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Cyclin D1 differential activation and its prognostic impact in patients with advanced breast cancer treated with trastuzumab

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ABSTRACT

Introduction We sought to determine the level of activation of the critical components of the cyclin D1-mediated pathway and to evaluate their prognostic significance across the different molecular subtypes of advanced breast cancer.

Patients and methods The study population comprised 219 female patients with advanced breast cancer who had been found to have human epidermal growth factor receptor 2 (HER2)-positive disease by local testing and were all treated with trastuzumab-based regimens. For all tumours, central testing for HER2 was performed, and cyclin D1 gene (*CCND1*) amplification, mRNA and protein expression were assessed by FISH, quantitative real-time-PCR and immunohistochemistry, respectively. Prognostic impact on clinical endpoints was evaluated with Cox regression analyses.

Results After central testing, only 134 (61.2%) of 219 patients were confirmed to have HER2 gene amplification by FISH and/or 3+ HER2 protein expression by immunohistochemistry. After a median follow-up time of 136.0 months (95% CI 123.3 to 148.9), 105 (78.4%) HER2-positive patients and 76 (89.4%) HER2-negative patients had died, while 80% of the former and 87.1% of the latter had experienced a disease relapse. Patients with positive oestrogen receptor/progesterone receptor status presented with higher cyclin D1 mRNA expression. In the HER2-negative subgroup, patients with negative cyclin D1 protein expression were at higher risk of progression (HR= 1.66, 95%CI 1.01 to 2.72, Wald's p=0.045). Among de novo metastatic patients, the risk of progression was higher for patients with non-amplified *CCND1* tumours (HR= 2.00, 95% CI 1.03 to 3.90, p=0.041).

Conclusion Aberrant activation of the cyclin D1-mediated pathway appears to reduce the risk of progression in HER2-negative tumours, but not in HER2-positive ones.

Key questions

What is already known about this subject?

- The cyclin D1-mediated molecular pathway exhibits significant crosstalk with the oestrogen receptor/progesterone receptor and human epidermal growth factor receptor 2 (HER2) pathways in patients with advanced breast cancer.
- Aberrant expression of the gene encoding cyclin D1 (*CCND1*) has been shown to correlate with increased tumour proliferation in breast carcinomas and has also been correlated with antitumour activity of hormonal therapy, including tamoxifen and aromatase inhibitors.

What does this study add?

- Patients with positive oestrogen receptor/progesterone receptor status presented with higher cyclin D1 mRNA expression.
- In the HER2-negative subgroup, patients with negative cyclin D1 protein expression were at higher risk for progression.
- Among de novo metastatic patients, the risk of progression was higher for patients with non-amplified *CCND1* tumours.

How might this impact on clinical practice?

- If our results are validated by prospective trials, further evaluation of the cyclin D1-mediated pathway might identify prognostic and therapeutic implications in patients with advanced breast cancer.

INTRODUCTION

Despite recent advances in molecular biology and therapeutics, breast cancer remains a highly lethal malignancy worldwide.[1] Breast cancer represents a heterogeneous disease entity that can be further categorised by the use of simple immunohistochemical molecular markers, including the oestrogen receptor (ER), the progesterone receptor (PgR), the epidermal growth factor receptor, the c-erbB2 (human epidermal growth factor receptor 2 (HER2)/neu) receptor, the

mitotic index Ki67 and the cytokeratins 5/6.[2] Classification of early breast cancer according to these criteria leads to five distinct immunophenotypical subtypes, namely the luminal A, luminal B, luminal-HER2, HER2-enriched and triple-negative tumour types, each one comprising a different constellation of markers.[3]

The mammalian cell cycle is driven by a complex interplay between cyclins and their associated cyclin-dependent kinase (CDK) partners, and dysregulation of this process is one of the hallmarks of breast cancer.[4] Today the cell cycle is viewed as an orderly progression of distinct phases (G1, S, G2, M), with various cyclin/CDK combinations being essential in regulating this process. Cyclin D1 is a member of the superfamily of cyclins and is an important regulator of the cell cycle, acting mainly as an effector of mitosis by activating the CDKs 4 and 6.[5] Evidence indicates that dysregulation of the cyclin D1–CDK 4/6 axis has a role in breast cancer, with some tumours overexpressing cyclin D1.[6 7] Additionally, while not necessary for normal mammary gland development, CDK 4 and cyclin D1 are required for induction of breast malignancies in mouse models.[8] Aberrant expression of the gene encoding cyclin D1 (*CCND1*) has been shown to correlate with increased tumour proliferation in breast carcinomas and has also been correlated with antitumour activity of hormonal therapy, including tamoxifen and aromatase inhibitors.[9 10] More recently, the development of selective CDK 4/6 inhibitors, including palbociclib,[11] ribociclib[12] and abemaciclib,[13] that show significant synergistic activity with hormonal therapy such as letrozole and fulvestrant in advanced breast cancer has provided further documentation of the important crosstalk between cyclin D1 and hormonal receptors and has fostered efforts to clarify the mechanisms that underlie these associations.

