Serum thymidine kinase activity in patients with hormone receptor positive and HER2 negative metastatic breast cancer treated with palbociclib and fulvestrant

Results from the single-arm PYTHIA trial (*subtitle*)

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ABSTRACT

BACKGROUND: Biomarkers for cyclin dependent kinase 4/6 (CDK4/6) inhibitors, like palbociclib, for patients with HR+/HER2- metastatic breast cancer (MBC) are lacking. Thymidine kinase is a proliferation marker downstream of the CDK4/6 pathway. We prospectively investigated the prognostic role of serum thymidine kinase activity (sTKa), in patients treated with Palbociclib+fulvestrant.

PATIENTS AND METHODS: PYTHIA was a phase II, single-arm, multicenter, trial that enrolled 124 post-menopausal women with endocrine resistant HR+/HER2- MBC. Serum samples were collected pre-treatment (pre-trt; n=122), at day 15 of cycle 1 (D15; n=108), during the one week-off palbociclib before initiating cycle 2 (D28; n=108) and at end of treatment (EOT; n=76). sTKa was determined centrally using Divitum®, a refined ELISA-based assay with a limit of detection (LOD) of 20 Divitum Units (Du)/L. The primary study endpoint was PFS, assessed for its association with pre- and on-treatment sTKa.

RESULTS: Data from 122 women were analyzed. Pre-treatment sTKa was not associated with clinical characteristics, and moderately correlated with tissue Ki-67. Palbociclib+fulvestrant markedly suppressed sTKa levels at D15, with 83% of patients recording levels below LOD. At D28, sTKa showed a rebound in 60% of patients. At each timepoint, higher sTKa was associated with shorter PFS (each $p<0.001$), with the strongest effect at D15.

CONCLUSIONS: sTKa is an independent prognostic biomarker in patients treated with palbociclib. High pre-treatment sTKa and its incomplete suppression during treatment may identify patients with poorer prognosis and primary resistance. This warrants validation in prospective comparative trials.

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Key words: Breast Cancer, Serum Markers, Thymidine Kinase, Palbociclib, Fulvestrant, Prognostic factors

INTRODUCTION

Cyclin dependent kinase 4 and 6 inhibitors (CDK4/6i) in combination with endocrine therapy (ET) are standard treatment options for both endocrine-sensitive and resistant hormone receptor-positive (HR+), HER2-negative (HER2-) metastatic breast cancer (MBC). The main antiproliferative mechanism of action of CDK4/6i relies on their inhibition of the retinoblastoma-associated protein (RB) and E2F family of transcription factors, ultimately halting progression through the G1-S transition of the cell cycle [1]. Although several mechanisms of *de novo* and acquired resistance to CDK4/6i have been described in breast cancer models, no clinically validated biomarker has emerged to allow for patient selection[2].
Thymidine kinase 1 (TK1) is an enzyme involved in the DNA salvage pathway, and is expressed predominantly in dividing cells, peaking during the S phase [3,4]. For this reason, it has long been recognized as a marker of cell proliferation. TK1 is also an E2F-dependent gene, existing downstream of the CDK4/6 pathway, which implies its potential role as a pharmacodynamic marker in the context of CDK4/6 inhibition[3].
Relative to normal controls, high levels of TK1 have been reported in many cancer types, including breast cancer [5]. TK1 activity (TKa) can be measured in serum and plasma samples. Both pre-treatment and on-treatment circulating TKa levels have proven prognostic in patients with MBC treated with ET alone, as well as with the CDK4/6i palbociclib [6–13]. Additionally, sTKa has been shown to reflect a reduction in tumor cell proliferation upon treatment with palbociclib, with strong correlation with matched Ki67 evaluation on tumor biopsy[14]. Serum TKa (sTKa) therefore represents an attractive biomarker for prognostic stratification and real-time monitoring of CDK4/6i activity. Based on these principles, we aimed to prospectively assess sTKa within the phase II biomarker discovery trial PYTHIA.

METHODS

Study design

PYTHIA (IBCSG 53-14 / BIG 14-04; NCT02536742) is an international, multicenter, prospective single-arm phase II biomarker discovery clinical trial. The primary objective assesses the association of progression-free survival (PFS) with a number of biomarkers including sTKa, in women with endocrine-resistant MBC. Safety and tolerability according to NCI CTCAE v4.0 is a secondary objective.

This trial is downstream of the AURORA program conducted by the Breast International Group (BIG 14-01; NCT02102165). AURORA is a pan-European molecular screening program which aims to improve the understanding of MBC through extensive profiling of paired primary tumors and metastatic samples, as well as circulating tumor DNA (ctDNA) extracted from plasma [15].

