REVIEW

Cyclin E1 and cyclin E2 in ER+ breast cancer: prospects as biomarkers and therapeutic targets

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This article was commissioned following sponsorship of the 7th PacRim Breast and Prostate Meeting by *Endocrine-Related Cancer*

Abstract

Cyclin E1 is one the most promising biomarkers in estrogen receptor positive (ER+) breast cancer for response to the new standard of care drug class, CDK4/6 inhibitors. Because of its strong predictive value, cyclin E1 expression may be used in the future to triage patients into potential responders and non-responders. Importantly, cyclin E1 is highly related to cyclin E2, and both cyclin E1 and cyclin E2 are estrogen target genes that can facilitate anti-estrogen resistance and can be highly expressed in breast cancer. However cyclin E1 and E2 are often expressed in different subsets of patients. This raises questions about whether the expression of cyclin E1 and cyclin E2 have different biological drivers, if high expressing subsets represent different clinical subtypes, and how to effectively develop a biomarker for E-cyclin expression. Finally, several pan-CDK inhibitors that target cyclin E-CDK2 activity have reached Phase II clinical trials. In this review, we outline the data identifying that different cohorts of patients have high expression of cyclins E1 and E2 in ER+ cancer and address the implications for biomarker and therapeutic development.

Key Words

- CDK4/6 inhibitors
- breast cancer
- endocrine therapy resistance
- cyclin E1
- cyclin E2

Endocrine-Related Cancer (2020) **27**, R93–R112

Cyclin E1 and E2 are core cell cycle proteins

The cell cycle is the core machinery that a cell engages in order to undergo a round of DNA replication, replicate its cellular contents, and undergo a cell division. Each cell cycle consists of functional phases: G_1 is the phase where cells ready themselves for DNA replication and commit to a cell cycle, during S phase cells undergo DNA replication, and G_2/M coordinates mitosis. The G_1 to S phase of the cell cycle has particular importance in cancer, as this is the point where cells commit to another round of cell division, and this threshold is weakened in many cancer cells. The gatekeeper for the G_1/S phase transition is the Retinoblastoma (Rb) protein which in its unphosphorylated form inhibits progression into S phase. During G_1/S phase, cyclin dependent kinases (CDKs) phosphorylate Rb; first CDK4/6 is activated by cyclin D1, D2, or D3 to phosphorylate Rb, followed by phosphorylation by CDK2 in complex with cyclin E1 or cyclin E2 (Table 1). Phosphorylated Rb releases E2F transcription factors to upregulate genes necessary for DNA replication and mitosis. The cyclin E1/E2-CDK2 complexes, as well as phosphorylating Rb, phosphorylate a large number of other target proteins involved in DNA replication and histone transcription. In this way the CDK2 kinase synchronises multiple cell cycle events to

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Table 1 Cyclin-CDK complexes in the cell cycle.

Phase of cell cycle	Cyclin	CDK binding partner	CDK inhibitors	References
G_1	Cyclin D1/D2/D3	CDK4 CDK6	$p16^{INK4A}$ $p15^{INK4B}$ $p18^{INK4C}$ $p19^{INK4D}$	(Sherr & Roberts 1999, Sheppard & McArthur 2013)
G_1 / S phase transition	Cyclin E1/E2	CDK2	$p21^{Cip1/Waf1}$	(Prall *et al.* 1997)
S phase	Cyclin E1/E2 Cyclin A2		$p27^{Kip1}$ $p57^{Kip2}$	
G_2 / M phase transition	Cyclin A2	CDK1 (CDC2)		(Yam *et al.* 2002)
M phase	Cyclin B1		WEE1 MYT1	(Fung & Poon 2005)

allow for successful DNA replication and cell division. A further level of control is added through the CDK inhibitor proteins $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, which can inhibit the CDK4, CDK6, and CDK2 complexes to fine tune their activity (Fig. 1).

In addition to their canonical S phase roles, cyclins E1 and E2 coordinate other cell cycle functions, often without a requirement for CDK2. Cyclin E1 and E2 physically localize to centrosomes (Rogers *et al.* 2015), where cyclin E1 binds to Minichromosome Maintenance Complex Component 5 (MCM5) (Ferguson & Maller 2008) and cyclins E1 and E2 facilitate loading of MCM replicative helicases in cells exiting quiescence (Geng *et al.* 2007). Cyclin E1 promotes proliferation in a CDK2 independent manner in hepatocytes and hepatocellular carcinoma, and this may be due to the kinase independent roles at the centrosome and during re-entry from quiescence (Geng *et al.* 2018).

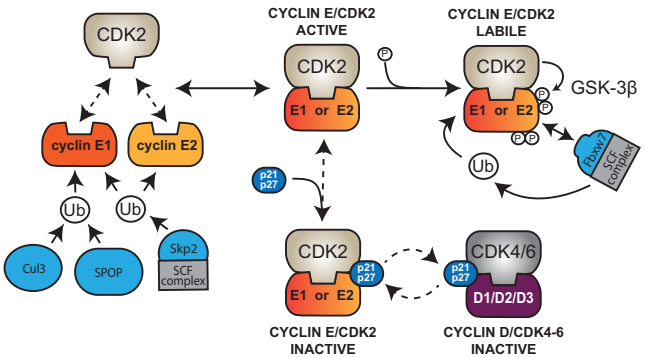

Figure 1
Activation and de-activation of cyclin E1/E2-CDK2. CDK2 binds with either cyclin E1 or cyclin E2 to form cyclin E-CDK2 complexes. Cyclin E1 monomers can be ubiquitinated by Cul3 and SPOP and cyclin E1/E2 monomers by SCF^{Skp2} complexes, leading to proteosomal degradation. Cyclin E-CDK2 complexes can be inhibited by CDK inhibitors $p21^{Waf1/Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$, but cyclin D-CDK4/6 complexes compete for these inhibitors. Active cyclin E-CDK2 complexes autophosphorylate cyclin E, priming for further GSK-3β mediated phosphorylation. Phosphorylated cyclin E is recognised for SCF^{Fbxw7} mediated ubiquitination, followed by proteosomal degradation.

The major isoform of cyclin E1 is a 395 amino acid protein transcribed from the 19q12 locus, and cyclin E2 encodes a 69.3% similar protein of 404 amino acids, transcribed from an independent gene located at chromosome 8q22. As we have described previously (Caldon & Musgrove 2010), the E-cyclin genes are conserved in vertebrates, but a single E-type cyclin is encoded in invertebrates such as *Drosophila melanogaster*. There are some critical differences in function between the two E-cyclins, the most striking of which is that cyclin E2-/- male mice are infertile, while cyclin E1-/- mice have normal fertility. There are also some examples of CDK targets that appear unique to either cyclin: SAMHD1, which controls dNTP cellular pools, is a specific target of cyclin E2-CDK2 (Hu *et al.* 2018), and NPAT, a master regulator of histone transcription, which preferentially co-localises with cyclin E2 (Rogers *et al.* 2015). Another key difference between cyclin E1 and E2 is that cyclin E1 can be cleaved into a low molecular weight (LMW) protein, which hyperactivates CDK2, has increased cytoplasmic localisation and altered substrate interactions (Caruso *et al.* 2018).

Despite these differences, cyclins E1 and E2 can substitute for many of each other's function (Geng *et al.* 2003) and show similar cell cycle expression (Gudas *et al.* 1999). What has become increasingly apparent is that cyclin E1 and E2 diverge in their expression in cancer (Caldon *et al.* 2009, 2013*a*,*b*, Caldon & Musgrove 2010). This could represent activation by distinct transcriptional cascades or regulators, independent amplification events, or it may represent differences in function.

Alterations to cyclin E1 and cyclin E2 in cancer

Cyclin E1 and cyclin E2 drive proliferation and genome instability in cancer

As core cell cycle regulators, cyclins E1 and E2 have the capacity to drive excessive proliferation when deregulated

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in cancer. Cyclin E1 can act as a bona fide oncogene to drive the formation of tumours when constitutively overexpressed in the mouse mammary gland, albeit with low penetrance (10%) and long latency (Bortner & Rosenberg 1997). In many cancer cell line models cyclin E1 overexpression will increase the proportion of cells in S phase, leading to increased Rb phosphorylation and cell proliferation (Hwang & Clurman 2005). Cyclin E2 overexpression has not been assessed in mouse models, but in cell line models its overexpression also leads to increased S phase fraction and proliferation (Zariwala *et al.* 1998, Gudas *et al.* 1999). Inhibition of CDK2 has a profound anti-proliferative effect (Horiuchi *et al.* 2012), and downregulation of either cyclin E1 or E2 can inhibit proliferation (Caldon *et al.* 2009).