To elucidate these aspects, we undertook a translational research study focusing on the relation of *CCND1* activation and/or cyclin D1 protein expression with other important determinants of breast cancer immunophenotypes. In particular, we sought to determine the level of activation of the critical components of the cyclin D1-mediated pathway and to evaluate their prognostic significance across the different molecular subtypes of advanced breast cancer.

PATIENT CHARACTERISTICS AND METHODS

Patient cohort

The study population comprised patients with advanced breast cancer who had been treated with trastuzumab-based combinations between March 1999 and June 2009 in all Hellenic Cooperative Oncology Group-affiliated clinical centres, as described previously.[2] Eligibility criteria for case inclusion in the present study were histologically confirmed advanced (de novo metastatic or recurrent) breast cancer; adequacy of clinical data on patients' medical records, demographics, tumour characteristics, treatment details (drug dosage, schedule of administration, adverse events) and clinical outcomes; availability of adequate fresh frozen parrafin embedded (FFPE) tumour tissue for biological marker evaluation; and HER2-positive disease assessed by local testing and trastuzumab-based treatment for relapsed or metastatic disease. Patients who had received trastuzumab as adjuvant or neoadjuvant treatment were excluded from the current analysis.

FFPE tissue processing

All patients had received trastuzumab based on HER2 assessment in local pathology laboratories. However, because of the broad period of patient recruitment during which ER, PgR and HER2 guidelines for breast tumour typing and patient stratification for trastuzumab treatment were repeatedly modified, all tumours were re-evaluated centrally for these basic breast cancer typing parameters, in the laboratory of Molecular Oncology of the Aristotle University of Thessaloniki School of Medicine. ER, PgR and HER2 were re-evaluated centrally according to the American Society for Clinical Oncology/Canadian Association of Pathologists guidelines as previously published.[14]

In total, 219 cases meeting the above eligibility criteria for patients and tissues were examined. Corresponding paraffin blocks were originated from the primary tumour, were obtained at diagnosis before any treatment initiation, and were histologically evaluated on H&E sections for tumour presence and marked for the most tumour-dense areas. Tumour cell content (TCC) was assessed as the ratio of cancer cells versus non-cancer cells in these areas, which was used for manual macrodissection for DNA/RNA extraction and, on a second H&E evaluation, for obtaining cores for tissue microarray (TMA) construction, in this order. For cases with low tumour tissue availability, inclusion of tumour tissue in TMAs was prioritised over DNA/RNA extraction. Manual macrodissection was performed on 10 µm thick unstained sections and processed for dual nucleic acid extraction with silica-coated magnetic beads (Versant Tissue Preparation Reagents, Siemens Healthcare Diagnostics, Tarrytown, New York, USA), according to the manufacturer's instructions. Based on the abundance of tumour tissue on blocks and the availability of thick sections, extracts were divided into two aliquots for storage at −20°C until use. DNase I was added to one aliquot per sample for removing DNA and ensuring the presence of pure RNA for gene expression analyses. TCC was ≥30% in 93% of these cases. Seventeen TMA blocks with 2×1.5 mm cores per tumour were constructed with a manual tissue microarrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA) for the implementation of in situ methods, that is, immunohistochemistry (IHC) and fluorescent in situ hybridisation (FISH). These methods were performed on 3 µm and 5 µm thick TMA sections, respectively.

CCND1 mRNA expression

We applied complementary DNA (cDNA) synthesis for 204 RNA samples, with random primers and SuperScript

III Reverse Transcriptase (Invitrogen, Paisley, UK; cat no 48190011 and 18080044, respectively), according to the manufacturer's instructions. cDNAs were assessed in 10 µL duplicate reactions in an ABI 7900HT system for 45 amplification cycles (default conditions). We used the premade TaqMan MGB assay Hs00765553_m1 (Applied Biosystems/Life Technologies) for the specific detection of *CCND1* mRNA transcripts (GenBank reference NM_053056.2, exons 3–4; transcript size 57 nucleotides), along with the endogenous reference assay 4333767F for GUSB (beta-glucuronidase) mRNA transcripts. We also used a commercially available reference RNA from multiple transformed cell lines (TaqMan Control Total RNA, cat no 4307281, Applied Biosystems) in multiple positions in each 384-well plate as positive control and for inter-run evaluation of the qPCR efficiency. To obtain linear relative quantification (RQ) values, we assessed relative expression as 40-dCT, whereby dCT (or delta cycle threshold, equivalent to Cq in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines) was calculated as ((average target CT) – (average GUSB CT)) from all eligible measurements under the same reading threshold. Inter-run RQ values for the reference RNA were <1 for the target assay. Samples were considered eligible for further analysis if GUSB CT is <36 and deltaRQ for each duplicate pair (intrarun variation) is <0.8.

