

Original Research Article

Pharmacodynamic biomarker of CDK4/6 inhibition: prospective evaluation of serum Cyclin D1, CDK4, and CDK6 in palbociclib-letrozole-treated metastatic breast cancer

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Abstract

Purpose: To evaluate the usefulness of serum cyclin D1, CDK4, and CDK6 as biomarkers of treatment response and progression-free survival (PFS) in metastatic breast cancer (MBC).

Methods: In a single-center prospective observational study, 74 women with hormone-receptor-positive (HR+)/ human epidermal growth receptor-2 (HER2)-negative metastatic breast cancer were recruited from the Oncology Hospital, Medical City in Baghdad, Iraq. The patients were treated with oral Palbociclib (125 mg) for 21 days, then continued with Letrozole (2.5 mg) for 7 days, and completed six treatment cycles. The candidates were divided into two groups based on therapeutic response: Group 1 responded to the palbociclib plus letrozole combination therapy, and Group 2 did not. Serum cyclin D1, CDK 4, and CDK 6 levels were measured using enzyme-linked immunosorbent assay (ELISA) after the 6th cycle. Treatment response was evaluated using RECIST 1.1 criteria. The study was designed and reported in alignment with STROBE guidelines.

Results: Serum cyclin D1 levels were higher in responders than non-responders ($p = 0.070$). Importantly, higher cyclin D1 levels were significantly associated with longer progression-free survival (PFS; $p = 0.027$). In contrast, serum CDK4 and CDK6 levels did not differ between groups and showed no predictive or prognostic value for PFS.

Conclusion: Serum cyclin D1 has demonstrated prognostic relevance in palbociclib-treated MBC, supporting its role as a pharmacodynamic biomarker of CDK 4/6 inhibitors. These findings highlight the limitations of circulating intracellular kinase proteins and support further investigation of dynamic, tumor-derived biomarkers for treatment monitoring.

Keywords: Metastatic breast cancer, Palbociclib, Cyclin D1, CDK4/CDK6, Biomarker

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INTRODUCTION

Cancer remains a leading cause of mortality worldwide, with carcinogenesis driven by the accumulation of genetic alterations in key cellular

proteins, influenced by genetic predisposition and prolonged environmental exposure [1]. Chronic inflammation and infection are established risk factors for malignant transformation, as reactive oxygen species

generated in inflamed tissues induce DNA damage and disrupt tissue integrity, thereby promoting tumorigenesis [2]. Chemotherapy-related toxicity and adverse drug reactions (such as nausea, vomiting, fatigue, and headaches) are frequent complications, substantially diminishing quality of life, prolonging hospitalization, and increasing the economic burden on affected individuals [3].

Metastatic breast cancer (MBC) remains a leading cause of cancer-related mortality worldwide, with the hormone receptor-positive (HR+)/human epidermal growth factor receptor 2-negative (HER2-) subtype accounting for the majority of cases. Estrogen and progesterone receptor expression are strongly correlated, underscoring their importance in predicting disease progression, survival outcomes, and guiding therapeutic selection. Cyclin E, encoded primarily by CCNE1, is a key regulator of the G1/S cell-cycle transition; its overexpression facilitates tumor growth and enhances invasiveness by promoting the transition from G1 to S phase [4].

According to recent epidemiological data from the American Cancer Society, HR+/HER2-breast cancer represents approximately 73 % of all breast cancer diagnoses. For decades, endocrine therapy (ET), such as Tamoxifen, which targets estrogen signaling pathways, has served as the cornerstone of management; however, the treatment landscape was fundamentally transformed by the advent of cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitors. Palbociclib, the first-in-class oral CDK4/6 inhibitor, binds selectively and reversibly to cyclin-dependent kinases 4 and 6 (CDK4/6) and inhibits them, which prevents phosphorylation of retinoblastoma (Rb) tumor suppressor protein. This inhibition maintains the Rb protein in its hypophosphorylated state, preventing the release of E2F transcription factor and blocking the G1/S transition. Consequently, palbociclib induces sustained G1 phase arrest, effectively blocking cancer cell proliferation and significantly improving progression-free survival (PFS) when combined with endocrine therapy in the pivotal PALOMA-2 and PALOMA-3 trials, establishing it as a standard-of-care regimen [5]. Letrozole, a potent aromatase inhibitor, competitively and reversibly binds to the heme iron center of cytochrome P450 19A1 (CYP19A1/aromatase), blocking the conversion of C19 androgens to C18 estrogen. It has demonstrated superior efficacy and safety compared to tamoxifen [6], validating its role in combination therapy. Despite these clinical advances, the heterogeneity in MBC leads to