The cyclic expression of cyclins E1 and E2 is important to its cell cycle functions, and perturbing the degradation of the E-cyclins in S phase can lead to genomic instability. Excess cyclin E1-CDK2 in S phase causes replication stress resulting in the under-replication of DNA in late S phase and genomic deletions (Teixeira *et al.* 2015). Persistent expression of cyclin E1-CDK2 at mitosis can then inhibit the APC^{Cdh1} complex and proper mitotic exit, leading to the misalignment of chromosomes at the metaphase plate. This results in chromosome mis-segregation and polyploidy (Keck *et al.* 2007). Excess mitotic cyclin E1-CDK2 also results in persistent CENPA Ser18 phosphorylation, which disrupts proper centromere formation and leads to mitotic defects (Takada *et al.* 2017).

Cyclin E2-CDK2 also causes genomic instability, but its mechanism is less well characterised (Caldon *et al.* 2013*b*). Unlike cyclin E1, genomic instability via cyclin E2 does not occur downstream of APC^{Cdh1} (Caldon *et al.* 2013*b*), but its overexpression causes the mis-segregation of chromosomes (Duffy *et al.* 2016).

Increased expression through increased transcription

The cyclin E1 and E2 genes, *CCNE1* and *CCNE2*, are frequently observed at high levels in solid cancers, as a result of gene amplification and gene deregulation. *CCNE1* occurs in the large 19q12 amplicon, and 19q12 amplification associated with cyclin E1 overexpression is believed to be a driver event in about 20% of high grade serous ovarian cancers (Karst *et al.* 2014). The role of cyclin E1 amplification as a driver in breast cancer is less certain (Natrajan *et al.* 2012). Chromosome region 8q22, which includes the *CCNE2* gene, is also frequently amplified, although several other genes from this region have been highlighted as driver oncogenes for this amplification event (Li *et al.* 2010).

Cyclins E1 and E2 undergo strong cyclical regulation due to their pivotal roles in cell cycle progression. In G_0 and G_1 the *CCNE1* and *CCNE2* genes are repressed via inhibitory deacetylation and methylation of their promoter regions (Caldon & Musgrove 2010). Both cyclin E1 and E2 are then upregulated during late G_1 following E2F transcription factor activation, along with the recruitment of co-factors such as CARM1, SRC, and CHD8 (reviewed in Caldon & Musgrove 2010). Consequently, cyclin E1 and E2 gene expression is responsive to growth factor signalling downstream of SRC, and other factors. Further differential expression of *CCNE1* and *CCNE2* expression is driven through miRNA networks that individually target each gene (reviewed in Caldon & Musgrove 2010).

Increased expression through altered degradation

As cyclical proteins, both cyclin E1 and cyclin E2 are downregulated during late S phase through ubiquitin-mediated proteosomal degradation (Fig. 1). This is first primed by phosphorylation of the cyclin E1 and cyclin E2 proteins on regions of the protein called 'phospho-degrons'. The phosphorylation sites of cyclin E1 have been described in detail (Welcker *et al.* 2003) and these sites facilitate recognition by the F-box protein FBXW7. Several sites are autophosphorylated as part of the cyclin E-CDK2 complex, and these modifications then prime for additional phosphorylation by GSK3β (Welcker *et al.* 2003). Downregulation of GSK3β via PI3K/Akt signaling can, in this way, disrupt cyclin E1 stability (Siu *et al.* 2012).

Despite these common phospho-degrons in the cyclin E1 and E2 proteins, the degradation of cyclins E1 and E2 is asynchronous, implying different mediators of degradation (Caldon *et al.* 2013*a*). Cyclins E1 and E2 are both targeted by Skp1-Cullin-F-box (SCF) E3 ubiquitin ligases, where the F-box adaptor protein recognises and binds the cyclin protein. The F-box protein FBXW7 (Klotz *et al.* 2009) binds to cyclin E-CDK2 complexes, though FBXW7 targeting of cyclin E2 is ineffective in breast cancer cells (Caldon *et al.* 2013*a*). Cyclin E1 monomers are additionally targeted by Skp2 (Nakayama *et al.* 2000), SPOP (Ju *et al.* 2019), and CUL3 (Davidge *et al.* 2019) for ubiquitination and subsequent proteosomal degradation. In the case of CUL3 this is specific to cyclin E1, as CUL3 does not ubiquitinate cyclin E2 or LMW cyclin E1 (Davidge *et al.* 2019). For cyclin E2 turnover, other ubiquitin ligases are likely to be important, as indicated

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by the cyclin E2-specific turnover mediated by calcium dependent signaling (Perez-Neut *et al.* 2015).

Failure to degrade cyclin E1 and cyclin E2 can lead to increased proliferation and genomic instability. Cyclin stabilisation can occur through changes to the ubiquitin ligases that target cyclins E1 and E2. *Fbxw7* can be disabled by mutation or gene methylation and occurs at low levels in ER+ breast cancer, although its loss is not particularly correlated with survival for these patients (Wei *et al.* 2012). SPOP is frequently reduced in cancer and associated with either mutation or loss of heterozygosity events (Li *et al.* 2011), whereas CUL3 is generally elevated in breast cancer and associated with advanced stage disease (Haagenson *et al.* 2012). SKP2 is also elevated in breast cancer (Ravaioli *et al.* 2008) and while this may increase cyclin E1/E2 turnover, this is counteracted by SKP2's other effects. SKP2 facilitates degradation of the cyclin E-CDK2 inhibitor protein $p27^{Kip1}$ (Nakayama *et al.* 2000), and it can transactivate the activity of E2F1 on the cyclin E1 promoter (Salon *et al.* 2007).

Relative expression and amplification of cyclin E1/cyclin E2 in ER+ breast cancers

ER+ breast cancers are highly dependent on estrogen to stimulate passage of cells through the G_1/S phase transition to commit to ongoing proliferation. Since CDK2 has a pivotal role in the G_1/S phase transition, cyclins E1 and E2 are central players in ER+ breast cancer. In this review we have performed primary analyses of the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset, and a metastatic breast cancer dataset, to illustrate the differences and similarities between cyclin E1 and E2 across ER+ breast cancer and discuss this in the context of previous findings (Figs 2, 3, 4 and 5). METABRIC is a genomic and transcriptomic study of >2000 primary breast cancers (available at http://www.ebi.ac.uk/ega/, under accession number EGAS00000000083) that includes 1175 samples of a luminal type and allows for a comprehensive analysis across the luminal A and luminal B types of ER+ breast cancer (Curtis *et al.* 2012).

Cyclin E1 mRNA has moderate expression in luminal B type ER+ breast cancers, but it is expressed at the highest level in the ER-negative (ER-) basal-like breast cancers (Fig. 2A). Likewise, 19q12 amplification is predominantly observed in ER- breast cancers (Natrajan *et al.* 2012), and it occurs in only 0.7% of ER+ cancers (Fig. 2B).

In contrast to cyclin E1, cyclin E2 mRNA occurs at its highest levels in luminal B ER+ breast cancers and also at high levels in basal-like and HER2 overexpressing breast cancers (Fig. 2A). The 8q22 chromosomal locus, which includes the *CCNE2* gene, is amplified in 15–20% of breast cancers (Li *et al.* 2010) and this amplification event occurs in 28.6% of luminal B breast cancers (Fig. 2B). 8q22 amplification is associated with increased risk of metastasis and anthracycline chemotherapy resistance via expression of the genes LAPTM4B and YWHAZ (Li *et al.* 2010). However, it should be noted that chemotherapy is not used as therapy in the majority of early stage ER+ breast cancer so other driver events, such as *CCNE2* amplification, are possible in ER+ breast cancer.