Immunohistochemistry

IHC staining for ER (clone 6F11, Novocastra, Leica Biosystems), PgR (clone 1A6, Novocastra), Ki67 (clone MIB-1, Dako, Glostrup, Denmark) and c-erbB2 (HER2/neu, A0485 polyclonal antibody, Dako) on each slide was performed as previously described,[14] while detection of cyclin D1 (clone SP4, Spring Bioscience, USA), phosphatase and tensin homolog (PTEN) (clone 6H2.1, code M3627, Dako) and mammalian target of rapamycin (mTOR) (clone 49F9, code 2976, Cell Signaling Technology, Danvers, Massachusetts, USA) proteins was performed as mentioned in earlier published studies.[2 15]

FISH for HER2 and *CCND1* gene status

We applied on TMA sections the ZytoLight SPEC *HER2/TOP2A*/CEN17 Triple Color Probe Kit for *HER2* (code Z-2073) and the ZytoLight SPEC *CCND1*/CEN11 Dual Color Probe (code Z-2071–200) (both from ZytoVision, Bremerhaven, Germany). Digital images were constructed with the specifically developed software for cytogenetics (XCyto-Gen, ALPHELYS, Plaisir, France).

Interpretation of the IHC and gene amplification results

ER and PgR positivity were defined as positive nuclear staining in at least 1% of cancer cells.[16] HER2 status was considered to be positive if HER2 was amplified (ratio >2.2 or copy number >6) by FISH and/or an HER2 score of 3+ was obtained by IHC.[17] For Ki67, 20% was used as cut-off to categorise low (<20%) and high (≥20%) protein status.[18] Using these criteria, we assigned the patients as luminal A (ER-positive and/or PgR-positive and Ki67 <20%), luminal B (ER-positive and/or PgR-positive and Ki67 ≥20%), luminal-HER2 (ER-positive and/or PgR-positive and HER2-positive), HER2-enriched (ER-negative and PgR-negative and HER2-positive) and triple-negative (ER-negative and PgR-negative and HER2-negative).

Positivity for cyclin D1 was evaluated using an all red score scale from 0 to 8 resulting from the sum of staining intensity and per cent of positive tumour cells while positive samples were considered those with score >4.[19] PTEN evaluation (cytoplasmic, nuclear or both) was based on staining intensity (0—negative, 1—mild, 2—moderate, 3—strong), and only moderate and strongly stained samples were considered positive.[20] For mTOR, cases expressing the protein in >1% of tumour cells were considered as positive.[15]

For the evaluation of *CCND1* gene status, we counted target gene locus and centromere signals in 60 non-overlapping cancer cell nuclei. Because there is no consensus for the assessment of *CCND1* gene status, we used cut-offs based on signal counting in normal breast tissues.[21] We used normal breast tissue sections from 20 women without cancer who had undergone reduction mastectomy and calculated the normal cut-offs from the mean counts in these normal nuclei plus 3XSD (SD). Thus, we considered abnormal *CCND1* and chromosome 11 status based on the following cut-offs: >3.37 for *CCND1* copy gain; >3.34 for CEN11 copy gain; and >2.19 for the ratio *CCND1*/CEN11.

The flow chart of the study including the corresponding sample numbers is presented in figure 1.

Statistical considerations

Follow-up information for all patients was updated in January 2017. Distribution characteristics of the examined markers were evaluated and presented for the entire study population, as well as for patients based on their HER2, ER/PgR and disease presentation status. Categorical data are presented as frequencies with corresponding percentages, while the median, minimum and maximum values are presented for the continuous variables. Group comparisons of categorical data were assessed using the χ^2 or Fisher's exact (where appropriate) test, while Wilcoxon rank-sum or Kruskal-Wallis tests were performed to detect differences between categorical and continuous variables.

Progression-free survival (PFS) was defined as the time from the date of the initiation of trastuzumab treatment for metastatic disease (with or without concurrent chemotherapy/hormonal therapy) to the date of the first documented disease progression, death or last contact (whichever occurred first). Survival was also measured from the initiation of trastuzumab treatment to the date of death. Alive patients were censored at the date of their last contact. Survival curves were estimated using the Kaplan-Meier method and compared across groups with the log-rank test. The prognostic value of cyclin D1 mRNA expression was examined with respect to PFS and survival using the median value as the optimal cut-off, and