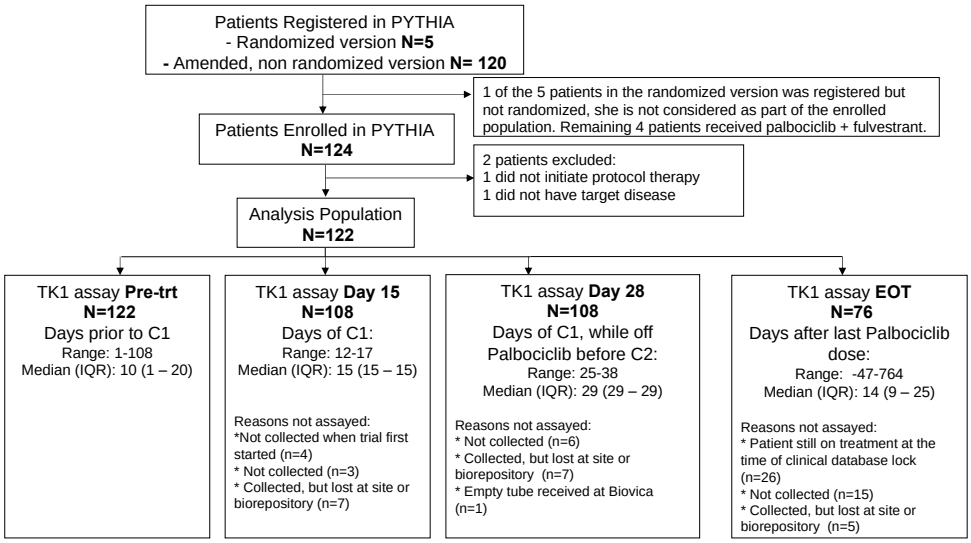
The PYTHIA trial commenced on 4 May 2016 as a randomized study, which originally included a control arm of fulvestrant plus placebo. On 23 November 2016, following the announcement of the imminent approval of palbociclib in combination with fulvestrant as standard treatment for the study population, the Data and Safety Monitoring Committee (DSMC) recommended to close the placebo arm and unblind treatment for the one patient randomized, which was palbociclib+fulvestrant. Health authorities permitted continuing accrual in the combination arm only while protocol amendment 1 was being implemented. This was distributed to sites on 20 February 2017, including the design change to a single-arm trial of 120 patients and the collection of a serum sample on Day 15 of Cycle 1. Additional details on study design are reported in Supplementary Appendix I.

Participants

PYTHIA enrolled post-menopausal women with HR+/HER2-negative, locally relapsed or MBC, who had received previous ET (i.e. relapsed while on or within 12 months after completion of adjuvant ET, or progressed on first-line ET for MBC) and 0-1 lines of chemotherapy for MBC. Eligibility criteria included: ECOG performance status of 0 or 1, measurable and non-measurable but evaluable disease according to RECIST 1.1, including bone-only disease. Additional details are reported in Supplementary appendix I.

Of the five patients registered under the original protocol between August 2016 and April 2017, one was not randomized and therefore was not considered as enrolled; the four patients who were enrolled, all received palbociclib+fulvestrant. Under the amended single-arm design, 120 patients were enrolled between May 2017 and June 2019. Two patients were excluded, one who did not start protocol therapy and another who did not meet trial eligibility. Thus 122 patients are included in the PYTHIA analysis population (**Figure 1a**).

Figure 1a. Flow diagram of PYTHIA patient participation and serum sample availability for TK1 assay



(Matched Pre-trt, Day 15 and Day 28 samples were available from N=101 patients, Matched D28 and EOT samples from N=68 patients)

All patients provided written informed consent. Local ethics committees and appropriate health authorities approved the protocol. The trial was conducted according to the ethical principles of the Declaration of Helsinki and Good Clinical Practices guidelines. PYTHIA was sponsored by the IBCSG and co-led by IBCSG and BIG. Pfizer provided financial support and drug supply. AstraZeneca provided drug supply. BIOVICA provided financial support for sample handling and assays. The IBCSG DSMC reviewed the trial at six-monthly intervals.

Treatment

Patients were treated with fulvestrant (500mg intramuscularly administered on Days 1 and 15 of Cycle 1, then on Day 1 of every 28-day cycle) plus palbociclib (125mg orally per day, continuously for three weeks followed by one week off; repeated for each subsequent cycle of 28 days). Patients received treatment until progression, unacceptable toxicity, or patient decision to cease therapy.

Assessments

Tumor measurements according to RECIST 1.1 criteria were performed prior to start of protocol therapy and every 12 weeks (±2) weeks until documented progression. If trial treatment ceased prior to disease progression, tumor measurements continued until progression, or for a maximum of 12 months after treatment cessation. Adverse events (AEs) were recorded according to CTCAE v4.0, at the end of each cycle from first dose of trial treatment, until 28 days following all treatment discontinuation.

Samples

Serum samples were taken before treatment start (pre-trt), on Day 15 (± 2 days) of Cycle 1 (D15), on Day 26-28 (+ max 7 days) before Cycle 2 treatment (D28), and at end-of-treatment.

Pre-treatment and end-of-treatment serum samples were derived from those originally drawn for AURORA, with "on-treatment" (D15 and D28) samples drawn specifically for the PYTHIA study. All samples were stored locally at the participating sites and shipped in batches to the AURORA biorepository. Out of 122 patients in the analysis population, serum samples were available for: all patients for pre-treatment, 108 patients for D15, 108 patients for D28 and 76 patients for end-of-treatment. One-hundred-one patients had matched pre-treatment, D15 and D28 samples. **(Figure 1a)**.

Thymidine kinase activity assay

Serum samples were retrieved from the AURORA biorepository (IBBL, INTEGRATED BIOBANK OF LUXEMBOURG, Dudelange, Luxembourg). Aliquots of 350 ul from eligible patients were shipped to the central laboratory of BIOVICA in Uppsala (Sweden) in two batches, labelled with an anonymised code. BIOVICA had no access to clinical data or timing of the sample. TK1 activity was determined by a refined ELISA based method, the DiviTum® assay (Biovica, Instructions for Use, www.biovica.com). The methodology of this assay is described in detail in the Supplementary Appendix I and elsewhere[16]. In brief, the assay measures Bromo-deoxyuridine (BrdU) incorporation into a synthetic DNA strand, revealed by an anti-BrdU monoclonal antibody. The signal is proportional to the TK-activity of the tested sample, and given as DiviTum units per liter (Du/L). Each sample was assayed in duplicate and the mean value of the two measurements was used. As a quality control measure, all samples (duplicates) must be below the limit of coefficient of variation (CV) of 20%. The median coefficient of variation of all samples analysed was 7,54%. Values below limit of detection (LOD) were imputed as 18 Du/L for all analyses.