varied outcomes, with intrinsic resistance in a subset of patients and acquired resistance developing in nearly all cases [7].

Palbociclib exerts its anti-proliferative effect by selectively inhibiting the CDK4/6-retinoblastoma (Rb)-E2F axis, thereby preventing Rb phosphorylation and arresting the cell cycle in G1 phase. While this targeted approach is highly effective, the emergence of resistance is complex, including a case of CCNE1 gene (Cyclin E1) overexpression [8]. Other mechanisms of acquired resistance are Rhenoblastoma-1 gene (RB1) loss-of-function mutations and high p16 expression [9]. Current evaluation of therapeutic efficacy relies primarily on serial radiological imaging using the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 criteria, which have notable limitations in evaluating non-measurable disease, such as a bone-only metastasis, which are frequent in HR+ breast cancer [10].

To overcome these limitations, dynamic and noninvasive biomarkers are urgently needed to accurately evaluate molecular response to CDK4/6 inhibitors earlier than traditional imaging. Liquid biopsy-based biomarkers have emerged as a promising strategy for real-time monitoring. Researchers demonstrated that serum and tissue biomarkers are interconnected, with each marker influencing the other, and these interactions play a critical role in determining the progression of breast cancer [11]. Some scientists established the utility of circulating tumor DNA (ctDNA), and failure to clear ctDNA or a rise in specific mutations after one cycle of treatment is strongly predictive of rapid disease progression [12].

Other parameters such as inflammatory cytokines and oxidative stress markers, serve as potential biomarkers for early disease detection and assessment of disease severity as they are significantly elevated in breast cancer [13]. In addition, thymidine kinase 1 (TK1) and other enzymes strictly regulated by the E2-promoter-binding factor (E2F) pathway have been investigated as critical biomarkers. Decreased plasma TK1 activity is seen in responders due to successful cell cycle arrest, whereas persistent activity elevation serves as an early warning of resistance, offering a functional readout of the CDK 4/6 pathways status in vivo. These findings suggest that integrating such biomarkers could guide more precise, adaptive therapeutic strategies in MBC [14]. Genetic polymorphisms in drug-metabolizing enzymes contribute to inter-individual variability in drug response and toxicity [15].

Serum cyclin D1 has never been validated as a predictive biomarker for CDK4/6 inhibitor efficacy before in any prospective clinical trial or retrospective biomarker analysis from major trials (PALOMA, MONALEESA, MONARCH).

This study aims to evaluate serum Cyclin D1, CDK4 and CDK6 levels after six treatment cycles as predictive biomarkers of treatment response and to assess their association with progression-free survival in patients receiving CDK4/6 inhibitors.

EXPERIMENTAL

Study design

This prospective observational study was conducted at the Medical City complex, Baghdad, Iraq, between February 2024 and March 2025. This study was reviewed and approved by the Ethics Committee at the University of Baghdad, College of Pharmacy, Baghdad, Iraq (approval no. RECAUBCP0525145A). All patients were informed about the purpose and procedures of this study, and their participation was voluntary and confidential. Verbal informed consent was obtained from each participant before enrollment, in accordance with the approved institutional protocol.