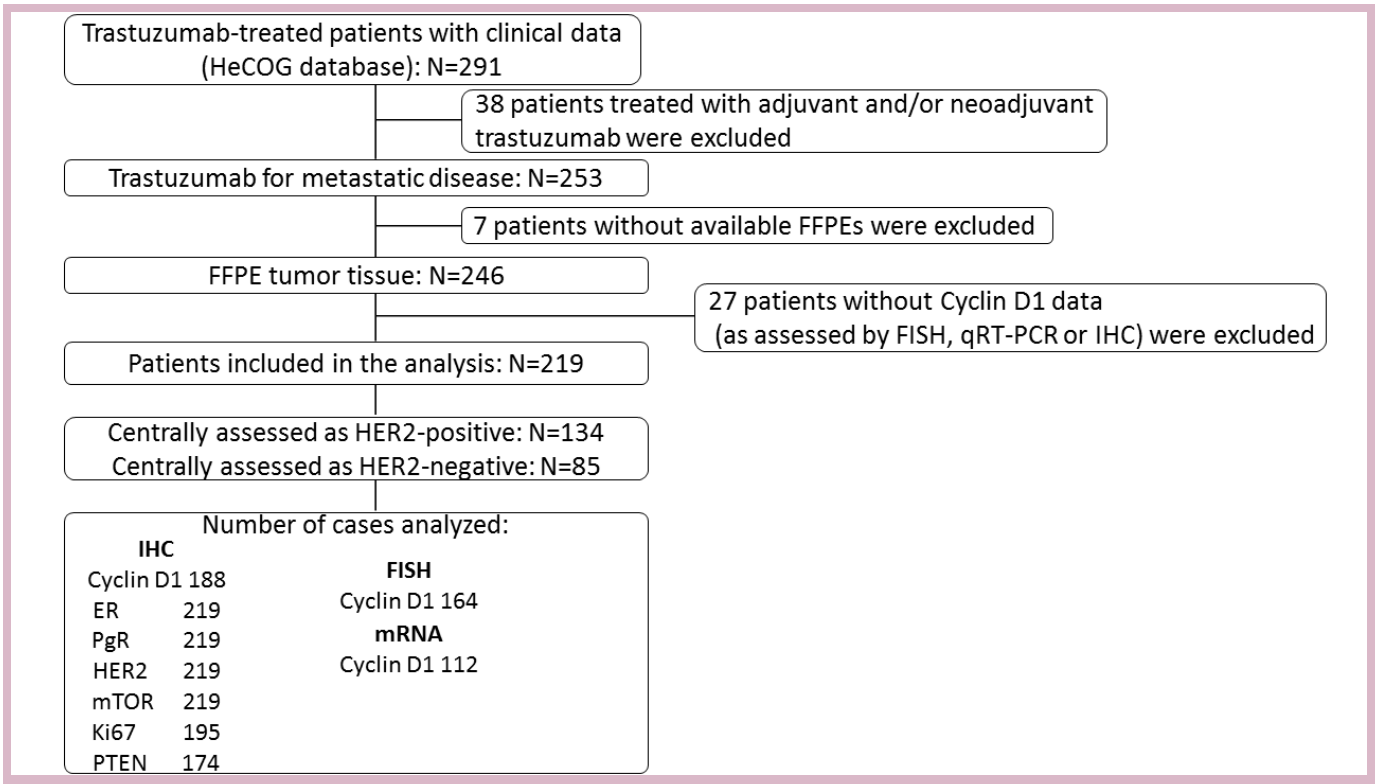

Figure 1 REMARK diagram. ER, oestrogen receptor; FFPE, fresh frozen paraffin embedded; FISH, fluorescent in situ hybridisation; HeCOG, Hellenic Cooperative Oncology Group; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; mTOR, mammalian target of rapamycin; PgR, progesterone receptor; PTEN, phosphatase and tensis homolog; qRT-PCR, quantitative reverse transcription-PCR.

if this was not significant the upper and lower quartiles were assessed, as possible thresholds.

All parameters were tested for proportionality using time-dependent covariates. The associations between the factors of interest and progression/mortality rates were evaluated with HR estimated with univariate Cox proportional hazard regression models. Multivariate Cox regression analyses were also performed, including the following clinicopathological parameters: menopausal status (reference: postmenopausal), performance status (reference: 0), subtype classification (reference: luminal B), PTEN status (reference: loss), as well as each of the markers that were found to be significant or revealed a trend towards significance in the univariate analysis ($p<0.10$).

The PFS analyses were conducted in the entire cohort as well as in the subgroups of patients with HER2-positive, HER2-negative, recurrent and de novo metastatic breast cancer (MBC). Survival analyses were performed in the subgroups of HER2-positive, recurrent and de novo metastatic patients, excluding patients with HER2-negative tumours from the latter two subgroups.

Results of this study are presented according to the reporting recommendations for tumour marker prognostic studies.[22] All tests are two-sided at an alpha 5% level of significance. Analyses were conducted using SAS V.9.3 software.

RESULTS

Patient flow

A total of 219 patients with advanced breast cancer treated with trastuzumab were included in the analysis. Among them, only 134 patients (61.2%) were found to have HER2 gene amplification by FISH and/or 3+ HER2 protein overexpression, by IHC according to central HER2 assessment. Thus, 85 (38.8%) patients had been treated with trastuzumab even though they were found to be HER2-negative by central re-evaluation. Selected patient and tumour characteristics for HER2-positive and HER2-negative patients are presented in table 1. Overall, there were 71 cases of stage IV breast cancer (patients with de novo MBC), while in the rest of the patients (67.6%) breast cancer was diagnosed at earlier stages of the disease (patients with recurrent breast cancer). A total of 185 (84.5%) patients were treated with first-line trastuzumab therapy (64 HER2-negative and 121 HER2-positive patients), while in the remaining 34 (15.5%) patients trastuzumab was administered later in the course of metastatic disease. Trastuzumab was administered in combination with chemotherapy in 198 (90.4%) patients, while 17 patients received the drug with concurrent hormonal therapy and 4 patients received trastuzumab as a monotherapy.