Statistical considerations

Enrollment of 120 patients was planned, assuming median PFS in the range of 9 to 10.5 months, with primary analysis after at least 80 PFS events were documented. This provided 80% power to detect a hazard ratio of 2.0 for association of PFS with a binary biomarker having 30-50% prevalence (two-sided α=0.05).

The primary endpoint in PYTHIA was PFS, defined as time from treatment initiation until documented investigator-assessed disease progression according to RECIST 1.1 criteria or death, whichever occurred first, or censored at the date of last disease assessment. The association of continuous log-transformed sTKa values with PFS were assessed using multivariable Cox proportional hazards. Hazard ratios with 95% confidence intervals were estimated with Wald tests. The distribution of PFS stratified by dichotomized log-transformed sTKa was estimated by Kaplan-Meier method; point estimates are provided with log-based 95% CI. Median cut-off for sTKa at each time-point was chosen for the pre-specified analysis. As exploratory analysis, the 200 Du/L cut-off, which is based on prior and current studies of sTKa in HR+/HER2- MBC patients[10,13], was used for pre-treatment and D28 time-points. Additional details are reported in Supplementary Appendix I.

All results are reported following REMARK (Reporting recommendations for tumour MARKer prognostic studies) criteria[17].

RESULTS

CLINICAL CHARACTERISTICS

The median age of the study population was 61 years (IQR, 55-69). Most participants (63.9%) had an ECOG performance status of 0. The majority (61.5%) had measurable disease at study entry. Overall, 86% of the patients had oligometastatic disease with one or two metastatic sites involved, 48.4% had visceral disease, 31.2% had bone-only metastases, and 20.5% had non-visceral disease. Sixty-two patients (51%) received palbociclib+fulvestrant treatment on study as their first-line endocrine therapy for MBC, and only 18% received one line of chemotherapy for MBC prior to study entry. All patients had endocrine-resistant disease, with the vast majority (78%) exhibiting secondary endocrine resistance (**Table 1**).

Table 1. Clinical characteristics of the PYTHIA cohort overall and according to pre-treatment sTKa status

Characteristic	**Overall**	**Pre-trt sTKa High**	**Pre-trt sTKa Low**	**p-value**
	N = 122	**N=61**	**N=61**	
Median age (IQR)	61 (55, 69)	61 (54, 71)	61 (55, 67)	0.87
ECOG PS n (%)				0.35
0	78 (64%)	36 (59%)	42 (69%)	
1	44 (36%)	25 (41%)	19 (31%)	
Prior ET for MBC n(%)				>0.99
No	62 (51%)	31 (51%)	31 (51%)	
Yes	60 (49%)	30 (49%)	30 (49%)	
Prior CT for MBC n (%)				0.81
No	100 (82%)	51 (84%)	49 (80%)	
Yes	22 (18%)	10 (16%)	12 (20%)	
Type of endocrine resistance n (%)				>0.99
Primary	27 (22%)	14 (23%)	13 (21%)	
Secondary	95 (78%)	47 (77%)	48 (79%)	
Presence of visceral disease n (%)				0.28
No	63 (52%)	28 (46%)	35 (57%)	
Yes	59 (48%)	33 (54%)	26 (43%)	
Bone-only disease n (%)				0.17
No	84 (69%)	46 (75%)	38 (62%)	

Yes	38 (31%)	15 (25%)	23 (38%)	
Nr of metastatic sites n (%)				>0.99
1-2	105 (86%)	53 (87%)	52 (85%)	
3+	17 (14%)	8 (13%)	9 (15%)	
Successfully included in AURORA n (%)				
YES	67 (55%)			
NO	55 (45%)			

TREATMENT, ADVERSE EVENTS AND OUTCOME

After a median follow-up of 24.5 months, the median number of administered treatment cycles was 11 (IQR, 6-22; range 2-42) with 92 of 96 patients discontinuing therapy for disease progression and 26 patients continuing treatment. In total, 67 (55%) participants had palbociclib dose reduction to 100mg and 28 (42%) patients had further dose-reduction to 75mg. Adverse events were in line with expectations (**etable 1 in the Supplementary Appendix**). The median PFS was 10.3 months (95% CI 8.4-15). Best overall response rates were: progressive disease (n=17; 13%), stable disease (n=80; 65.5%), partial response (n=20; 16.4%), with six patients experiencing complete response (5%). In patients with responsive disease, the median duration of response was 7.8 months (95% CI 3.6-11.3).

THYMIDINE KINASE ACTIVITY

Exploratory data analysis and visual inspection of the distribution plots did not reveal apparent batch effects in sTKa measurements (**eFigure 1 in the Supplementary Appendix**).

Median pre-treatment sTKa was 87 Du/L (range <20- 14,510), with only 17 patients (14%) having sTKa below the assay LOD (20 Du/L). A striking drop in median sTKa was observed upon treatment with palbociclib+fulvestrant. At D15, the median sTKa was <20 Du/L (range <20- 7,060), with 83% (90/108) of patients with sTKa below LOD. At D28, following the week off palbociclib and before starting Cycle 2, only 29% (31/108) had sTKa below LOD and median sTKa was 52 Du/L (range <20 – 3,533). At end of treatment (EOT), median sTKa was to 515 Du/L (range <20 – 59,847) and 3/76 (4%) had sTKa below LOD. The dynamic change of log-transformed sTKa across timepoints is depicted in **Figure 1b-e**.