The study was designed and reported in alignment with Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines [16]. A total of 74 women were enrolled, aged 35 to 60 years, with confirmed diagnosis of HR+/HER2- metastatic breast cancer. The participants were divided into two groups: Group 1 was for patients who responded to the palbociclib plus letrozole combination therapy, while Group 2 did not respond to this treatment regimen. This cohort was ethnically homogenous (Middle Eastern). All subjects initiated first-line combination therapy consisting of letrozole (2.5 mg/day) orally and palbociclib (25 mg/day) orally administered on a 21-days-on/7-days-off schedule per 28-day cycle. Only subjects who completed at least six consecutive treatment cycles were included in the analysis. Clinical evaluation was conducted according to routine institutional workflow, consisting of clinical assessment and PET scan to determine treatment response. Response was categorized according to RECIST 1.1 criteria into responders, graded as complete response, partial response, or stable disease, and non-responders depicted as progressive disease. On the other hand, progression-free survival was defined as the time from the first dose to the

earliest occurrence of radiologically or clinically documented disease progression or death from any cause during the 6 treatment cycles.

Patients with other breast cancer subtypes (e.g., triple-negative or HER2+) and those who did not complete six cycles of palbociclib–letrozole therapy were excluded from the study.

Blood collection and biomarker assay

Peripheral venous blood (5 mL) samples were collected for biomarker analysis at the follow-up visit after administration of the 6th cycle of palbociclib-letrozole. Blood was collected into gel tubes and allowed to clot for 20 - 30 minutes at room temperature. Samples were then centrifuged at 3000 rpm for 15 minutes to separate the serum. The resulting serum was carefully separated, aliquoted into cryovials to avoid repeated freeze-thaw cycles and subsequently stored at -80 °C until batch biomarker analysis.

Measurement of serum cyclin D1, CDK4, and CDK6

Serum concentrations of cyclin D1, CDK4, and CDK6 were estimated using commercially available sandwich ELISA kits (SEA585Hu, SEB233Hu, SEC059Hu, and SEC823Hu; USCNk Life Science INC., Wuhan, China) according to the manufacturer's instructions.

Briefly, standards and serum samples were added to antibody-coated wells and incubated. After washing to remove unbound material, wells were exposed to horseradish peroxidase (HRP)-conjugated detection antibody and subsequently treated with a chromogenic substrate. The reaction was terminated, and absorbance was read at 450nm using a microplate reader, and concentrations were calculated from the standard curve.

Data analysis

Data analyses were conducted using the Statistical Package for the Social Sciences (SPSS) for Windows version 26.0. Mann-Whitney U test was used to compare the distribution of a continuous variable between two independent groups (responders vs non-responders). Receiver Operating Characteristics (ROC) curve analysis assessed the discriminative capacity of the biomarker to distinguish between two outcomes and to identify an optimal diagnostic cut-off. Kaplan Meier survival analysis estimated the time-to-event outcomes across different patients. Log-rank test was used to compare

survival distribution and significant difference in time to event outcomes between groups. Hazard ratio estimation was used to quantify the relative risk of an event over time.

RESULTS

Cyclin D1

Serum cyclin D1 levels were higher in responders compared with non-responders (14.22 vs 9.02 ng/mL). The between-group difference was not conventionally significant according to Mann-Whitney U test ($U = 825.0$, $p = 0.070$; Table 1), though a trend toward a higher mean cyclin D1 in the responder group was observed. The result of ROC analysis was used to determine positive outcome of the treatment. Cyclin D1 demonstrated modest discrimination in identifying responders, with an AUC of 0.63 (95 % CI: 0.49 - 0.75; $p = 0.055$). The Youden-optimized cut-off of approximately 9.65 ng/mL yielded sensitivity and specificity of 50 and 80 %, respectively, for predicting response. This indicates moderate classification performance despite the favorable trend. In contrast, time-to-event analysis demonstrated a more apparent prognostic effect on PFS. When patients were dichotomized at the median cyclin D1 level (8.0 ng/mL), the higher cyclin D1 group experienced fewer progression events (6/37 vs 14/37) and a longer mean PFS (7.9 vs 5.6 months) than the lower cyclin D1 group. Kaplan-Meier analysis confirmed statistically significant improvement in the PFS for patients with higher mean cyclin D1 levels (log-rank $Z = -2.21$, $p = 0.027$; Table 2), with an estimated hazard ratio of approximately 0.30, consistent with a favorable prognostic impact of elevated cyclin D1.