One of the earliest publications on cyclin E2 reported a high correlation between cyclin E1 and E2 mRNA in ten breast cancer samples (Payton *et al.* 2002). With the availability of much larger datasets such as METABRIC, we can now see that there is only a moderate correlation in mRNA expression between cyclin E1 and cyclin E2 in ER+ cancers (r=0.470, Fig. 3A). *CCNE2* expression correlates

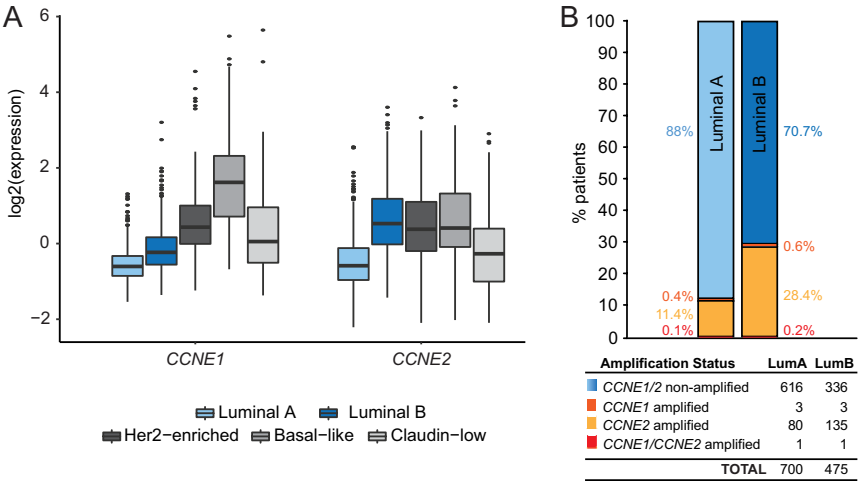

Figure 2
Expression and amplification of *CCNE1* and *CCNE2* in primary breast cancer. (A) Gene expression data (Illumina HT-12 v3 BeadChip) was downloaded from https://www.ebi.ac.uk/ega/studies/EGAS00000000083 and relative expression of *CCNE1* and *CCNE2* was plotted across breast cancer subtypes (luminal A, luminal B, Her2-enriched, basal-like, and claudin-low) as annotated in METABRIC (Curtis *et al.* 2012). (B) Gene amplification data were obtained from 'Breast Cancer METABRIC' via cBioPortal (http://cbioportal.org) for *CCNE1* and *CCNE2* and determined for luminal A (n = 700) and luminal B (n = 475) cancers. The percentage of *CCNE1* and *CCNE2* amplification is plotted for both ER+ breast cancer subtypes.

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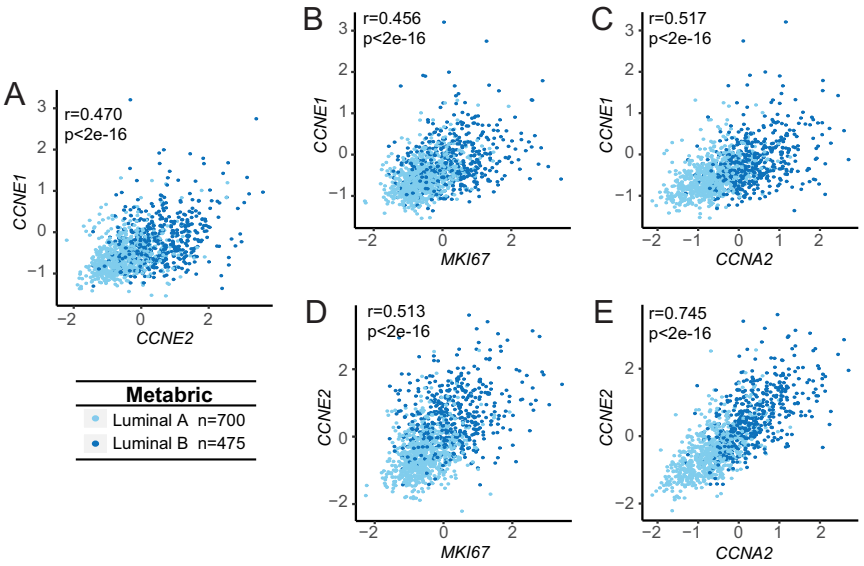

Figure 3
Relative expression of *CCNE1* and *CCNE2* in ER+ breast cancer. Relative *CCNE1* and *CCNE2* expression across luminal A (light blue) and luminal B (dark blue) ER+ breast cancers compared to one another and to genes associated with proliferation: *CCNA2* (cyclin A2) and *MKI67* (Ki67). (A) *CCNE1* vs *CCNE2*, (B) *CCNE1* vs *MKI67*, (C) *CCNE1* vs *CCNA2*, (D) *CCNE1* vs *MKI67*, and (E) *CCNE1* vs *CCNA2*. Gene expression data were downloaded from https://www.ebi.ac.uk/ega/studies/EGAS00000000083. Scatter plots axes show expression log intensity level z-scores for each gene across ER+ breast cancer subtypes. The association between genes was measured using the Pearson correlation coefficient (*r*) and respective computed *P-value*. Analysis was performed in the *R* environment using the packages *ggpubr* and *ggplot2*.

more with the proliferation markers *CCNA2* and *MKI67* than it does with *CCNE1*, and *CCNE1* correlates better with *CCNA2* than it does with *CCNE2* expression (Fig. 3B, C, D and E). This indicates that cyclin E1 and cyclin E2 are not tightly co-regulated in ER+ breast cancer, and we speculate that they could be driving the proliferation of different subsets of ER+ cancers.

Cyclin E1 and cyclin E2 in endocrine resistance

Due to the strong mitogenic effects of estrogen, endocrine therapies in the form of anti-estrogens (tamoxifen, fulvestrant) and aromatase inhibitors have been a standard of care for ER+ breast cancer for over 30 years. Resistance to these therapies (endocrine therapy resistance) occurs in up to 30% of patients. This is an important clinical problem: it is a very large cohort of patients, and once patients fail endocrine therapy they are usually refractory to standard chemotherapies. As a result, biomarkers of therapeutic resistance and therapeutic targets are required to identify and treat these patients.

Cyclins E/CDK complexes are acutely responsive to estrogen and anti-estrogens (Caldon *et al.* 2009) and have thus been investigated as drivers and biomarkers of endocrine therapy resistance. Both the *CCNE1* and *CCNE2* genes are stimulated by E2F-mediated transcription (El Messaoudi *et al.* 2006, Caldon *et al.* 2009), which is a major conduit of estrogen-induced proliferative signaling (Miller *et al.* 2011). Cyclin E1-CDK2 activity is, however, more downstream of c-Myc activation,

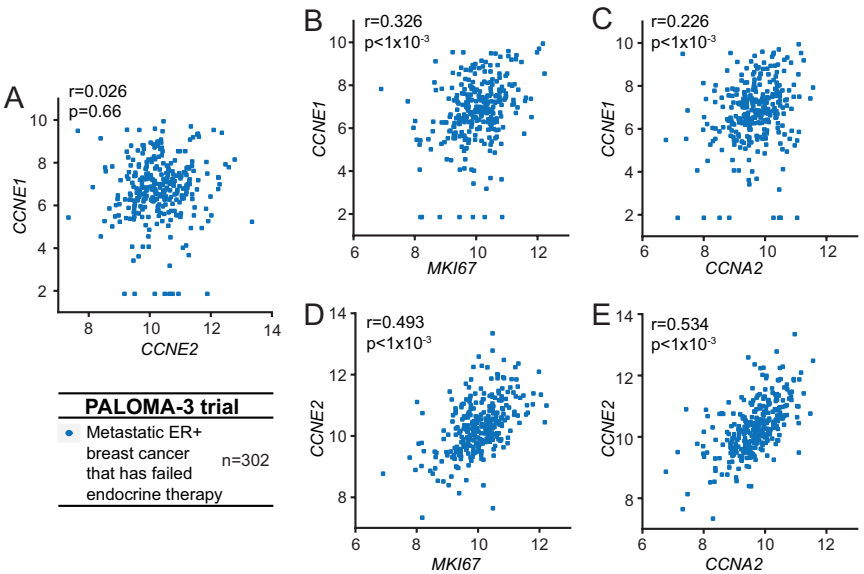

Figure 4
Relative expression of *CCNE1* and *CCNE2* in metastatic, endocrine therapy refractory, ER+ breast cancer. Relative *CCNE1* and *CCNE2* expression across metastatic, endocrine therapy refractory, ER+ breast cancers from the PALOMA-3 trial. *CCNE1* and *CCNE2* expression was compared to one another and to genes associated with proliferation: *CCNA2* (cyclin A2) and *MKI67* (Ki67). (A) *CCNE1* vs *CCNE2*, (B) *CCNE1* vs *MKI67*, (C) *CCNE1* vs *CCNA2*, (D) *CCNE1* vs *MKI67*, and (E) *CCNE1* vs *CCNA2*. Gene expression data were obtained from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) for dataset GSE128500. Scatter plots axes show normalised counts for each gene from targeted RNA sequencing. The association between genes was measured using the Pearson correlation coefficient (*r*) and respective *P-value*. The analysis was performed in the *R* environment using the packages *ggpubr* and *ggplot2*.