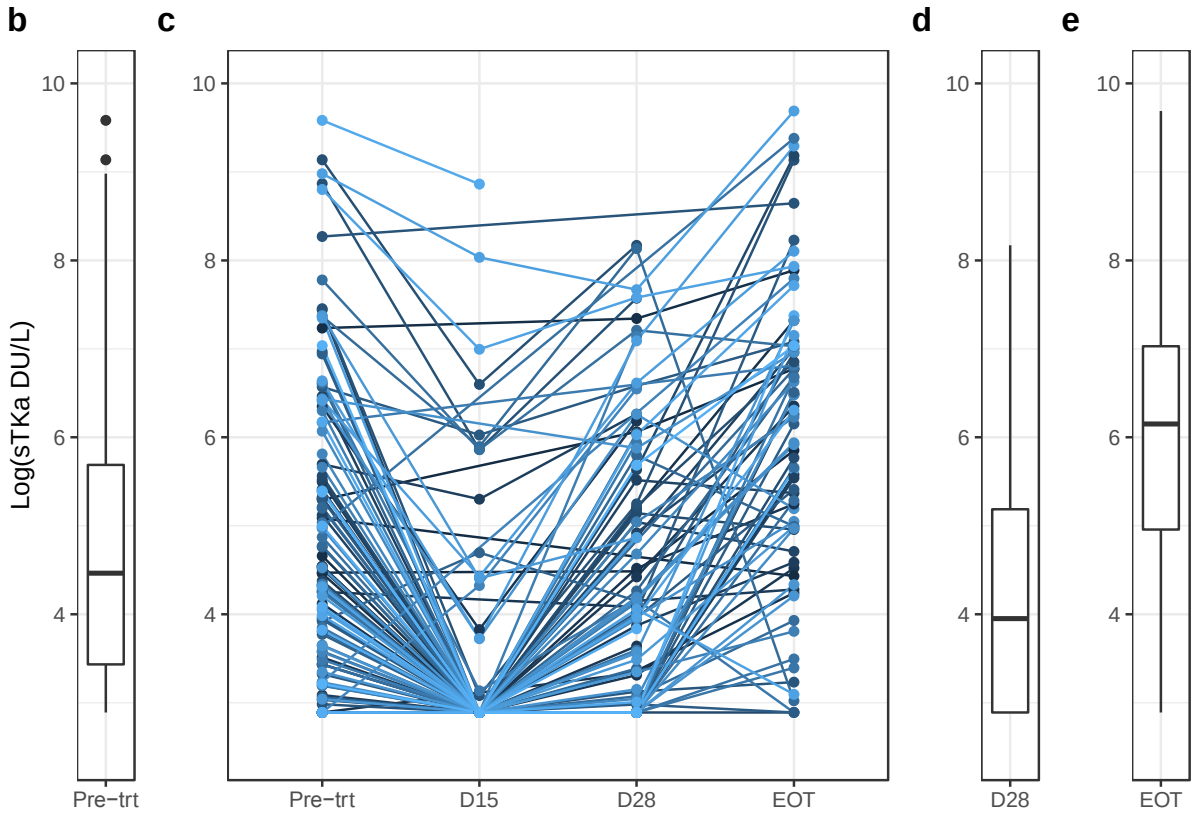


Figure 1 (b-e). sTKA distribution. (b) Boxplot of log-transformed sTKa values at baseline. (c) Spaghetti plot of individual log-transformed sTKa values across timepoints for each patient (n=122). (d) Boxplot of log-transformed sTKa values at D28. (e) Boxplot of log-transformed sTKa values at EOT.
In the boxplots the lower and upper hinges correspond to the first and third quartiles, the upper whisker extends from the hinge to the largest value no further than 1.5 * IQR from the hinge, the lower whisker extends from the hinge to the smallest value at most 1.5 * IQR of the hinge, data beyond the whiskers is plotted as points, horizontal line across the box is the median.

Pre-treatment sTKa, when dichotomized at the median value of the distribution, was not associated with potentially prognostic clinicopathological variables (Table 1). A moderate association was observed between sTKa and log-transformed Ki-67 measured in primary tissue or metastatic biopsies (Spearman ρ=0.44 and 0.38, respectively) **(Figure 2).**