Cyclin-dependent kinase 4 (CDK4)

Mean CDK4 concentrations were broadly similar in responders and non-responders (2.24 vs 2.58 ng/mL, respectively). The Mann-Whitney U test revealed no significant difference between the two groups ($U = 545.0$, $p = 0.207$; Table 1). The ROC analysis, with treatment non-response defined as a positive class, yielded an AUC of 0.59 (95 % CI: 0.45 - 0.72, $p = 0.20$), indicating weak, non-significant discrimination. At the

Youden-derived cut-off (2.8 ng/mL), sensitivity (53 %) and specificity (73 %) were insufficient for reliable clinical prediction. Consistently, CDK4 did not meaningfully stratify patients by PFS. The dichotomization at the median CDK4 value (2.35 ng/mL) resulted in comparable progression rate (11/37 vs 9/37) and similar mean PFS (6.4 vs 7.2 months) in the high and low CDK4 groups. The log-rank test was non-significant ($Z = 0.51$, $p = 0.61$; Table 2), and the estimated hazard ratio for high versus low CDK4 was approximately 1.26, indicating no clear association between CDK4 levels and PFS.

Cyclin-dependent kinase 6 (CDK6)

Serum CDK6 levels tend to be slightly higher in non-responders than in responders (7.11 vs 5.88 ng/mL), but this difference was clearly non-significant on the Mann-Whitney test ($U = 642.0$; $p = 0.847$; Table 1). In ROC analysis, when the non-responders group was treated as the positive outcome, CDK6 showed no significant discriminative capacity, with an AUC of 0.51 (95 % CI: 0.37 - 0.66, $p = 0.84$). The sensitivity (37 %) and specificity (84 %) for non-responders did not have practical diagnostic usefulness at the Youden-optimized threshold (8.21 ng/mL). Similarly, PFS analysis did not reveal a prognostic effect for CDK6.

The median dichotomization (5.34 ng/mL) generated a similar progression rate in the high and low CDK6 groups (11/37 vs 9/37) and nearly identical mean PFS (6.8 vs 6.7 months). The log-rank test was non-significant ($Z = 0.41$, $p = 0.68$; Table 2), with an estimated hazard ratio of 1.2 for high versus low CDK6, indicating no applicable impact of CDK6 on PFS in this cohort. The ROC analysis demonstrated that cyclin D1 moderately discriminated responders from non-responders (AUC = 0.63, 95 % CI: 0.49 - 0.75, $p = 0.055$). The optimal Youden cut-off of 9.65 ng/mL yielding 50 % sensitivity and 80 % specificity. Stratification by the median cyclin D1 level (8.0 ng/mL) revealed a significant association with progression-free survival (PFS). Patients with high expression had longer mean PFS (7.9 vs 5.6 months) and fewer progression events (6/37 vs 14/37), with a significant log-rank test.

Table 1: Response status of palbociclib-letrozole treatment on serum cyclin D1, CDK4, and CDK6

Biomarker	Responders (mean, ng/mL)	Non-responders (mean, ng/mL)	Mann-Whitney U	P-value
Cyclin D1	14.22±13.1	9.02±7.67	825.0	0.070
CDK4	2.24±1.09	2.58±1.23	545.0	0.207
CDK6	5.88±3.02	7.11±4.12	642.0	0.847