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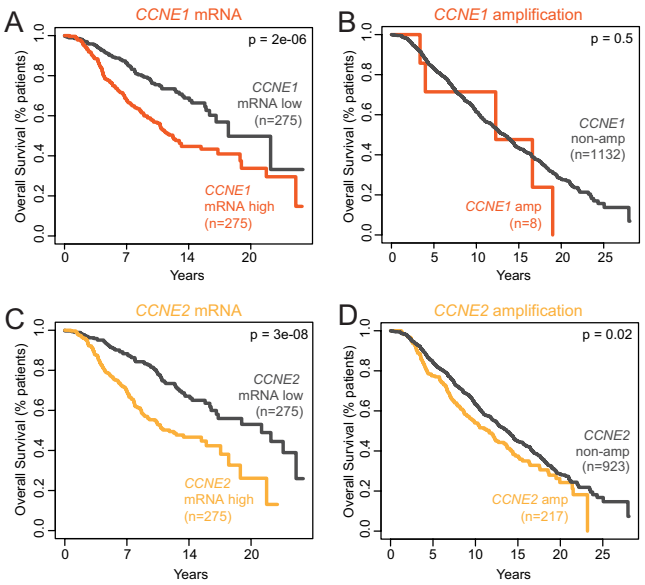

Figure 5

Patient overall survival associated with CCNE1 and CCNE2 in the METABRIC ER+ breast cancer cohort. Kaplan–Meier curves show estimated survival over time associated with *CCNE1/CCNE2* gene expression or gene amplification in patients with ER+ breast cancers ($n = 1140$). (A) High *CCNE1* (top quartile, $n = 275$) vs low *CCNE1* (bottom quartile, $n = 275$). (B) *CCNE1* amplified ($n = 8$) vs *CCNE1* non-amplified ($n = 1132$). (C) High *CCNE2* (top quartile, $n = 275$) vs low *CCNE2* (bottom quartile, $n = 275$). (D) *CCNE2* amplified ($n = 217$) vs *CCNE2* non-amplified ($n = 923$). Gene expression data and survival outcomes were downloaded from https://www.ebi.ac.uk/ega/studies/EGAS00000000083. To determine the significant differences between the two independent groups, we computed the *P-value* using the Kaplan–Meier (log rank) test. The analysis was performed in the *R* environment using the packages *survival* and *survminer*.

whereas *CCNE2* lies downstream of estrogen-mediated activation of Cyclin D-CDK4 (Caldon *et al.* 2009). This is because of the co-activation of *CCNE2* by the chromatin remodeller CHD8, which has an important role in steroid hormone control of G1/S phase genes (Caldon *et al.* 2009, Menon *et al.* 2010). By contrast, the *CCNE1* gene is co-activated by CARM1 (El Messaoudi *et al.* 2006), which is an ER co-activator that drives proliferation (Al-Dhaheri *et al.* 2011).

Data from experimental models have provided strong evidence that cyclins E1 and E2 have roles in endocrine therapy resistance. The proliferation of tamoxifen-resistant cells is inhibited by RNAi-mediated knockdown of cyclin E1, cyclin E2, or CDK2 (Caldon *et al.* 2012), and cyclin E1 and cyclin E2 overexpression can reduce antiestrogen sensitivity *in vitro* (Dhillon & Mudryj 2002, Hui *et al.* 2002, Akli *et al.* 2004, Caldon *et al.* 2012). The LMW form of cyclin E1 appears more effective at preventing fulvestrant-mediated arrest than the full length version (Akli *et al.* 2004) and sustaining S phase entry when aromatase is inhibited (Akli *et al.* 2010). The downregulation of CDK inhibitors $p21^{Cip1/Waf1}$ or $p27^{Kip1}$ also abrogates anti-estrogen mediated arrest (Cariou *et al.* 2000). Notably, cyclin E2 is commonly detected at elevated levels in models of anti-estrogen resistance compared to parental models (Huang *et al.* 2011, Caldon *et al.* 2012, Tu *et al.* 2013). Cyclin E1 is occasionally elevated (Louie *et al.* 2010), or it is reported as unchanged (Post *et al.* 2019).

Using the data available from the PALOMA-3 trial (NCT01942135), we were able to compare expression of *CCNE1* and *CCNE2* in patients with metastatic breast cancer who have progressed on endocrine therapy, derived from primary samples and metastatic samples ($n=159$ primary, $n=142$ metastatic, $n=302$ total: data to identify individual patient subsets is not available) (Turner *et al.* 2019). In this cohort, there was no correlation between *CCNE1* and *CCNE2* expression ($r=0.026$, Fig. 4A). Both *CCNE1* and *CCNE2* correlated to proliferative markers: *CCNE1* correlates weakly with *CCNA2* ($r=0.326$) and *MKI67* ($r=0.226$) (Fig. 4B and C), whereas *CCNE2* has a moderate correlation with both these markers (*CCNA2*: $r=0.493$, *MKI67*: $r=0.534$; Fig. 4D and E). For this cohort, we conclude that the regulation of *CCNE1* and *CCNE2* is dominated by individual regulatory networks rather than co-regulation, but each gene is involved in proliferation independently. In addition, there are a significant proportion of cancers which are either *CCNE1* high/*CCNE2* low or *CCNE1* low/*CCNE2* high. This aligns with the data from *in vitro* models, where either cyclins E1 and E2 can act as putative drivers of endocrine therapy resistance.

Cyclin E1 as a biomarker for ER+ breast cancer and endocrine resistance

CCNE1 mRNA is generally predictive of poor outcome for ER+ patients (Fig. 5A). High *CCNE1* was also predictive across three cohorts of tamoxifen treated patients, including shorter disease-free interval for patients on adjuvant therapy (Span *et al.* 2003, Desmedt *et al.* 2006) and time to progression (Jansen *et al.* 2012). However, in a composite cohort of patients treated with either tamoxifen or goserelin, *CCNE1* was not predictive of a shorter disease-free interval (Caldon *et al.* 2012). It is unlikely that *CCNE1* amplification has a significant role in ER+ breast cancer: only a very small proportion of patients show *CCNE1* amplification (Fumagalli *et al.* 2016), and this is not associated with improved overall survival (Fig. 5B).

Surprisingly, high cyclin E1 protein has not been indicative of poor survival across ER+ cohorts of patients. In a study of 2020 patients, high cyclin E1 did not predict

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poorer overall survival or disease-free survival in ER+ or ER- patients, using a nuclear staining cyclin E antibody (Porter *et al.* 2006). Using a nuclear/cytoplasmic staining antibody (raised against the HE12 epitope), Cyclin E1 did not predict for poor outcome in a cohort of 51 ER+ patients (Agarwal *et al.* 2009), nor did it predict tamoxifen response (Berglund *et al.* 2008).

There are several caveats to the interpretation of studies on cyclin E1 protein which may explain the conflicting results from studies on *CCNE1* mRNA and cyclin E1 protein in ER+ breast cancer. First, not all antibodies have been demonstrated to be specific to cyclin E1, and these antibodies may also detect cyclin E2. Second, the LMW isoform of cyclin E1 is not detected by all cyclin E1 antibodies (Karakas *et al.* 2016). The LMW isoform of cyclin E1 is detected in ~22% of ER+ breast cancers and associated with increased disease recurrence (Akli *et al.* 2010). Fortunately most studies, including standard RPPA analysis in the TCGA datasets, use the antibody raised against the HE12 epitope, which does not detect cyclin E2 (Caldon *et al.* 2009) and is able to detect LMW cyclin E1 (Akli *et al.* 2010, Karakas *et al.* 2016). In addition, LMW cyclin E1 preferentially localises to the cytoplasm. Cytoplasmic cyclin E, when used as a marker in combination with phosphorylated CDK2, associates strongly with poor recurrence-free survival (Karakas *et al.* 2016). Consequently, if cohorts are scored solely on nuclear staining, the outcome of patients may be biased toward effects that are more associated with the full length cyclin E1 protein.

Cyclin E2 as a biomarker for ER+ breast cancer and endocrine resistance

Cyclin E2 mRNA (*CCNE2*) expression is associated with poor outcome in ER+ breast cancer (Desmedt *et al.* 2006, Sieuwerts *et al.* 2006) (Fig. 5C), although in subgroup analyses of the tamoxifen-only treated patients of one cohort (Desmedt *et al.* 2006), high *CCNE2* levels did not predict treatment resistance. In other large cohorts, *CCNE2* is included in gene signatures that predict disease progression in either tamoxifen-resistant breast cancer or metastatic breast cancer, whereas cyclin E1 does not feature in these same signatures (van't Veer *et al.* 2002, Sotiriou *et al.* 2006, Bosco *et al.* 2007, Musgrove *et al.* 2008, Huang *et al.* 2011). Our previous analysis of a composite cohort of six studies showed that high *CCNE2* predicted poor metastasis-free survival in endocrine therapy treated patients (Caldon *et al.* 2012).

Despite its high prevalence (18.5% of ER+ cancers, Fig. 2B), Cyclin E2 gene amplification has not been independently examined as a prognostic marker in ER+ breast cancer to date. However, our analysis of METABRIC shows that *CCNE2* amplification is significantly associated with poor outcome (Fig. 5D). Given the large percentage of luminal B ER+ cancers that are *CCNE2* amplified (28.6%, Fig. 2B) and the propensity for luminal B cancers to be intrinsically endocrine resistant (Ades *et al.* 2014), it is feasible that *CCNE2* amplification has a role in endocrine therapy resistance and disease recurrence.