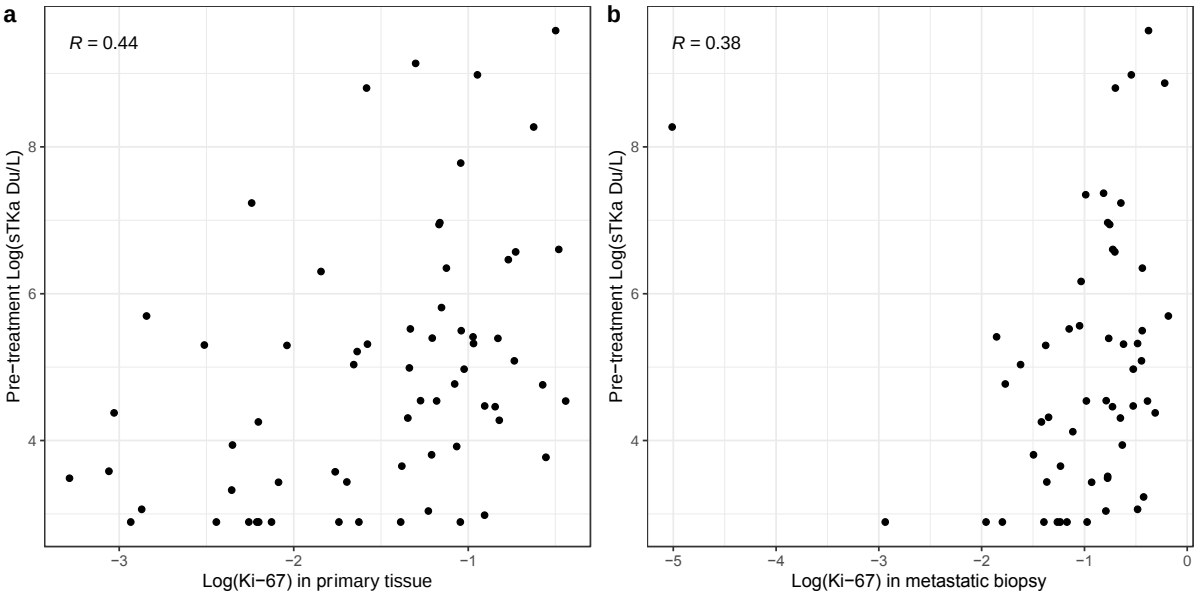


Figure 2. Distributions of log-transformed sTKa at baseline and log-transformed tissue Ki67 measured in (a) primary breast tissue (n=65) or (b) pre-treatment metastatic biopsy tissue (n=55). Spearman correlation coefficients are shown.

PROGNOSTIC ROLE OF PRE-TREATMENT sTKa

Using the median value of pre-treatment sTKa as a cut-off, patients with low sTKa had a median PFS of 17 months (95% CI: 14 – 28) versus 7.4 months (95% CI: 5.5-8.7) in patients with high sTKa (**Figure 3a**).

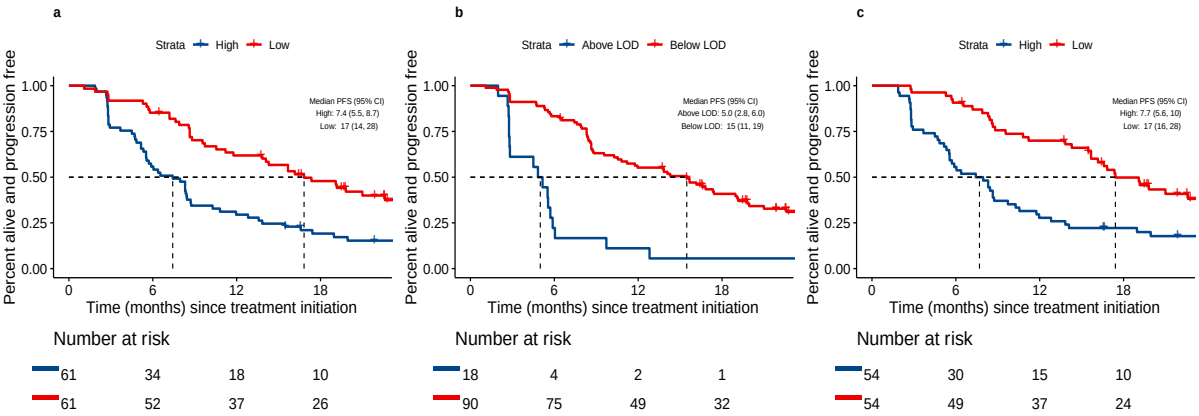


Figure 3. Distribution of PFS according sTKa values. (a) pre-treatment median-dichotomized sTKa (n=122); (b) D15 below-above LOD dichotomized sTKa (n=108); (c) D28 median-dichotomized sTKa (n=108).

Kaplan Meier estimates of PFS at three and six months were 77% (95% CI 67%-88%) and 56% (95% CI 45%-70%) for sTKa high, compared to 92% (95% CI 85%-99%) and 85% (95%CI 77%- 95%) for sTKa low, respectively. In multivariable analyses, log-transformed continuous pre-treatment sTKa values were significantly associated with PFS (HR 1.39; 95% CI: 1.23-1.59 p<0.001; **Table 2**). These results were generally consistent in the exploratory analysis using the 200 Du/L cut-off (eFigure 2 in the Supplementary Appendix).

Table 2: Multivariable Cox PH regression analysis of sTKa in relation to PFS.

	Pre-trt (n=122)			**D15 (n=108)**			**D28 (n=108)**		
Characteristic	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	**p-value**
Age ≥ 65	0.81	0.52 – 1.28	0.37	0.86	0.54 – 1.37	0.52	0.85	0.51 – 1.38	0.52
Visceral disease	2.53	1.37 – 4.66	0.003	2.51	1.26 – 5.00	0.009	2.31	1.19 – 4.47	0.01
Bone-only disease	0.92	0.44 – 1.92	0.83	0.85	0.38 – 1.89	0.69	0.88	0.40 – 1.93	0.75
Nr metastatic sites	0.97	0.74 – 1.28	0.83	0.91	0.67 – 1.25	0.56	1.03	0.76 – 1.38	0.87
Prior treatment for MBC	0.65	0.42 – 1.00	0.05	0.78	0.49 – 1.24	0.30	0.88	0.54 – 1.45	0.62
Secondary endocrine resistance	0.63	0.037 – 1.06	0.08	0.75	0.42 – 1.33	0.32	0.72	0.40 – 1.32	0.29
Log(sTKa)	**1.41**	**1.24 – 1.60**	**<0.001**	**1.50**	**1.23 – 1.82**	**<0.001**	**1.42**	**1.20 – 1.67**	**<0.001**

ROLE OF sTKa at D15 and D28

At D15, 18 patients (16.7%) did not experience a reduction of sTKa below LOD. Of these, one patient discontinued palbociclib during cycle 1, while all others received study treatment as scheduled. For this group, the median PFS was only 5 months (95% CI: 2.8-6.0) versus 15 months (95% CI: 11-19) in the larger group of 90 patients with a drop of sTKa below LOD (**Figure 3b**). Kaplan Meier estimates for PFS at three and six months were 61% (95% CI 42%-88%) and 22% (95% CI 9.4%-53%) for sTKa remaining above LOD, versus 91% (95% CI 85%-97%) and 83% (95% CI 76%-91%) for sTKa below LOD, respectively. sTKa at D15 was significantly associated with PFS in multivariable analysis (HR 1.48; 95% CI 1.22-1.78; p<0.001; **Table 2**).

