125mg daily for three weeks, followed by one 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 enrolment (oral anastrozole 1mg/day or letrozole 2.5mg/day or exemestane 25mg/day, or intramuscular fulvestrant 500mg every four 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 pre-planned translational sub-study, "c-TREnd", designed to identify potential biomarkers, was run in parallel with TREnd under approval from the independent local ethics committees of each participating centre. Separate informed written consent was prospectively obtained from patients joining in this sub-study.

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 four weeks from baseline). TREnd defined mPFS as the median time from randomisation to radiological disease progression or death on study. Additionally, pTKa was measured in a number of patients at the point of disease progression on trial, and correlated to time to treatment failure (TTF) on subsequent post-study 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 (Rossi).

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=44) and after disease progression on trial, prior to starting a new line of therapy (T2; N= 34). Overall, 32 patients had available, valid samples collected at all three time points. Blood samples were processed within one hour from withdrawal by centrifugation at 1600g for 10 min at 4°C, followed by a second centrifugation at 14000rpm for 10 min 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 Figure 1.

Analysis of pTKa using DiviTum assay

TKa was determined by the DiviTum® assay, a refined ELISA based method, at Biovica laboratories in Uppsala, Sweden. Analysis was performed with no access to, nor any knowledge of, patient or tumour characteristics. Each sample was diluted 1/10 in a dilution buffer and then incubated with a reaction mixture on the assay microtiter plate. Bromo-deoxyuridine (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 monoclonal antibody 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 is proportional to the enzymatic TKa of each sample, that is 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 is 20-4000 Du/L; at 100 Du/L, the coefficient of variation is <20%.

Statistical analysis (c-TREnd)

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

Ethics statement

The presented 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 if palbociclib affects TK1 expression, in hormone receptor-positive (HR+) cell lines, we analysed gene expression profiles of PDS cells treated with drug vehicle (untreated PDS) or with palbociclib STC for three days (PDS treated) and PDR cells continuously receiving palbociclib 1μM. Data demonstrated that, among E2F target genes, TK1 was one of the most differentially expressed genes between PDR and PDS treated cells (Figure 2a). In PDS-treated cells compared to 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 to untreated PDS cells despite the presence of the drug (Figure 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 HR+/HER2-negative and a HR+/HER2-positive cell line, MCF7 and BT474, respectively, and analysed TKa in cell lysates extracted from PDS and PDR cells treated for three days with different doses of palbociclib. TKa was significantly reduced in PDS cells treated with palbociclib compared to vehicle ($p<0.05$), even at the lower dose (50 nM) (Figure 3a). In accordance with the expression data, TKa response to palbociclib was more dramatic in the HER2-positive model BT474 as compared to MCF7. As expected, cell proliferation of PDS models was inhibited by treatment with palbociclib (Figure 3c), with a significant reduction of proliferation rate being observed only after six days of exposure to the drug. Conversely, no significant alterations in TKa (Figure 3b) or proliferation rate (Figure 3d) were observed in PDR cells, at any dose of palbociclib.

Patient characteristics in c-TREnd

The baseline characteristics of the patients analysed in c-TREnd were representative of the overall cohort of the original TREnd study (Table 1), and were well-balanced across randomised 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 enrolment in the monotherapy arm, compared to 46% in the combination arm. Characteristics according to baseline pTKa levels (high versus low), and pTKa after one cycle of treatment (rise or no rise) are presented in Table 2.

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, 20-4302). When the median value was employed as the defined cut-off between "high" and "low" levels of baseline pTKa, no notable difference in the median PFS (mPFS) was seen between groups. When the upper quartile was used as the cut-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) (Figure 4). Additionally, there was no significant difference between pTKa levels at baseline and CBR on treatment (Table 2).

Prognostic role of on-treatment pTKa

The overall median pTKa at timepoint T1 was 36 Du/L (range, 20-4504). When comparing matched T0 and 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). The majority of patients exhibited either a decrease or no significant change in pTKa at T1 compared to baseline.. In this group (N=33), median pTKa at T1 was 21 Du/L (range, 20-2780) and the mPFS was 9.0 months (95% CI 5.8-12.0). Interestingly, a small group of patients (N=9; 21%) recorded a rise in pTKa at T1. In this group, median pTKa at T1 was 169 Du/L (range, 30-4503) and mPFS was significantly shorter when compared to the group with decreased or stable reading (mPFS 3.2 months, 95% CI 2.7-NA, p=0.027) (Figure 5). Additionally, within the group of patients with increasing pTKa during treatment, only 3 patients (33%) achieved CB on study, as compared to 73% of patients in the other group

(p=0.05) (Table 2). A greater proportion of patients randomised to the palbociclib monotherapy arm demonstrated a rise in pTKa on treatment (78%) compared to those assigned to receive palbociclib plus endocrine therapy (22%, p=0.005) (Table 2). Figure 6 illustrates pTKa dynamics according to allocated trial treatment arm.