Currently there are no reports of cyclin E2 immunohistochemistry across ER+ breast cancer, most likely due to the lack of suitable antibodies.

Cyclin E driven CDK2 activity in CDK4/6 inhibitor sensitivity and resistance

The current standard of care for metastatic ER+ breast cancer is CDK4/6 inhibitors in combination with endocrine therapy (Portman *et al.* 2019). Inhibitors such as palbociclib, ribociclib, and abemaciclib are now used routinely in metastatic breast cancer that has progressed on endocrine therapy, after the demonstration in multiple phase III trials that these inhibitors extend progression-free survival (Finn *et al.* 2015, Hortobagyi *et al.* 2016, Goetz *et al.* 2017). While CDK4/6 inhibitors have a significant effect on disease progression, resistance is inevitable in most patients (Portman *et al.* 2019).

The current predictive biomarker for use of CDK4/6 inhibitors is ER, but there has been a concerted effort to identify further biomarkers that could refine the patient cohort into responders and non-responders. CDK4/6 inhibitor resistance is also now emerging as a significant clinical problem, and there are not yet effective biomarkers for the emergence of resistance (Portman *et al.* 2019). Both *CCNE1* and *CCNE2* have been identified as potential biomarkers that alter sensitivity to combination endocrine therapy and CDK4/6 inhibitors and could indicate an increased likelihood of resistance (Arnedos *et al.* 2018, Turner *et al.* 2019), which we discuss in detail.

CDK4/6 inhibitor resistance is associated with increase in CDK2 activation

Current efforts to understand the emergence of CDK4/6 inhibitor resistance have shown that numerous mechanisms converge to drive proliferation through CDK2 activation, including upregulation of cyclin E1 and cyclin E2. Studies across laboratory cell line models,

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mainly performed in the ER+ MCF-7 and T-47D breast cancer cell lines, have identified cyclin E1 and E2 mRNA and protein upregulation in multiple models (Table 2). The overexpression of either cyclin E1 or cyclin E2 is able to reduce sensitivity to palbociclib (Caldon *et al.* 2012). Although full length cyclin E1 overexpression does not affect all models, LMW cyclin E1 overexpression can be more effective at reducing sensitivity to palbociclib (Doostan *et al.* 2017).

Other pathways that converge on CDK2 activation are changes to the CDK inhibitor proteins $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. This includes reduction of $p21^{Cip1/Waf1}$ (Iida *et al.* 2019) and $p27^{Kip1}$ protein (Dean *et al.* 2010). Increased expression of components of the cyclin D-CDK4/6 complexes such as CDK6, observed in (Yang *et al.* 2017) and (Cornell *et al.* 2019), could also increase CDK2 activity by engaging the $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ inhibitory proteins and hence remove these inhibitors from CDK2 complexes (Tsubari *et al.* 1999). Since CDK4/6 inhibitors bind in the ATP pocket of CDK4/6, $p21^{Cip1/Waf1}$/$p27^{Kip1}$ binding is not prevented in the presence of drug (Paternot *et al.* 2014), allowing CDK4/6 to sequester $p21^{Cip1/Waf1}$ and $p27^{Kip1}$.

The role of CDK2 in the transition to senescence must also be considered, as CDK4/6 inhibitors drives breast cancer cells into a senescent growth arrested state (Klein *et al.* 2018). Cellular senescence is a state of permanent proliferative arrest and exit from the cell cycle which is characterised by enlarged cellular morphology and production of markers such as senescence-associated β-galactosidase (Campisi 2013). It is mediated by the CDK inhibitors $p16^{INK4a}$ and $p21^{Cip1/Waf1}$ and tumour suppressors p53 and Rb in response to oncogenic stresses or DNA damage (Coppe *et al.* 2010). In this response, cyclin-CDK complexes are inactivated by CDK inhibitor binding: $p16^{INK4a}$ binds to cyclin D-CDK4/6 and $p21^{Cip1/Waf1}$ to cyclin E-CDK2 and cyclin D-CDK4/6 (Table 1) (Prall *et al.* 1997, Sherr & Roberts 1999, Sheppard & McArthur 2013). CDK2 transcription is also downregulated downstream of p53 and Rb as part of the senescence response (Zalzali *et al.* 2015). Downregulation of CDK2 is necessary for senescence entry (Zalzali *et al.* 2015), but conversely high levels of CDK2 can inhibit senescence entry (Campaner *et al.* 2010). Residual CDK2 activity may, however, be useful to drive cells into senescence following a DNA damage-induced G2 arrest (Müllers *et al.* 2017). In light of these findings, the persistence of high CDK2 in CDK4/6 inhibitor resistance setting would most likely prevent cells from entering senescence and promote continual proliferation.

Cyclin E1 and cyclin E2 as biomarkers of response to CDK4/6 inhibitors

The PALOMA-2 and PALOMA-3 trials show that irrespective of the expression levels of *CCNE1* and *CCNE2* in the pre-treated primary tumour samples, the addition of palbociclib to endocrine therapy demonstrates a benefit on recurrence-free survival compared to endocrine therapy alone. However, those patients with the longest and shortest recurrence-free survival can be differentiated by the expression of *CCNE1* (Table 3). In both the MONALEESA-2 (letrozole/ribociclib) and the PALOMA-3 (fulvestrant/palbociclib) trials, high expression of *CCNE1* mRNA was associated with poor progression-free survival (Formisano *et al.* 2019, Turner *et al.* 2019). There was a trend for *CCNE1*-high patients to have poor progression-free survival in the MONALEESA-7 and CLEE011X2106 trials. Finally, patients expressing cytoplasmic cyclin E1 protein, thought to be representative of LMW cyclin E1, were found to have worse survival than patients expressing only nuclear cyclin E1 protein (Vijayaraghavan *et al.* 2017). In PALOMA-3, high pre-existing *CCNE2* mRNA expression was not associated with any difference in progression-free survival (Turner *et al.* 2019). *CCNE2* was not included in the gene panels for MONALEESA-2, MONALEESA-7, or CLEE011X2106.

The degree of change of proliferative markers in response to CDK4/6 inhibitors can be correlated to either *CCNE1* or *CCNE2* across several studies. In the POP trial of pre-operative palbociclib, *CCNE2* expression was significantly decreased in anti-proliferative responders vs non-responders measured over 15 days ($P=0.006$) (Arnedos *et al.* 2018). The POP study also showed a non-significant trend for *CCNE1* to be high in non-responders ($P<0.09$) (Arnedos *et al.* 2018). Similar observations were made in the NeoPalAna study (*CCNE1* was high in patients who maintained high Ki67 after 15 days of treatment) (Ma *et al.* 2017) and the PALLET study (low Ki67 at 14 weeks was significantly less frequent in *CCNE1* high patients) (Martins *et al.* 2019). *CCNE2* was not measured in the NeoPalAna or PALLET studies.

It is unlikely that cyclin E1 amplification predisposes to CDK4/6 inhibitor resistance, as the frequency is not high in ER+ breast cancer (0.7%, Fig. 2B). There is no evidence that *CCNE1* amplification is acquired during progression to resistance: in the PALOMA-2 trial it was noted that there were insufficient *CCNE1* amplified patients to examine the relationship with resistance (Finn *et al.* 2019), and in the PALOMA-3 trial *CCNE1* amplification was not acquired in 28 patients who progressed on palbociclib plus fulvestrant

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Table 2 Pre-clinical HR+ HER2- breast cancer models of CDK4/6 inhibitor resistance.