Similarly, using the median cut-off at D28, high sTKa was strongly associated with worse outcomes on treatment with palbociclib+fulvestrant. Median PFS in the group with low sTKa was 17 months (95% CI: 16-28) versus 7.7 months (95% CI: 5.6-10) in the group with high sTKa. Estimated HR for continuous log-transformed sTKa in multivariable analysis was 1.41 (95% CI: 1.20-1.66, p < 0.001; **Figure 3c and table 2**). Also at D28, these results were generally consistent in the exploratory analysis using the 200 Du/L cut-off (eFigure 2 in the Supplementary Appendix).

Out of 90 patients with sTKa below LOD at D15, matched information at D28 were available for 84. As exploratory analysis, this group was further stratified according to the change in sTKa at D28. Forty-six (54.7%) experienced sTKa rebound 20% above LOD at D28, while 38 had persistent suppression. For the latter group, median PFS was 17 months (95% CI: 14-25) versus 13 months (95% CI: 8.7-28) in those with rebound (**eFigure 3a in the Supplementary Appendix)**. Two patients in each group had palbociclib dose interruption within cycle 1 (n=3 adverse events; n=1 erroneous dosing).
When sTKa dynamics at D15 and D28 were stratified by pre-treatment levels using median cut-off, it was evident that the group whose sTKa did not drop below LOD at D15 also had high pre-treatment sTKa. Conversely, high or low pre-treatment sTKa did not seem to add information either in the group with sTKa rebound at D28, or in the group without rebound (e**Figure 3b in the Supplementary Appendix**). However, this analysis must be interpreted with caution due to the small sample size of the groups.
In the group of 108 patients with matched pre-treatment and D28 sTKa results, no significant difference in outcome was observed between those with an increase >10% of sTKa at D28 from pre-treatment (n=15) compared to those with stable or decreased sTKa (n=93) (eFigure 4 in the Supplementary Appendix).

sTKa at end of treatment

Of the 76 patients with EOT data, 76 and 67 had matched pre-treatment and D28 sTKa data available, respectively. At EOT most of these patients displayed higher sTKa values compared to pre-treatment (55/76, 72%) and D28 (56/67, 84%). sTKa at EOT was not associated with clinical characteristics at study inclusion such as presence of bone-only disease or visceral disease (data not shown).

DISCUSSION

Tumor cell proliferation rate represents an important feature of HR+/HER2- breast cancer as it allows discrimination between Luminal A and B subtypes, with prognostic implications in early breast cancer[18]. Currently, immunostaining of Ki-67 is the most widely used biomarker of cell proliferation, notably in the setting of early breast cancer, where it can help to guide clinical decision-making[19]. In the metastatic setting wherein tissue from metastatic sites is commonly unavailable, the prognostic value of tumor tissue proliferation is rarely assessed and utilized. TKa is a proliferative marker that can be non-invasively quantified in blood, allowing for repeated measurements of tumor cell proliferation[6,7].
Studies on samples obtained from patients with HR+/HER2- MBC treated with ET alone have consistently shown TKa as a strong prognostic marker[8,9,10]. Thymidine kinase has been shown among the top E2F-dependent genes differentially regulated in palbociclib-resistant cell lines compared to sensitive counterparts[20]. These data support the investigation of TKa as a dynamic biomarker in patients treated with palbociclib and potentially other CDK4/6i.

PYTHIA is the first multicenter study to prospectively assess the association of pre-treatment and on-treatment sTKa levels with PFS in HR+/HER2- MBC treated with ET plus palbociclib. Our data show sTKa measured before commencing treatment is a strong and independent prognostic factor. Similar findings have been shown in the single-center, retrospective ALCINA study (NCT02866149) where pre-treatment sTKa assessed in plasma samples from patients with ER+/HER2- MBC treated with ET and palbociclib was an independent prognostic factor for both PFS and overall survival (OS)[11]. Although the patient population in ALCINA differed from PYTHIA in being more heavily pre-treated, having more patients with visceral metastases (66% vs 48%) and a higher median pre-treatment sTKa (292 vs 87 Du/L), the prognostic effect of TKa was remarkably similar in the two studies. Similarly to ALCINA, we also did not observe any prognostic role for the change between baseline and D28 sTKa using the same cut-off.

In PYTHIA, the availability of sTKa data at D15 captured early treatment-induced changes in sTKa. At D15, sTKa was markedly low in 83% of the patients, with a subsequent rebound observed in half of them. With only four patients interrupting treatment due to adverse events during cycle 1, it is not likely this rebound is attributable to treatment interruptions. This pattern has been similarly described in the NeoPalAna trial, a pre-operative study of anastrozole+palbociclib for patients with early-stage HR+/HER2- BC[14]. In this trial, a marked reduction of sTKa was observed after two weeks of palbociclib-containing treatment, with a significant rebound during preoperative wash-out, which was not observed in a small group of patients who continued palbociclib until surgery[21]. Palbociclib has a terminal half-life of 25.9 hours (REF: Flaherty et al CCR 2012 10.1158/1078-0432.CCR-11-0509). Therefore, its anti-proliferative effect may be attenuated during the one-week treatment break. The observed sTKa rebound after palbociclib interruption is compatible with a recovery in tumor cell proliferation during the scheduled one-week break and suggests a pharmacodynamic role for sTKa in patients treated with palbociclib.