Table 2: ROC and PFS analysis for Cyclin D1, CDK4, and CDK6

Biomarker	Positive class in ROC	AUC (95% CI)	ROC p-value	Youden cut-off (ng/mL)	Sensitivity (%)	Specificity (%)	PFS grouping (median cut-off (ng/mL))	Progression events (high vs low)	Mean PFS, months (high vs low)	Log-rank Z	Log-rank P-value
Cyclin D1	Responder	0.63 (0.49–0.75)	0.055	9.65	50	80	8.0	6/37 vs 14/37	7.9 vs 5.6	-2.21	0.027
CDK4	Non-responder	0.59 (0.45–0.72)	0.20	2.8	53	73	2.35	11/37 vs 9/37	6.4 vs 7.2	0.51	0.61
CDK6	Non-responder	0.51 (0.37–0.66)	0.84	8.21	37	84	5.34	11/37 vs 9/37	6.8 vs 6.7	0.41	0.68

By contrast, CDK4 (AUC = 0.59, $p = 0.20$) and CDK6 (AUC = 0.51, $p = 0.84$) showed limited discriminatory capacity, and their serum levels were not significantly associated with PFS (CDK4: $p = 0.61$; CDK6: $p = 0.68$), indicating no meaningful prognostic values in this cohort.

DISCUSSION

In this cohort of HR + HER2- advanced breast cancer treated with palbociclib and letrozole, the prognostic and predictive values of circulating cyclin D1, CDK4 and CDK6 have been explored. The findings provide preliminary insight into the biological behavior of different components of CDK4/6-cyclin D-axis in the context of CDK4/6 inhibition.

Elevated serum cyclin D1 in responders, in addition to the significantly long progression-free survival and fewer progression events, indicates that circulating cyclin D1 is a strong prognostic biomarker rather than a predictive one in metastatic breast cancer treated with palbociclib and letrozole. This incident could be described as the “drain is clogged” effect. Elevated intracellular cyclin D1 in palbociclib responders reflects the fundamental mechanism of cell cycle arrest induced by CDK4/6 inhibition. Under normal conditions, Cyclin D1 expression is tightly regulated throughout the cell cycle and is highly expressed during the G1 phase, facilitating progression toward the S phase. However, upon transition into S phase, cyclin D1 becomes subjected to rapid proteasomal degradation through GSK3 β -mediated phosphorylation, a critical mechanism that prevents uncontrolled replication. In responsive tumors, palbociclib induced G1 arrest, preventing cells from entering S phase, thereby blocking the degradation signal that normally terminates cyclin D1 expression. While *de novo* cyclin D1 synthesis continues unabated, the forestalling of S phase entry precludes the proteolytic clearance mechanisms, consequently resulting in substantial intracellular accumulation of the protein. This paradoxical elevation of cyclin D1 in treatment responders serves as a pharmacodynamic biomarker of successful CDK4/6 inhibition and G1 checkpoint enforcement, rather than an indicator of aggressive proliferation or treatment resistance [17]. However, the magnitude of discrimination for response was only moderate and confidence intervals were wide, so cyclin D1 should currently be regarded as a hypothesis-generating biomarker rather than a clinically actionable test. The prognostic utility of serum cyclin D1 is modest compared with validated functional biomarkers. TK1 activity at cycle 2 showed a strong association with PFS and rising TK1

anticipated radiologic progression by 2 - 6 months, while early ctDNA clearance in PALOMA-3 predicted significantly longer survival outcomes. Unlike TK1 and ctDNA, which reflect dynamic pathway activity or human-specific genomic alterations, total serum protein likely includes substantial non-tumor contributions [12]. Some studies that assessed serum cyclin D1 reported a high sensitivity and specificity, and a diagnostic accuracy of 96 % for breast cancer detection in cross-sectional analysis [18-19].

The findings of this study align with a study in 2021 that analyzed 1,003 Middle Eastern breast cancer patients and found that cyclin D1 overexpression was an independent predictor of favorable overall survival [20]. Similarly, another study revealed that patients with cyclin D1 overexpression had a higher tumor-free survival rate and a longer cumulative tumor-free survival time, so the overexpression of cyclin D1 is used as an indicator for evaluating the prognosis of breast cancer. High cyclin D1 expression in breast cancer is strongly associated with luminal A estrogen receptor+ subtypes. These tumors are dependent on estrogen signaling and less aggressive, as their growth relies on a hormone-driven pathway rather than on uncontrolled proliferation mediated by alternative oncogenic mechanisms [21].