	Study	ER+ Model	Generation of CDK4/6 resistance	*CCNE1*/cyclin E1	*CCNE2*/cyclin E2
In vitro					
Abemaciclib	(Yang *et al.* 2017)	MCF-7	Acquired resistance to abemaciclib (100 nM) after 21 weeks (resistant cells maintained with 200 nM)	No change to *CCNE1* mRNA; Cyclin E1 protein not reported	Increased expression of cyclin E2 protein downstream of CDK6 amplification No change to *CCNE2* mRNA
		T47D	Acquired resistance to abemaciclib (500 nM) after 21 weeks (resistant cells maintained with 1 µM)	No change to *CCNE1* mRNA; Cyclin E1 protein not reported	Increased expression of cyclin E2 protein downstream of CDK6 amplification No change to *CCNE2* mRNA
		CAMA-1	Acquired resistance to abemaciclib (100 nM) after 21 weeks (resistant cells maintained with 100 nM)	Significant overexpression of *CCNE1* mRNA; Cyclin E1 protein not reported	No change in cyclin E2 protein expression
Palbociclib	(Herrera-Abreu *et al.* 2016)	MCF-7	Acquired resistance to palbociclib (3–4 months; 1 µmol/L)	Amplification of *CCNE1* (on chromosome 19q12); increase in cyclin E1 protein expression; sensitive to *CCNE1* siRNA in the presence of palbociclib	Sustained cyclin E2 protein in the presence of palbociclib Not sensitive to *CCNE2* siRNA
		T47D		No change	Sustained cyclin E2 protein in the presence of palbociclib Not sensitive to *CCNE2* siRNA
	(Vijayaraghavan *et al.* 2017)	Panel of cell lines classified as being sensitive or resistant to palbociclib		High *CCNE1* expression in cell lines intrinsically resistant to palbociclib	Not reported
		MCF-7	6 days of palbociclib (1 µM) and letrozole (1 µM) treatment	Overexpression of LMW cyclin E1 mediated resistance to palbociclib+letrozole	
		T47D	6 days of palbociclib (1 µM) treatment	Overexpression of LMW cyclin E1 mediated resistance to palbociclib	
	(Michaloglou *et al.* 2018)	MCF-7	Acquired resistance to palbociclib (increasing concentration up to 1 µM) after 4–9 months	Not reported	Not reported
	(Lypova *et al.* 2019)	MCF-7	Acquired resistance to palbociclib (0.1–4 µM) after 6 months (resistant cells maintained with 1 µM)	Increase in full-length cyclin E1 AND LMW cyclin E1	Not reported
	(Ji *et al.* 2019)	MCF-7	Acquired resistance to palbociclib (increasing concentration up to 30 µM) after 1 year	Elevated expression of cyclin E1 downstream of AR activation by DHT treatment	Sustained expression of cyclin E2 protein in the presence of palbociclib shAR decreased cyclin E2 protein in resistant cells Enzalutamide + palbociclib reduced cyclin E2 protein expression
	(Kong *et al.* 2019)	MCF-7	Acquired resistance to palbociclib (increasing concentration up to 900 nmol/L) after 8 weeks	High cyclin E1 protein in 6/11 MCF-7 resistant clones	Not reported
	(Kettner *et al.* 2019)	MCF-7	Acquired resistance to palbociclib (increasing concentration up to 5 µmol/L) after 6 months	Increased cyclin E1 kinase activity; increase in LMW cyclin E1	Not reported
		T47D		CNV amplification of *CCNE1*; increase in LMW cyclin E1	

(*Continued*)

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Table 2 Continued

	Study	ER+ Model	Generation of CDK4/6 resistance	*CCNE1*/cyclin E1	*CCNE2*/cyclin E2
	(Cornell *et al.* 2019)	T47D	Acquired resistance to palbociclib (100–500 nM) after 12 weeks	No significant decrease in *CCNE1* mRNA (100 nM palbociclib); no significant variation in *CCNE1* copy number; small increase in cyclin E1 protein expression with increasing concentration of palbociclib (200–300nM); significant decrease in *CCNE1* mRNA when resistant cells are cultured with no palbociclib (7 weeks) compared to continuous treatment	Small (but not significant) decrease in *CCNE2* mRNA (100 nM palbociclib)
Ribociclib	(Jansen *et al.* 2017)	MCF-7 T47D HCC1428	Acquired resistance to ribociclib (increasing concentration up to 1 μM)	Higher levels of 'cyclin E' protein in resistant cell lines mediated by upstream PI3K/PDK1 signaling pathway	
	(Iida *et al.* 2019)	Estrogen-deprived MCF-7-E10	Acquired resistance to ribociclib (100 nM) after 7 months	Unchanged cyclin E1 protein expression	Not reported
In vivo					
Palbociclib	(Formisano *et al.* 2019)	TM00386 (ER+ HER2- *FGFR1*-amplified breast cancer PDX)	Treatment with fulvestrant (5 mg/week; s.c.) + palbociclib (30 mg/kg/day; oral gavage) (3 weeks) supplemented with 21-day release of 0.25 mg 17β-estradiol	Downregulation of *CCNE1* expression using NanoString 770-gene nCounterPanCancer Pathways panel	Downregulation of *CCNE2* expression using NanoString 770-gene nCounterPanCancer Pathways panel
Ribociclib	(Herrera-Abreu *et al.* 2016)	PDX244 (CDK4/6-sensitive PDX)	Acquired resistance to ribociclib (100 days; 75 mg/kg, 6 days/week) supplemented with continuous treatment with 17β-estradiol (1 uM)	Not reported	Sustained expression of cyclin E2 protein in resistant tumours

Treatment: Enzalutamide (second generation AR antagonist).
AR: androgen receptor; CNV: copy-number amplification; DHT: 5-α-dihydrotestosterone; IHC: immunohistochemistry; LMW cyclin E1: low-molecular weight cyclin E1.

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Table 3 Clinical trials with CDK4/6 inhibitors in combination with endocrine therapy for HR+HER2- breast cancer.

Trial	Study	Phase	Enrolment	Treatment history	Description	Outcome measured	*CCNE1*	*CCNE2*
Palbociclib								
PALOMA-2 NCT01740427	(Finn *et al.* 2016)	III	*n* = 666	ER+ HER2- ABC *No prior treatment for advanced disease* *Postmenopausal*	palbociclib + letrozole vs letrozole	PFS: 24.8 months vs 14.5 months (HR: 0.58; 95% CI: 0.46–0.72; *P* < 0.001)	Not reported	Not reported
	(Rugo *et al.* 2019)					38 month follow up PFS: 27.6 months vs 14.5 months (HR: 0.563; 95% CI: 0.461–0.687; *P* < 0.0001)		
	(Finn *et al.* 2019)				Comprehensive biomarker analysis using patient tissue	Data were correlated with PFS to determine markers of sensitivity and resistance to combination therapy	• Irrespective of baseline *CCNE1* expression levels, palbociclib + letrozole demonstrated a benefit vs placebo + letrozole • *CCNE1* amplification is rare in ER+ luminal breast cancer	Irrespective of baseline *CCNE2* expression levels, palbociclib + letrozole demonstrated a benefit vs placebo + letrozole
PALOMA-3 NCT01942135	(Turner *et al.* 2015)	III	*n* = 521	HR+ HER2- ABC *Progression on prior endocrine therapy* *≤ 1 line of chemotherapy for advanced/ metastatic disease* *Any menopausal status*	palbociclib + fulvestrant vs fulvestrant	PFS: 9.2 months vs 3.8 months (HR: 0.42; 95% CI: 0.32–0.56; *P* < 0.001)	Not reported	Not reported
	(Cristofanilli *et al.* 2016)					~8.9 month follow up PFS: 9.5 months vs 4.6 months (HR: 0.46; 95% CI: 0.36–0.59; two-sided *P* < 0.0001)		
						ctDNA analysis of *PIK3CA* mutation at baseline		
	(Turner *et al.* 2018)					OS: 34.9 months vs 28 months (HR: 0.81; 95% CI: 0.64–1.03; *P* = 0.09)		
	(O'Leary *et al.* 2018)				ctDNA analysis in paired baseline, 15 days of treatment and EOT plasma samples	• Early *PIK3CA* ctDNA levels predicts sensitivity and PFS to combination therapy • Early *ESR1* ctDNA levels did not predict sensitivity and PFS to combination therapy		
	(O'Leary *et al.* 2018)				ctDNA analysis of paired baseline and EOT plasma samples	Clonal evolution occurs frequently during combination therapy (*RB1* mutations in 6/127 patients) and *PIK3CA* (*P* = 0.00069) and *ESR1* (*P* = 0.0037) driver mutations after treatment in both arms	3/24 patients had existing *CCNE1* amplifications, but no patients acquired *CCNE1* amplification at EOT (palbociclib + fulvestrant; *n* = 24)	4/18 patients had existing CCNE2 amplifications, but no patients acquired *CCNE2* amplification at EOT (palbociclib + fulvestrant; *n* = 18)
	(Turner *et al.* 2019)	Retrospective study	Samples analysed: *n* = 194 (combination arm) *n* = 108 (placebo)		Biomarker analysis using EdgeSeq Oncology 2543-gene panel on primary and metastatic tumour samples	mRNA expression correlated with PFS to determine markers of resistance to combination therapy	High expression of *CCNE1* was associated with reduced response to palbociclib • PFS: 7.6 months (palbociclib arm) vs 4 months (placebo) • (HR: 0.85; 95% CI: 0.58–1.26) • Low expression of *CCNE1* associated with greater efficacy of palbociclib • PFS: 14.1 months (palbociclib arm) vs 4.8 months (placebo) (HR: 0.32; 95% CI: 0.2–0.5)	Not reported

(*Continued*)