The proof of principle that early changes in TKa levels might have a role in predicting outcome in patients with HR+/HER2- MBC treated with CDK4/6i has been explored in a small cohort from the TREnd trial (NCT02549430)[23]. In this study, an increase of >10% in TKa after one month of palbociclib treatment, identified a group of patients with an extremely poor outcome[24].

The additional insights that PYTHIA brings to established data are two-fold. Firstly, PYTHIA suggests that the dynamic changes in sTKa detected as early as 15 days on treatment may indeed have prognostic implications. Specifically, the group of patients who did not have a sTKa below LOD at D15 fared comparatively poorly, with a median PFS of only 5 months, and only 22% remaining free from progression at 6 months. Randomized trials investigating CDK4/6i in combination with fulvestrant (PALOMA 3[25], MONARCH 2[26] and MONALEESA 3[27]) have consistently shown that 5-15% of patients show primary resistance to the experimental therapy, with disease progression within three months from randomization. Our data suggest that the group of patients with incomplete response in sTKa at D15 may identify those with primary resistance to palbociclib+fulvestrant. Secondly, PYTHIA showed that patients who experienced a rebound of sTKa at D28 (after a drop below LOD at D15), might have a slightly worse PFS as compared to those with no rebound. These results are provocative, and highlight the importance of assessing sTKa dynamic

changes at multiple, rather than single, timepoints. However, due to the exploratory nature of this analysis and the small sample size, confirmation in larger studies is needed. In this regard, data from the phase IIIb BioItaLee trial have recently been communicated[13] which validate our findings. In this single-arm, biomarker discovery trial, pre- and on-treatment sTKa was confirmed as a strong prognostic biomarker in post-menopausal patients with previously untreated HR+/HER2- MBC, receiving first-line ribociclib and letrozole. Median pre-treatment sTKa in this study was 74.8 Du/L, which is very close to 87 Du/L in PYTHIA; also, similarly in the two studies, a significant reduction in median sTKa at D15 of treatment was observed, with only 15.1% of the patients reporting sTKa above LOD (vs 16.7% in PYTHIA) at this time-point. A subsequent rebound above LOD at day 1 of cycle 2 was observed in 71,4% of the patients (vs 54.7% in PYTHIA). Interestingly, a statistically significant prognostic effect for baseline and D28 sTKa (both using the median and the 200 Du/L cut-offs), and of D15 sTKa (using the LOD cut-off) was observed in BioItaLee. These effects were of similar magnitude as those observed in our study. These data underline the robustness of our observations and suggest that sTKa has similar potential as a biomarker across different lines of treatment in the metastatic setting, endocrine sensitivity status and different CDK4/6i used.

Data of Ki-67 labelling on primary and/or metastatic archived tissue were available for a subset of patients, with moderate correlation between sTKa and tissue Ki-67 observed. In the pre-operative setting, high concordance between changes in sTKa and tumor Ki-67 was observed[21]. Ki-67 labelling on metastatic tumor biopsies can interrogate only a minimal amount of tumor cells, while sTKa can potentially capture information from all metastatic sites in a single measure. This may explain why the correlation observed between tissue Ki-67 and sTKa was not strong.

Other potential prognostic circulating biomarkers in patients treated with CDK4/6i have been proposed[28]. Investigators from the PALOMA 3 trial used ctDNA to show that a reduction in the variant allele frequency of mutations in the *PIK3CA* gene after 15 days of treatment with palbociclib+fulvestrant correlated with good prognosis[29]. This was not observed for mutations in the *ESR1* gene, suggesting that only clonal variants may be useful dynamic biomarkers[29]. Our data with sTKa confirm that capturing signals of reduced cell turnover very early during treatment may inform longer-term prognosis. In this context, sTKa has an advantage over ctDNA-based approaches in being a technologically simpler and cheaper approach, with results obtainable in virtually every patient, not only in those harboring a given clonal variant. The data reported here on sTKa at end of treatment suggest that, as expected, sTKa increases upon radiologically documented disease progression, supporting the hypothesis that increased sTKa during treatment may be a signal of disease progression. Further studies to assess sTKa role in this setting are ongoing and prospective trials investigating the clinical utility of sTKa as a biomarker to guide therapy decisions are warranted.

LIMITATIONS AND STRENGTHS

Our study is limited by the sample size, which does not permit exploration of subgroups within the different dynamic patterns of sTKa during treatment. Another limitation is the lack

of a control arm with fulvestrant alone, which would have permitted the exploration of the predictive value of pre-treatment sTKa in this setting. However, these centrally assessed results strongly suggest a role for sTKa as a stratification tool, and furthermore give novel information on the optimal timing of the assay and the potential interpretation of its results. The availability of matched genomic and transcriptomic data (arising from the AURORA program) for patients enrolled in PYTHIA will allow future integration of sTKa data, with the potential to investigate the biology of breast cancer according to sTKa levels and dynamics.

CONCLUSIONS

sTKa represents a potential novel circulating biomarker that appears to be prognostic in patients with HR+/HER2 negative MBC treated with palbociclib+fulvestrant. Pre-treatment assessment of sTKa is independently prognostic. Dynamic changes as early as 15 days on treatment and subsequent changes after the week-off palbociclib offer unique information and allow for independent risk stratification. These data warrant further validation in larger patient populations.