The median duration of follow-up was 136.0 months (95% CI 123.3 to 148.9) for the entire study population,

Table 1 Selected patient and tumour characteristics according to HER2 status

	HER2 status	
	Negative (n=85)	**Positive (n=134)**
Age (years)*		
Median (min–max)	59.0 (31.8–78.8)	54.7 (28.4–95.0)
	n (%)	**n (%)**
Menopausal status*		
Premenopausal	18 (21.2)	35 (26.1)
Postmenopausal	67 (78.8)	97 (72.4)
Unknown	0 (0.0)	2 (1.5)
Performance status*		
0	60 (70.6)	95 (70.9)
1–2	24 (28.2)	39 (29.1)
Unknown	1 (1.2)	0 (0.0)
Histological grade		
I–II	38 (44.7)	54 (40.3)
III	40 (47.1)	72 (53.7)
Unknown	7 (8.2)	8 (6.0)
Subtype classification		
Luminal A	15 (17.6)	0 (0.0)
Luminal B	51 (60.0)	0 (0.0)
Luminal-HER2	0 (0.0)	87 (64.9)
HER2-enriched	0 (0.0)	47 (35.1)
TNBC	12 (14.2)	0 (0.0)
Unknown	7 (8.2)	0 (0.0)
Number of metastatic sites*		
1–2	61 (71.8)	101 (75.4)
≥3	23 (27.1)	33 (24.6)
Unknown	1 (1.2)	0 (0.0)
Number of trastuzumab lines*		
1	32 (37.6)	44 (32.8)
2	19 (22.4)	31 (23.1)
3	13 (15.3)	23 (17.2)
≥4	21 (24.7)	36 (26.9)
Visceral metastases*		
Yes	51 (60.0)	93 (69.4)
No	32 (37.6)	40 (29.9)
Unknown	2 (2.4)	1 (0.7)
De novo MBC	28 (32.9)	43 (32.1)
Recurrent breast cancer	57 (67.1)	91 (67.9)
Adjuvant CT†	50 (87.7)	73 (80.2)
CMF-based adjuvant CT†	29 (50.9)	43 (47.3)
Taxane-based adjuvant CT†	15 (26.3)	34 (37.4)
Anthracycline-based CT†	31 (54.4)	62 (68.1)
Adjuvant HT†	42 (73.7)	64 (70.3)
Adjuvant RT†	30 (52.6)	47 (51.6)

*At the time of trastuzumab initiation.
†Only for patients with recurrent metastatic breast cancer.
CMF, cyclophosphamide/methotrexate/5 fluorouracil; CT, chemotherapy; HER2, human epidermal growth factor receptor 2; HT, hormonal therapy; MBC, metastatic breast cancer; RT, radiotherapy; TNBC, triple-negative breast cancer.

while HER2-positive and HER2-negative patients were followed up for a median of 144.0 (95% CI 122.8 to 152.3) and 125.9 (95% CI 109.4 to 136.0) months, respectively. In total, 105 (78.4%) of the 134 HER2-positive patients and 76 (89.4%) of the HER2-negative patients died, while 80% of the HER2-positive population and 87.1% of the HER2-negative patients experienced a disease progression throughout the study.

The median PFS was 14.0 months (95% CI 11.4 to 17.8) for HER2-positive and 8.9 months (95% CI 7.8 to 11.6) for HER2-negative patients, while the median survival of HER2-positive patients was 48.1 months (95% CI 37.2 to 54.0). HER2-positive patients experienced longer PFS compared with patients with HER2-negative tumours (log-rank p=0.016).

Biomarker distribution and associations

The distribution of the examined markers in the entire study population is presented in table 2, while the markers' distribution by HER2, ER/PgR and disease presentation status is presented in online supplementary tables S1, S2 and S3, respectively, in the online supplementary appendix. Slightly more than half of the patients

Table 2 Distribution of markers in the entire study population

	n (%)
Cyclin D1 protein expression	
Negative	86 (45.7)
Positive	102 (54.3)
Cyclin D1 mRNA expression	
Median (min–max)	42.5 (37.6–46.1)
High*	59 (52.7)
Low*	53 (47.3)
CCND1 gene amplification	
Non-amplified	118 (72.0)
Amplified	46 (28.0)

*The median value was used as the cut-off value.

had tumours with high cyclin D1 protein expression (54.3%) and almost three-quarters with non-amplified *CCND1* (72.0%). No significant differences were observed in the cyclin D1 expression either between patients with HER2-positive and HER2-negative tumours or between patients with de novo MBC and recurrent breast cancer (online supplementary tables S1 and S3). In contrast, compared with ER/PgR-negative tumours, ER/PgR-positive tumours presented with higher cyclin D1 mRNA expression and were more frequently *CCND1* non-amplified (Wilcoxon rank-sum p=0.014 and Fisher's p=0.003, respectively).

Cyclin D1 mRNA expression was higher in patients with *CCND1* gene amplification, as compared with those with non-amplified tumours (median cyclin D1 mRNA expression: 43.50 (min–max: 38.86–46.14) vs 42.31 (min–max: 37.60–45.49), Wilcoxon rank-sum p<0.001), as well as in patients with high cyclin D1 protein expression, as opposed to patients with lack of cyclin D1 protein expression (median cyclin D1 mRNA expression: 43.17 (min–max: 40.80–46.14) vs 41.90 (min–max: 37.60–43.86), p<0.001). In addition, lack of cyclin D1 protein expression was more frequent in patients with *CCND1* non-amplified tumours (χ^2 p=0.019).