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

At the point of disease progression on TREnd (T2), the overall median pTKa was 249 Du/L (range, 20-3653). Using this median value as a cut point, we correlated pTKa values (high versus low) with outcome on the treatment received immediately after exiting TREnd. On next-line treatment, the mPFS 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 mPFS was 8.8 months (95% CI 4.6-14.0) in patients with pTKa less than 249 Du/L at T2, versus 3.0 months (95% CI 2.3-3.9) in patients with values in excess of 249 Du/L (p=0.039) (Figure 7).

Discussion

The link between tumour cell proliferation and serum TKa (evaluated by the DiviTum assay) has previously been reported within the neoadjuvant NeoPalAna trial (Bagegni 2017). Patients with clinical stage 2/3 ER-positive, HER2-negative BC 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 two 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 continued palbociclib until surgery. Overall, there was a high concordance rate (89.9%) between the direction of palbociclib-induced TKa changes and that of tumour Ki67 levels, leading the investigators to conclude that serum TKa may serve as a pharmacodynamic marker of the antiproliferative effect of CDK4/6 inhibition. 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 down-regulation across models upon treatment. This suggests that, among E2F targets, *TK1* levels may serve a sensitive indicator of pathway inhibition in response to palbociclib. Concordantly with TK1 down-regulation, a reduction in TKa in response to palbociclib was observed exclusively in palbociclib-sensitive cells 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 three days of drug exposure, whereas it took six 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 (Bonechi, McCartney), 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 whilst 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 recognise patients who are unlikely to derive benefit at a time point 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 (Im 2019), 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 ten who receives CDK4/6 inhibition plus endocrine therapy in the first line setting for advanced breast cancer. Progression-free survival analyses of patients randomised to receive palbociclib and letrozole as a part of PALOMA-2 revealed 49 censored observations made by investigators within the first three months of the trial, increasing to 60 at central assessment (Finn 2016). 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 two months of trial entry (Hortobagyi 2016), and of those on the abemaciclib arm of MONARCH-3, 17% incurred censored observations within the first four months (Goetz 2017). 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 add another agent which inhibits an alternative target (eg an inhibitor of PI3K, mTOR or MEK) to the pre-existing endocrine therapy plus CDK4/6 inhibitor doublet.

Unlike our previous studies conducted within the context of endocrine therapy (Bonechi, McCartney), baseline pTKa in the c-TREnd study did not prove prognostic in the setting of CDK4/6 inhibition. 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 tumour cell proliferation measured by Ki67 on archival or baseline tumour samples is not predictive of benefit from CDK4/6 inhibitors (Finn 2016). Similarly, other studies suggest there is not a significant interaction between intrinsic BC subtype (luminal A versus the more proliferative luminal B type) and treatment effect of palbociclib (Turner 2019). Cumulatively, these data seem to suggest that the dynamic measurement of tumour cell proliferation during treatment with CDK 4/6 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 tumour cell proliferation (Bagegni). In our study, the T1 sample was obtained approximately four 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.

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 post-trial treatment failure (mTTF) of 3.8 months (Rossi). This phenomenon was consistent, irrespective of previous lines of treatment prior to trial enrolment, randomised arm allocation, evidence of response on trial or type of post-treatment received. Similarly, exploratory analysis of the duration of immediate subsequent line of post-progression 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 (Turner 2018). There are a number of currently ongoing trials exploring optimum treatment choices following progression on CDK4/6 inhibitors, including potentially continuing or re-challenging with these agents beyond progression. There is limited real-world (Eziokwu 2019) evidence to suggest that this approach may be feasible, though no biomarkers currently exist to select patients best suited for re-challenge. In view of the prognostic nature of point-of-progression

pTKa levels shown in this study, it might be hypothesised 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.

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