AUTHORS' DISCLOSURES

L. Malorni reports receiving support from Fondazione AIRC per la Ricerca sul Cancro (22869) for conduct of the PYTHIA trial. F. Hilbers reports receiving funding (to previous Institution affiliated with) from Pfizer for conduct of the PYTHIA trial. M. Ignatiadis reports receiving research grants (to Institution) from Roche, Natera Inc, and Pfizer and serving an advisory board role (honoraria) for Novartis and Seattle Genetics. M. Colleoni reports receiving a research grant (to Institution) from Roche. G. Jerusalem reports grants (to Institution), personal fees and non-financial support from Novartis, grants, personal fees and non-financial support from Roche, grants, personal fees and non-financial support from Pfizer, personal fees and non-financial support from Lilly, personal fees and non-financial support from Amgen, personal fees and non-financial support from BMS, personal fees and non-financial support from Astra-Zeneca, personal fees from Daiichi Sankyo, personal fees from Abbvie, non-financial support from Medimmune, and non-financial support from Merck KGaA. K. Papadimitriou reports serving an advisory board role (honoraria) for Roche, Pfizer, Lilly, and Novartis. F.P. Duhoux reports serving an advisory role (support to Institution) for Roche, Pfizer, AstraZeneca, Lilly, Novartis, Amgen, Daiichi Sankyo, Pierre Fabre, Mundipharma, Seagen, and Teva. I. R. MacPherson reports serving as a consultant for Roche, Novartis, Pfizer, Eli Lilly, Pierre Fabre, Daiichi Sankyo, and Astrazeneca, and reports receiving travel /conference registration support from Roche, Eli Lilly, and Daichi Sankyo. A. Thomson reports receiving speaker fees from Novartis, Roche, Exact Sciences, and Lilly, reports serving on the advisory board for Novartis and MSD, and reports receiving support for attending conferences from BMS, Astellas, MSD, Ipsen, and EUSA. M. Bergqvist reports being an employee and holding stock in Biovica. G. Zoppoli reports receiving travel grants from Novartis and Roche, and reagents from ThermoFisher Scientific and Cytiva. J.M. Bliss reports receiving research funding from AstraZeneca, Merck Sharp & Dohme, Puma Biotechnology, Clovis Oncology, Pfizer, Janssen-Cilag, Novartis, Roche, and Eli Lilly. H. De

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FUNDING

PYTHIA received financial support for trial conduct from Pfizer and the IBCSG. Pfizer and AstraZeneca provided drug supply. BIOVICA supplied support for sample handling and assays. Pfizer and AstraZeneca did not have a role in the reporting or interpretation of the trial. Support for the coordinating group, IBCSG: Frontier Science, Swiss Group for Clinical Cancer Research, Cancer Research Switzerland, Oncosuisse, Cancer League Switzerland, and the Foundation for Clinical Cancer Research of Eastern Switzerland. Support for the AURORA study: Breast Cancer Research Foundation (BCRF, BCRF-19-186 and ELFF-19-00) as the main funder, Foundation Cancer (Luxembourg), National Lottery (Belgium), Foundation NIF, Barrie and Dena Webb, Candriam, Fondation Futur 21, Sogerim, Think Pink Belgium (SMART Fund) and many individual donors. AURORA has also been supported by the Fund Friends of BIG, managed by the King Baudouin Foundation. Dr. Malorni is supported by a grant from the Fondazione AIRC per la Ricerca sul Cancro (22869).

DATA SHARING

Access to de-identified individual participant data used in this study may be requested by researchers by submitting a research proposal (pythia@bigagainstbc.org), which will be reviewed for scientific merit and feasibility in accordance with PYTHIA Policy for Access to Study Data and Biological Samples.

Legends:

Figure 1 (b-e). sTKA distribution. (b) Boxplot of log-transformed sTKa values at baseline. (c) Spaghetti plot of individual log-transformed sTKa values across timepoints for each patient (n=122). (d) Boxplot of log-transformed sTKa values at D28. (e) Boxplot of log-transformed sTKa values at EOT.
In the boxplots the lower and upper hinges correspond to the first and third quartiles, the upper whisker extends from the hinge to the largest value no further than 1.5 * IQR from the hinge, the lower whisker extends from the hinge to the smallest value at most 1.5 * IQR of the hinge, data beyond the whiskers is plotted as points, horizontal line across the box is the median.

Figure 2. Distributions of log-transformed sTKa at baseline and log-transformed tissue Ki67 measured in (a) primary breast tissue (n=65) or (b) pre-treatment metastatic biopsy tissue (n=55). Spearman correlation coefficients are shown.

Figure 3. Distribution of PFS according sTKa values. (a) pre-treatment median-dichotomized sTKa (n=122); (b) D15 below-above LOD dichotomized sTKa (n=108); (c) D28 median-dichotomized sTKa (n=108).

APPENDIX

PYTHIA (IBCSG 53-14/ BIG 14-04) Investigators and the International Breast Cancer Study Group (IBCSG) Participants

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CHU de Liège au Sart-Tilman, Liège (G Jerusalem: 5 enrolled)
Italy
Istituto Europeo di Oncologia (IEO) IRCCS, Milan (M Colleoni: 23 enrolled);
Instituti Clinici Maugeri, Pavia (A Bernardo: 4 enrolled);
Ospedale di Prato, Prato (L Malorni: 4 enrolled);
Ospedale Degli Infermi, Ponderano (E Seles: 3 enrolled)

European Organization for Research and Treatment of Cancer (EORTC):
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Cliniques Universitaires Saint-Luc, Brussels (F Duhoux: 2 enrolled)
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