Table 3 Continued

Trial	Study	Phase	Enrolment	Treatment history	Description	Outcome measured	*CCNE1*	*CCNE2*
PALLET NCT02296801	(Johnston *et al.* 2019)	II	*n* = 307	ER+ HER2- EBC *Neoadjuvant therapy: no prior treatment Postmenopausal*	(A) palbociclib (2 weeks) → palbociclib +letrozole (14 weeks) (B) letrozole (2 weeks) → palbociclib + letrozole (14 weeks) (C) palbociclib + letrozole (14 weeks) (D) letrozole (14 weeks)	Anti-proliferative response (Ki67; IHC) between baseline and EOT • (A–C) –4.1 vs (D) –2.2 (one-sided $P < 0.001$) • CRR • (A–C) 54.4% patients had complete or partial response vs (D) 49.5% patients had complete or partial response ($P = 0.20$)	Not reported	Not reported
	(Martins *et al.* 2019)					Biomarker analysis of tumour samples at baseline and EOT (14 weeks) by IHC and/or FISH: *n* = 124 patients (A–C) and *n* = 64 (D) 90% vs 59% achieved CCCA (KI67 < 2.7%)	• (A-C): At baseline high CCNE1 levels correlated with lower CCCA indicating resistance (OR: 50.34; 95% CI: 5.12–495.34; $P = 0.001$) • At EOT, CCNE1 levels were significantly supressed by all treatments: geomeans –74.7% (A–C) vs –68.2% (D) with no significant different occurring between treatments (A–C) • High CCNE1 could be a marker of poor Ki67 response to combination therapy	
NCT01037790	(DeMichele *et al.* 2015)	II	*n* = 33	HR+ HER2+/- Rb+ ABC *Unlimited prior therapies*	palbociclib	CBR: 7/33 patients (21%) had stable disease for ≥6 months. CB was seen exclusively in patients (7/24; 29%) who progressed on ≥2 prior lines of hormonal therapy Biomarker analysis of tumour tissue by IHC (Rb, p16, *CCND1*, Ki67) was not associated with CB or PFS	Not reported	Not reported
	(DeMichele *et al.* 2016)		*n* = 1 (patient recurred after 33 months of treatment)	Serial biopsies obtained from 67 year old patient with ER+ HER2+ MBC	palbociclib (50 mg)	784-gene custom NanoString array: no evidence of *RB1* loss or alterations in *CDKN2A*, *CCND1*, *CDK4*, *PIK3CA* or *ESR1*. Genomic profile did not change between primary and recurrent tissue samples	Not reported	• *CCNE2* increased by more than 2-fold at progressive disease • Increased activation of genes that could be downstream of FOXM1 (*PLK1*, *TOP2A*, *CDK1*, *BUB1*, *CDC20*, *CCNA2*, *CCNB1*, and *BIRc5)*
POP NCT02008734	(Arnedos *et al.* 2018)	II	*n* = 74 palbociclib *n* = 26 control	HR+ HER2+/- EBC *No prior treatment Pre- and postmenopausal*	palbociclib (2 weeks) → surgery vs surgery	Anti-proliferative response (Ki67) between baseline and EOT: higher Ki67 decrease following palbociclib treatment (58% vs 12%; $P < 0.001$)	*CCNE1* expression was not significantly more decreased in anti-proliferative responders vs non-responders ($P = 0.9$) Persistent elevated expression of *CCNE1* at EOT indicates resistance	*CCNE2* expression was significantly more decreased in anti-proliferative responders vs non-responders ($P = 0.006$) but not in the HR+ HER2- subgroup ($P = 0.43$)
	(Turner *et al.* 2019)	Retrospective study	*n* = –61		Gene expression analysis using GeneChip Human Gene Array ST2.1	mRNA expression correlated with anti-proliferative response *CCNE1* expression in luminal A tumours was significantly lower than in luminal B tumours ($P < 0.001$)	High *CCNE1* expression was associated with lower Ki67 in response to palbociclib High *CCNE1*: 36%, intermediate *CCNE1*: 79%; Low *CCNE1*: 80% ($P = 0.005$)	Not reported

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	NeoPalAna NCT01723774	(Ma *et al.* 2017)	II	*n* = 50	ER+ HER2- invasive breast cancer *Neoadjuvant therapy: no prior treatment* *Pre- and postmenopausal*	Anastrozole (4 weeks) → palbociclib + anastrozole (16 weeks) → palbociclib + anastrozole (1.5 weeks if no side effects) → surgery	Anti-proliferative response (Ki67) between baseline, day 1 and 15 of palbociclib treatment and surgery CCCA was higher on day 1 (87%) vs day 15 of palbociclib treatment (26%) ($P < 0.001$)	*CCNE1* expression was significantly elevated during palbociclib resistance (Ki67>2.7%, 5/41 patients) on day 15 of palbociclib treatment ($P = 2.25e^{-6}$)	Not reported
		(Formisano *et al.* 2019)	Retrospective study	*n* = 34	ER+ HER2- BC	palbociclib + endocrine therapy	Post-progression ctDNA analysed from patients using Guardant360 assay	• 2/14 patients had *CCNE1* amplification • 2/34 patients who progressed on palbociclib had *CCNE1* amplification. Status prior to treatment was unknown	Not reported
		(Kettner *et al.* 2019)	Prospective patient database from MDACC	*n* = 25 patients who progressed on therapy	ER+ HER2- MBC	palbociclib + endocrine therapy	IHC analysis of patient samples pre- and post-progression	LMWE increased from 8/25 patients (pre-treatment) to 15/25 patients (post-progression) ($P = 0.023$)	Not reported
		(Vijayaraghavan *et al.* 2017)	Prospective patient database from MDACC	*n* = 78	ER+ HER2- ABC	palbociclib + letrozole	IHC analysis of patient samples pre- and post-progression	• PFS: Rb+/LMWE- and Rb+/LMWE+ patients (>36.5 months vs 17 months) • 13/35 patients with progressive disease had RB+/MLWE+ tumours	Not reported
				n = 31		palbociclib + fulvestrant		• PFS: Rb+/LMWE- and Rb+/LMWE+ patients (10.7 months vs 4.7 months) • 13/35 patients with progressive disease had RB+/MLWE+ tumours	
Ribociclib									
	MONALEESA-1 NCT01919229	(Curigliano *et al.* 2016)	II Window of opportunity	*n* = 14	HR+ HER2- EBC *No prior therapy* *Postmenopausal*	Ribociclib (400 mg (A) or 600 mg (B)) + letrozole vs letrozole (C) (2 weeks) → surgery	Anti-proliferative response (Ki67) between baseline and EOT Ki67 levels decreased from baseline: 96% (range 78–100%; *n* = 6) (A), 92% (range 75–100%; *n* = 3) (B), 69% (range 38–100%; *n* = 2) (C) Biomarker analysis of paired tumour samples using NanoString 542-gene targeted panel	Decreased expression of *CCNE1* was observed following ribociclib treatment	Not reported
	MONALEESA-2 NCT01958021	(Hortobagyi *et al.* 2016)	III	*n* = 668	HR+ HER2- MBC *No prior therapy for advanced disease* *Postmenopausal*	Ribociclib + letrozole vs letrozole	PFS: median not reached vs 14.7 months (HR: 0.56; 95% CI: 0.43–0.72; $P < 0.00000329$)	Not reported	Not reported
		(Hortobagyi *et al.* 2018)					~26.4 month follow up PFS: 25.3 months vs 16 months (HR: 0.568; 95% CI: 0.457–0.704; $P = 0.0000000963$) Exploratory biomarker analysis of ctDNA in baseline and treated tumour samples (Ki67, *CCND1*, *CDKN2A*, p16, *ESR1*)		
		(Andre *et al.* 2017)				Biomarker analysis of tumour samples at baseline: Rb, p16, Ki67, *CDKN2A*, *CCND1*	Benefit from combination therapy was observed irrespective of baseline Rb, p16, Ki67 levels or *CDKN2A* and *CCND1* expression levels		
		(Formisano *et al.* 2019)	Retrospective study	*n* = 391		Archival patient samples pre- and post-progression on CDK4/6i	Tumour samples assessed for mRNA expression using NanoString 230-gene nCounter GX Human Cancer Reference panel	Ribociclib + letrozole treated patients with high *CCNE1* but not *CDK2* mRNA had statistically shorter PFS than patients with low *CCNE1*	Not reported

(*Continued*)