Association of cyclin D1 with clinicopathological parameters

Non-amplified *CCND1* was associated with the luminal-HER2 subtype (Fisher's p=0.014), while patients with non-amplified *CCND1* were of lower performance status (χ^2 p=0.026). The association of cyclin D1 expression with several biomarkers of pertinent molecular pathways including Ki67, PTEN protein status, mTOR protein status and PIK3CA mutations, as well as the combined PTEN/mTOR and PIK3CA/mTOR status, was also examined (data on these biomarkers in the same cohort of patients have been previously published[21]). No significant correlations were observed between Ki67 and cyclin D1 expression, while low cyclin D1 protein expression was associated with PTEN loss (χ^2 p=0.002). In addition, low cyclin D1 protein expression was associated with a combined loss of PTEN and mTOR protein expression (p=0.005) (table 3).

Association of cyclin D1 with clinical outcomes

Cyclin D1 (by any method) did not reach any prognostic significance in terms of PFS or survival either in the entire study population (online supplementary table S4) or in the subgroup of patients with centrally HER2-positive tumours (online supplementary table S5). Among patients with HER2-negative tumours, patients with negative cyclin D1 protein expression were at higher risk of progression compared with those with positive cyclin D1 protein expression (HR=1.66, 95% CI 1.01 to 2.72, Wald's p=0.045) (figure 2A, online supplementary table S6). The interactions between the markers of interest and ER/PgR status were assessed with respect to PFS and survival, but no significant interactions were detected (data not shown). Among patients with de novo MBC, the risk of progression was higher for patients with non-amplified *CCND1* tumours compared with patients with *CCND1* gene amplification (HR=2.00, 95% CI 1.03 to 3.90, p=0.041) (figure 2B), while none of the examined markers reached statistical significance in terms of survival. A trend for increased risk of progression was also observed for high cyclin D1 mRNA expression (using the upper quartile as a cut-off) (HR=1.74, 95% CI 0.97 to 3.13, p=0.062) among patients with recurrent breast cancer.

On multivariate analyses, none of the cyclin D1-related biomarkers studied had independent prognostic significance in the multivariate model encompassing established prognostic factors (table 4). In the subgroup of patients with HER2-negative tumours, subtype classification was the only parameter that was found to affect PFS (overall p<0.001), with patients with luminal A tumours presenting with lower risk for progression and patients with triple-negative breast cancer tumours presenting with higher risk of disease progression compared with those with luminal B tumours (HR=0.35, 95% CI 0.14 to 0.89, p=0.027 and HR=3.04, 95% CI 1.36 to 6.80, p=0.007, respectively), while low cyclin D1 protein expression did not retain its unfavourable prognostic significance for PFS (p=0.38). Among patients with recurrent breast cancer, after adjusting for the clinicopathological parameters (described in the Statistical considerations section) and cyclin D1 mRNA expression (using the upper quartile as a cut-off), cyclin D1 mRNA expression was not found to be of prognostic significance for PFS (p=0.28). Among de novo patients, none of the parameters included in the multivariable model reached statistical significance for PFS.

DISCUSSION

In the current study, one of the largest to our knowledge to assess the cyclin D1-mediated molecular pathway in advanced breast cancer, we aimed to evaluate whether the crosstalk of the cyclin D1 pathway with the ER/PR, HER2 and other molecular pathways has prognostic impact on patients treated with trastuzumab for presumably HER2-positive disease. We found that the cyclin D1 protein as assessed by IHC was overexpressed in slightly more than half of patients with advanced breast cancer (54.3%) and the *CCND1* gene was amplified in slightly more than a quarter (28.0%), as assessed by FISH. Both are compatible with recent evidence from the literature reporting cyclin D1 IHC expression ranging between 44% and 52%, and *CCND1* gene amplification in 9%–30%.[23–28] Of note, patients with hormone receptor-positive disease presented with higher cyclin D1 mRNA expression and *CCND1* gene amplification compared with those with negative ER/PgR tumours, which was expected given the strong association between the cyclin D1 molecular pathway and the ER/PgR-mediated pathways reported consistently in the literature and confirmed in a recently reported study.[27] These results suggest that hormone receptor signalling in breast cancer can be mediated

Table 3 Association of cyclin D1 protein expression and *CCND1* gene amplification with clinicopathological parameters