Serum CDK4 and CDK6 concentrations did not show a clinically significant signal in any of the analytical frameworks applied; that is, there is no indication that they contribute to treatment response prediction, prognostic stratification, or PFS discrimination. The mean CDK4 level was comparable between responders and non-responders, and the corresponding ROC analysis showed only a limited, statistically nonsignificant separation, consistent with weak discriminatory performance. Even at the empirically optimized decision threshold, CDK4 achieved approximately 53 % sensitivity and 73 % specificity for identifying non-responders, falling short of what would be required for reliable clinical application.

In parallel, serum CDK6 behaved essentially as a non-informative biomarker. Its ROC performance was indistinguishable from random classification, and the optimized cut-off was sensitive in only about 37 % of non-responders, despite relatively high specificity (84 %). Effectively, these findings suggest that circulating CDK4 and CDK6 are unlikely to be clinically helpful in this context. These findings are biologically consistent with the understanding that CDK4 and CDK6 are intracellular (nuclear and cytoplasmic) kinases, not routinely secreted into the circulation, and

serum levels slightly reflect contributions from multiple non-tumor tissues (bone marrow, lymphocytes, and proliferating epithelium) rather than tumor-specific origins. In addition, CDK4 exists as multiple protein forms due to alternative splicing and post-translational modifications, which complicates accurate quantification and may render tumor-specific signal ambiguous [22]. The failure of serum CDK6 to epitomize tissue-based prognostic associations is particularly informative. While a study demonstrated that high tissue CDK6 expressions (> 1 % by immunohistochemistry) were significantly associated with worse PFS in the PARSIFAL trial, serum measurements of CDK6 expression in this study showed no prognostic effect [23]. This discordance indicates that serum CDK6 protein does not accurately reflect tumor tissue CDK6 expression, because circulating protein originates more likely from non-tumor sources.

Strength and limitations of this study

The strength of this study lies in its novelty as the first systematic evaluation of serum CDK4/6 protein in Iraq in this context. The findings suggest that serum CDK4/6 levels are unlikely to have clinical usefulness as circulating biomarkers; hence, further studies on alternative assay modalities based on tumor-derived measurements are warranted. However, several limitations exist; first, ELISA specificity is limited; the ELISA kits used measure total protein but cannot distinguish active (phosphorylated) forms, splice variants, or tumor-derived versus host-derived protein. Second, static baseline measurements overlook critical pharmacodynamic changes that have been proven productive in TK1 and ctDNA studies. Third, pre-analytical variables (sample handling) may impact protein stability, and modest sample size limits statistical power for subtle subgroup analysis.

CONCLUSION

This study has revealed that serum cyclin D1 level has prognostic value, whereas serum CDK4/6 level determination showed no reliable prognostic or predictive importance in CDK4/6 inhibition. These findings buttress the biological rationale that serum proteins derived from intracellular kinases do not reliably reflect tumor-specific activity, unlike established functional biomarkers such as TK1 activity and ctDNA kinetics. Overall, this study highlights the need to emphasize functional and tumor-derived biomarkers over kinase-related serum proteins in disease therapy and progression.

DECLARATIONS

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Use of Artificial intelligence/Large language models

No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions.

Ethical approval

The study was approved by the Institutional Ethics Committee, College of Pharmacy, University of Baghdad (RECAUBCP0525145A).

Availability of data and materials

The data sets generated and analyzed during the current study are not publicly available due to patient privacy and institutional regulations of the College of Pharmacy, University of Baghdad, but are available from the corresponding author on reasonable request and after approval by the institutional ethics committee.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Adnan Mustafa Ismail: Design of the study, sample and data collection, analysis, and writing. Eman Saadi Saleh: Study design, revision of analysis, and final approval to publish. Ahmed Zuhair Alsammarraie: Revising the work, patients' follow-up.

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