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Table 3 Continued

Trial	Study	Phase	Enrolment	Treatment history	Description	Outcome measured	*CCNE1*	*CCNE2*
MONALEESA-7 NCT02278120	(Tripathy *et al.* 2018)	III	*n* = 672	HR+ HER2- ABC *No prior endocrine therapy for advanced disease* *≤1 line of chemotherapy for advanced disease* *Pre/perimenopausal*	Ribociclib + tamoxifen / NSAI + goserelin vs tamoxifen / NSAI + goserelin	PFS: 23.8 months vs 13 months (HR: 0.55; 95% CI: 0.44–0.69; one-sided $P < 0.001$) OS at 48 months: 70.2% vs 46% (HR: 0.71; 95% CI: 0.54–0.95; $P = 0.00973$)	Not reported	Not reported
	(Im *et al.* 2019)							
	(Lu *et al.* 2019)		*n* = 360		Gene expression analysis of baseline tumour mRNA	Customised NanoString nCounter GX 800-gene panel	PFS benefit seen with ribociclib also trended to be greater in patients with low vs high expression of *CCNE1* (HR: 0.38 vs 0.65)	Not reported
CLEE011X2106 NCT01857193	(Bardia *et al.* 2018)	Ib/II	*n* = 132	ER+ HER2- ABC Resistant to NSAI *No prior CDK4/6i (cohort 1) or progressed within 1 month of CDK4/6i therapy (cohort 2)* *Postmenopausal*	Ribociclib (300 mg) + everolimus + exemestane vs ribociclib + exemestane	Baseline tumour samples were assessed using NanoString 230-gene nCounter GX Human Reference panel	Cohort 2: trend for higher *CCNE1* and/or *CDK2* expression in patients with progressive disease compared to stable disease	Not reported

Treatment administration: Abemaciclib: every 12 h for 28 days (oral); Anastrazole: 1 mg daily (oral); Everolimus: 2.5 mg daily (oral); Exemestane: 25 mg daily (oral); Fulvestrant: 500 mg every 14 days for three cycles and then every 28 days (i.m. injection); Goserelin: 3.6 mg every 28 days (s.c. injection); Letrozole: 2.5 mg daily (oral); Palbociclib: 125 mg daily for 21 consecutive days, followed by 7 days off (oral); Ribociclib: 600 mg daily for 21 consecutive days, followed by 7 days off (oral); Tamoxifen: 20 mg daily (oral).
ABC: advanced breast cancer; CB: clinical benefit; CBR: clinical benefit rate; CCCA: complete cell cycle arrest; CDK4/6i: cyclin-dependent kinase 4/6 inhibitor; CI: confidence interval; CRR: clinical response rate; ctDNA: circulating tumour DNA; EBC: early breast cancer; ER+: estrogen receptor; EOT: end of treatment; FISH: florescent *in situ* hybridisation; HER2: human EGF receptor 2; HR: hazard ratio; HR+: hormone receptor; IHC: immunohistochemistry; LMW: low-molecular weight cyclin E1; MBC: metastatic breast cancer; MDACC: MD Anderson Cancer Centre (University of Texas); NCT: ClinicalTrials.gov Identifier; NSAI: nonsteroidal aromatase inhibitors, for example, letrozole, anastrozole; ORR: objective response rate; OS: overall survival; PFS: progression-free survival.

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(O'Leary *et al.* 2018). In the study of Formisano *et al.* (2019), 2/34 patients had *CCNE1* amplifications following progression on palbociclib (*CCNE2* status was not measured). However, this cohort was not analysed for pre-existing *CCNE1* amplifications, so it is not known if this was acquired during the development of resistance. *CCNE2* amplification is much more prevalent in ER+ breast cancer, so it could predispose to resistance or be acquired to facilitate resistance. *CCNE2* amplification was observed in 4/18 patients at commencement of the PALOMA-3 trial, but no further amplification events occurred during disease progression (O'Leary *et al.* 2018).

Overall, there is a growing body of evidence that *CCNE1* could be a significant biomarker for poorer response to CDK4/6 inhibitors combined with endocrine therapy. Given the poor performance of cyclin E1 protein as a biomarker for endocrine therapy resistant breast cancer, it seems unlikely that full length cyclin E1 protein could be used as an immunohistochemistry marker in the context of CDK4/6 inhibitor resistance. Insufficient data has been collected regarding the potential of *CCNE2* as a biomarker in CDK4/6 inhibitor resistance, but given the prevalence of gene amplification and strong correlations with proliferation and resistance (DeMichele *et al.* 2016, Arnedos *et al.* 2018), further investigation is warranted.

Emerging cyclin E-CDK2 inhibition strategies

CDK2 activity is potentially a major node of resistance or insensitivity for patients treated with CDK4/6 inhibitors, and this could be targeted by CDK2 inhibitors (Tadesse *et al.* 2019*a*). CDK2 inhibition would target most activities of LMW cyclin E1, full length cyclin E1 and cyclin E2. Specific CDK2 inhibitors are not yet available, although several pan-CDK inhibitors are available which target other CDKs alongside CDK2. Of these, Dinaciclib (CDK1, CDK2, CDK5, CDK9, and CDK12), SNS-032 (CDK2, CDK5, CDK7, and CDK9), and CYC065 (CDK2, CDK5, and CDK9) have been evaluated in clinical trials (NCT00732810, NCT00292864, and NCT02552953), and each has demonstrated preclinical efficacy against CDK2 (Conroy *et al.* 2009, Scaltriti *et al.* 2011, Danilov *et al.* 2016). Unfortunately, pan-CDK inhibitors often have a high level of associated toxicities because of their effects on the other CDKs (Malumbres *et al.* 2008). Targeting of CDK2 could thus be improved by generating more specific CDK2 inhibitors or pan-CDK inhibitors with a narrower spectrum of inhibition and less toxicity (Tadesse *et al.* 2019*b*).

In the context of CDK4/6 inhibition, it will be interesting to see how resistance emerges after treatment with abemaciclib, which has a wider spectrum of kinase inhibitory activity compared with palbociclib and ribociclib (Hafner *et al.* 2019). Abemaciclib has a minor off-target effect on CDK2 (Hafner *et al.* 2019), so it is possible that abemaciclib-treated cancers will not develop resistance via CDK2 activity. Also under development are pan-CDK2/4/6 inhibitors (Hall *et al.* 2019), and these inhibitors may be useful for CDK4/6 inhibitor resistant metastatic breast cancer.

Another possibility is to directly target cyclin E1 or cyclin E2 by RNAi. RNAi based therapies are in clinical trial, but none have yet reached the market. Currently these therapies are best placed to treat disease in the liver, where packaged RNAis accumulate, or for organs that allow topical application (Wittrup & Lieberman 2015). Cyclin E1 has unique kinase independent functions in the liver and is associated with liver fibrosis and hepatocellular carcinoma (Bangen *et al.* 2017), so *CCNE1* RNAi therapy may be useful in those diseases. It is less likely that RNAi based therapies will be successful in breast cancer, as the primary and disseminated disease is generally at sites inaccessible to RNAi therapy. Moreover, cyclin E1 RNAi therapies will not target the high expression of cyclin E2 is prevalent in ER+ breast cancer.

Conclusions

Metastatic ER+ breast cancer remains a clinical challenge because of the high frequency of resistance to endocrine therapies and the emerging resistance to CDK4/6 inhibitors. These drug classes directly or indirectly target CDK4/6 activity in the G_1 to S phase transition of the cell cycle, meaning that the re-routing of proliferation to rely more heavily on CDK2 activity is a prominent mechanism of resistance. Cyclin E1 and E2 are both dysregulated in ER+ breast cancer, providing a potential route to increased CDK2 activity. As shown in the preceding sections, there is now substantial evidence that dysregulation of these two proteins does occur in both endocrine therapy and CDK4/6 inhibitor resistance, but often not concurrently.

An unresolved issue in understanding the biology of cyclins E1 and E2 is the detection of their respective proteins in tissue samples. The detection of cyclin E1 is hampered by difficulties in completely distinguishing the full length and truncated (LMW) versions of the protein which can have different activities. Cyclin E2 has not yet been effectively examined by immunohistochemistry

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across ER+ patient cohorts. The development of better tools for the detection of each protein would aid in understanding the effect of each protein on ER+ breast cancer biology pre-, during-, and post-treatment with endocrine therapies and CDK4/6 inhibitors.

Irrespective of cyclin E1/E2 expression in metastatic ER+ breast cancers, patients will benefit from CDK4/6 inhibitor therapy (Finn *et al.* 2019). There is also no evidence that high expression of cyclins E1 and E2 would prevent any benefit from adjuvant endocrine therapy. However, the identification of pre-existing high expression of cyclin E1/E2 may identify those patients who will recur earlier and require another line of therapy. When patients stop responding to CDK4/6 inhibitors, high cyclin E1 and E2 could be alternative pathways to high CDK2 activity in resistant disease. Consequently, it would be ideal for future studies to include both biomarkers to detect potential perturbations of cyclin E-CDK2 activity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This work was supported by the National Breast Cancer Foundation Australia (ECF17-002 CEC) and the Cancer Institute of NSW (ID: ECF171156 HHM).

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Received in final form 11 February 2020
Accepted 13 February 2020
Accepted Manuscript published online 14 February 2020