	Cyclin D1 protein expression			*CCND1* gene amplification		
	Negative	**Positive**	**P value**	**Non-amplified**	**Amplified**	**P value**
Ki67						
Median (min–max)	40 (1–85)	40 (1090)	0.52	40 (1–90)	40 (10–90)	0.81
Performance status						
0	61 (70.9)	72 (70.6)	0.96	92 (78.0)	28 (60.9)	**0.026**
1–2	25 (29.1)	30 (29.4)		26 (22.0)	18 (39.1)	
Subtype classification						
Luminal A	5 (6.0)	8 (7.9)	0.22	7 (6.0)	1 (2.2)	**0.014**
Luminal B	16 (19.0)	32 (31.7)		31 (26.5)	13 (28.3)	
Luminal-HER2	35 (41.7)	39 (38.6)		43 (36.8)	28 (60.9)	
HER2-enriched	21 (25.0)	18 (17.8)		28 (23.9)	4 (8.7)	
TNBC	7 (8.3)	4 (4.0)		8 (6.8)	0 (0.0)	
PIK3CA status						
Mutated	11 (15.9)	20 (24.4)	0.20	20 (20.8)	6 (15.0)	0.43
Wild-type	58 (84.1)	62 (75.6)		76 (79.2)	34 (85.0)	
mTOR protein expression						
Negative	41 (47.7)	35 (34.3)	0.063	45 (38.1)	16 (34.8)	0.69
Positive	45 (52.3)	67 (65.7)		73 (61.9)	30 (65.2)	
PTEN protein expression						
Loss	53 (68.8)	39 (44.8)	**0.002**	63 (56.3)	22 (48.9)	0.40
No loss	24 (31.2)	48 (55.2)		49 (43.8)	23 (51.1)	
PIK3CA/mTOR status						
PIK3CA mutated/mTOR negative	4 (5.8)	6 (7.3)	0.17	5 (5.2)	2 (5.0)	0.79
PIK3CA mutated/mTOR positive	7 (10.1)	14 (17.1)		15 (15.6)	4 (10.0)	
PIK3CA wild-type/mTOR negative	27 (39.1)	19 (23.2)		31 (32.3)	12 (30.0)	
PIK3CA wild-type/mTOR positive	31 (44.9)	43 (52.4)		45 (46.9)	22 (55.0)	
PTEN/mTOR status						
PTEN loss/mTOR negative	24 (31.2)	17 (19.5)	**0.005**	28 (25.0)	9 (20.0)	0.77
PTEN loss/mTOR positive	29 (37.7)	22 (25.3)		35 (31.3)	13 (28.9)	
PTEN no loss/mTOR negative	12 (15.6)	13 (14.9)		16 (14.3)	6 (13.3)	
PTEN no loss/mTOR positive	12 (15.6)	35 (40.2)		33 (29.5)	17 (37.8)	

Significant p values are shown in bold.
PI3KCA, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; TNBC, triple-negative breast cancer; mTOR, mammalian target of rapamycin.

through collateral pathways, including cell proliferation operated by cyclin D1, as shown recently by the clinical activity of CDK inhibitors (CDK 4/6 inhibitors) in reversing resistance when added to aromatase inhibitors in women with hormone-sensitive advanced breast cancer.[11–13] A similar concept is currently being explored in trials combining CDK 4/6 inhibitors with trastuzumab and other HER2-targeting agents, such as the PATRICIA and the MONARCHER studies.[12 13]

It should be emphasised that in 38.8% of the samples in our study, initial HER2 positivity assessed in the local setting by means of either IHC or FISH was not confirmed by central lab testing. The fact that more than a third of patients presumed to be HER2-positive and treated with trastuzumab accordingly were finally deemed to be HER2-negative by central testing is worrisome and emphasises the need for quality control in molecular testing. It should be noted, however, that through the 12 years of patient accrual to the trials (from 1998 to 2010), experience on HER2 testing has substantially improved, as well as quality control. As expected, patients deemed to be HER2-negative and treated with trastuzumab had significantly worse clinical outcomes (PFS and overall survival) compared with patients deemed to be HER2-positive and treated with trastuzumab, exemplifying the need for an individualised therapeutic approach based on robust

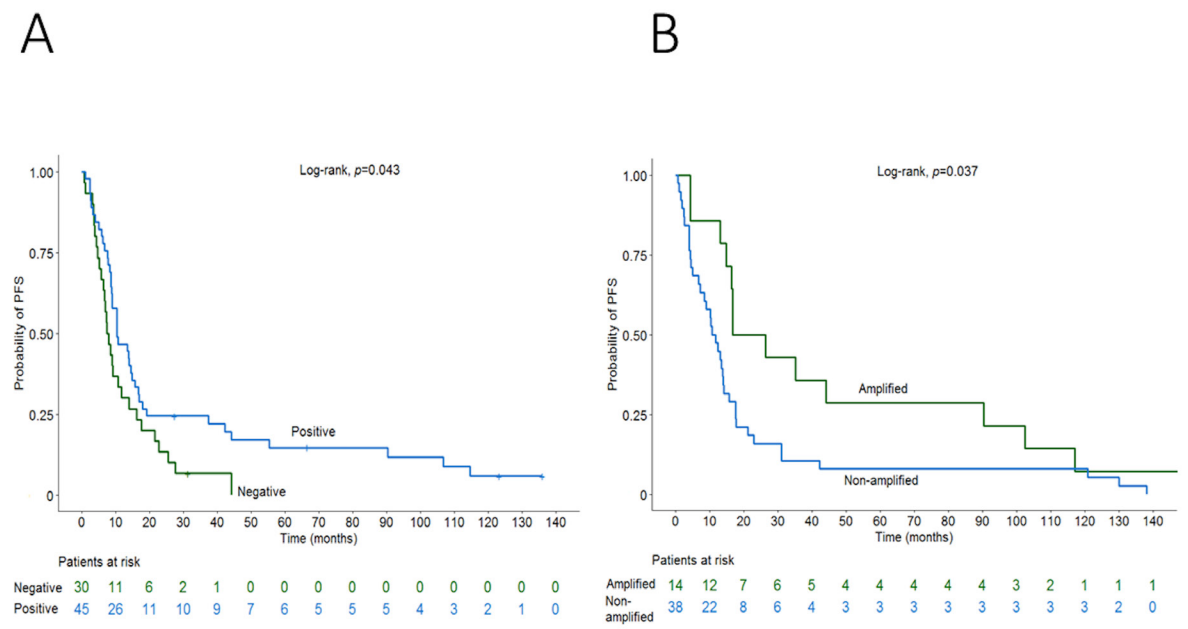

Figure 2 Kaplan-Meier curves with respect to PFS according to (A) cyclin D1 protein expression in patients with HER2-negative tumours and (B) *CCND1* gene amplification in patients with de novo metastatic breast cancer treated with trastuzumab. HER2, human epidermal growth factor receptor 2; PFS, progression-free survival.

molecular testing, in order to avoid unnecessary and expensive treatments and to optimise clinical outcomes. To add on the above observation, the recently reported NSABP-B47 trial clearly showed that, even in patients with early-stage breast cancer with low levels of HER2, defined as IHC 1-positive or IHC 2-positive and/or in situ hybridisation-negative, adding trastuzumab to standard adjuvant chemotherapy did not improve invasive disease-free survival.[28]

We found that low cyclin D1 protein expression was associated with both PTEN loss (χ^2 p=0.002) and with a combined loss of PTEN and positive mTOR protein expression (p=0.005). It is well established that loss of function of the tumour suppressor protein PTEN leads to activation of the phosphatidyl-inositol-3-kinase-Akt-mammalian target of rapamycin (PI3K-Akt-mTOR) pathway.[21] Consequently, it can be hypothesised that in the absence of cyclin D1, cancer cells use the PI3K-Akt-mTOR pathway

Table 4 Effect of cyclin D1 on PFS among patients with (A) HER2-negative, (B) recurrent and (C) de novo metastatic breast cancer: results of the multivariate models

		Univariate			Multivariate		
Parameter	**Category**	**HR**	**95% CI**	**P value**	**HR**	**95% CI**	**P value**
(A) HER2-negative patients							
Cyclin D1 protein expression	Positive	1.00			1.00		
	Negative	1.66	1.01 to 2.72	**0.045**	1.32	0.71 to 2.44	0.38
(B) Patients with recurrent breast cancer							
Cyclin D1 mRNA expression (upper quartile as cut-off)	Low	1.00			1.00		
	High	1.74	0.97 to 3.13	0.062	1.43	0.75 to 2.71	0.28
(C) Patients with de novo MBC							
CCND1 gene amplification	Amplified	1.00			1.00		
	Non-amplified	2.00	1.03 to 3.90	**0.041**	0.53	0.23 to 1.19	0.12

Significant p values are shown in bold.
HER2, human epidermal growth factor receptor 2; MBC, metastatic breast cancer; PFS, progression-free survival.

as a collateral pathway to obviate the absence of cell proliferation proteins and thus circumvent resistance.

Importantly, we found that patients with low cyclin D1 protein expression were at 66% significantly higher risk of progression compared with those with high expression, but only in the HER2-negative cohort of patients, whereas the same correlation was not true for HER2-positive patients. One hypothetical explanation for this is that trastuzumab obscures the effect of cyclin D1 activation only in patients with true HER2-positive disease, whereas in HER2-negative tumours the lack of a true target abolishes the effect of trastuzumab and renders cyclin D1 a reliable biomarker of activity for hormonal treatment. In these patients, it could be possible that the crosstalk between cyclin D1 and hormone receptors becomes more apparent and renders patients more susceptible to hormonal treatment with either tamoxifen or aromatase inhibitors.

Our study has some limitations. The collection and study of tumour samples were performed in a retrospective manner; however, the pathological review of each case and molecular testing were centralised and data regarding clinical outcomes were derived from prospective clinical trials with strict protocol criteria regarding evaluation of clinical endpoints. Notably, the fact that patients finally deemed to be HER2-negative by central testing had received trastuzumab appears as a paradox that may obscure interpretation of the data, but on the other hand it offers a unique opportunity to study treatment effects on both the presence and the absence of the true target, which can provide useful and clinically relevant information. Of course, reproduction and validation of these results will require robustly designed and well-conducted prospective trials incorporating evaluation of the appropriate biomarkers in biological samples obtained during and after the enrolment of patients.

In conclusion, we found that aberrant activation of the cyclin D1-mediated pathway reduces the risk of progression in HER2-negative tumours, but not in HER2-positive ones treated with trastuzumab. These results support the rationale that in HER2-negative patients, the crosstalk between cyclin D1 and other molecular pathways such as the hormone receptors and the PI3K-Akt-mTOR is more evident and confers therapeutic opportunities with various combinations of aromatase inhibitors, CDK 4/6 inhibitors and mTOR inhibitors. If our results are validated by large prospective translational trials, further evaluation of the cyclin D1-mediated pathway may offer important prognostic and therapeutic opportunities in patients with advanced breast cancer.

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