Circulating tumor DNA markers for early progression on fulvestrant with or without palbociclib in ER+ advanced breast cancer

Authors: Ben O'Leary MBBS PhD[1,2], Rosalind J Cutts PhD[1], Xin Huang PhD[3], Sarah Hrebien BSc[1], Yuan Liu PhD[3], Fabrice André MD PhD[4], Sibylle Loibl MD PhD[5], Sherene Loi MBBS PhD[6], Isaac Garcia-Murillas PhD[1], Massimo Cristofanilli MD[7], Cynthia Huang Bartlett MD PhD[3], Nicholas C Turner MBBS PhD[1,2,*]

Affiliations: 1 Breast Cancer Now Toby Robins Research Centre, The Institute of Cancer Research, Fulham Rd, London, SW3 6JB. 2 Breast Unit, Royal Marsden Hospital, London, UK.

3 Pfizer, 235 E 42nd St, New York, NY, 10017, USA. (CHB now an ex-employee)

4 Université Paris Sud and Department of Medical Oncology, Institut Gustave Roussy, 94800 Villejuif, France.

5 German Breast Group, Martin Behaim-Strasse 12, 63263 Neu-Isenburg, Germany

6 Division of Research and Cancer Medicine, University of Melbourne, Peter MacCallum Cancer Centre, Melbourne, VIC 3000, Australia.

7 Robert H Lurie Comprehensive Cancer Centre, Feinberg School of Medicine, 675 N St. Clair, Chicago, IL, 60611, USA.

***Corresponding author:** Prof Nicholas Turner – Tel +442073528133, email:

nicholas.turner@icr.ac.uk

Postal address: The Royal Marsden, Fulham Road, London SW3 6JJ

Keywords: palbociclib, fulvestrant, circulating tumor DNA

Abstract

Background

There are no established molecular biomarkers for patients with breast cancer receiving combination endocrine and CDK4/6 inhibitor (CDK4/6i). We aimed to determine whether genomic markers in circulating tumor DNA (ctDNA) can identify patients at higher risk of early progression on fulvestrant therapy with or without palbociclib, a CDK4/6i.

Methods

PALOMA-3 was a phase III multicenter double-blind randomized controlled trial of palbociclib plus fulvestrant (n=347) versus placebo plus fulvestrant (n=174) in patients with endocrine pre-treated ER+ breast cancer. Pre-treatment plasma samples from 459 patients were analyzed for mutations in 17 genes, copy number in 14 genes, and circulating tumor fraction. Progression free survival (PFS) was compared in patients with circulating tumor fraction above or below a pre-specified cut off of 10%, and with or without a specific genomic alteration. All statistical tests were two-sided.

Results

Patients with high ctDNA fraction had worse PFS on both palbociclib plus fulvestrant (HR = 1.62, 95%CI 1.17–2.24, p=.004) and placebo plus fulvestrant (HR = 1.77, 95%CI 1.21–2.59, p=.004. In multivariable analysis high circulating tumor fraction was associated with worse PFS (HR = 1.20 per 10% increase in tumor fraction, 95%CI 1.09–1.32, p<.001, as were *TP53* mutation (HR = 1.84, 95%CI 1.27– 2.65, p=0.001 and *FGFR1* amplification (HR = 2.91, 95%CI 1.61–5.25, p<0.001. No interaction with treatment randomization was observed.

Conclusions

Pre-treatment ctDNA identified a group of high-risk patients with poor clinical outcome despite the addition of CDK4/6 inhibition. These patients might benefit from inclusion in future trials of escalating treatment, with therapies that may be active in these genomic contexts.

CDK4/6 inhibitors (CDK4/6i) now play a key role in the treatment of advanced, estrogen receptor positive (ER+) breast cancers[1], with established efficacy in combination with endocrine therapy in both first and second line treatment[2-8]. However, a substantial proportion of patients progress early on treatment and there is a clinical need to identify patients at risk of early progression.

There are a number of established molecular markers associated with poor outcome in early ER+ breast cancer, most notably the risk-classifiers based on gene expression assessed in tumor biopsies which are now routinely used to augment clinical decision-making[9]. Genomic markers other than *HER2* amplification associated with poorer outcome in primary disease include mutations in *TP53[10, 11]*, amplifications in *FGFR1*[12], which may contribute to endocrine therapy resistance[13], and amplification of *MYC[14]*. Less is known of the associations between common genomic aberrations in advanced ER+ breast cancer and clinical outcome, particularly in the updated therapeutic landscape that includes combination CDK4/6i treatments.

Recent work has identified a number of potential genomic mechanisms of resistance to CDK4/6i, notably amplification of *CCNE1*, mutations in *FAT1,* CDK6 over-expression, and loss of *RB1*[15, 16], with emerging data for immune signatures and other oncogenic signaling[17, 18]. Of these there are clinical data supporting acquisition of *RB1* mutations in a minority of cancers progressing on CDK4/6 inhibitors[19, 20], with pre-existing loss of functional RB1 associated with poor prognosis on CDK4/6i therapy. Loss of *FAT1* was also associated with poor outcome on CDK4/6i therapy[21], although inactivating mutations in *FAT1* are rare in advanced ER+ breast cancer. We have shown previously that mutations in *PIK3CA* and *ESR1* in advanced ER+ breast cancer previously treated with endocrine therapy, do not predict response to palbociclib[22].

Circulating tumor DNA (ctDNA) is found in the plasma of a substantial majority of patients with advanced cancer, and presents a source of cancer DNA for non-invasive

analysis of tumor somatic genetic features. In addition, circulating tumor fraction, the fraction of plasma DNA that is derived from the tumor, may be a biological marker that reports both on tumor bulk and tumor aggressiveness[23], and is associated with poorer clinical outcome in triple negative breast cancer[24].

In conducting this analysis we hypothesized that genomic aberrations identified at baseline, including mutations, copy number and circulating tumor fraction, could be predictive or prognostic of clinical outcome for patients with advanced ER+ breast cancer receiving fulvestrant with or without palbociclib, investigating this using a multi-modal ctDNA sequencing analysis of plasma DNA from the PALOMA-3 trial.

Methods

Full details of the methods can be found in the Supplementary Methods.

Study design and patients

The design of the PALOMA-3 trial (NCT01942135) and clinical outcome data has been previously reported[2]. Patients with advanced, ER+ breast cancer that had previously progressed on endocrine therapy were randomized 2:1 to receive palbociclib plus fulvestrant or placebo plus fulvestrant.

Plasma collection and DNA extraction

Blood was collected in EDTA tubes on day 1 of treatment and within 30 minutes was centrifuged at 3000g for 10 minutes before plasma separation. Samples were then stored at -80°C prior to DNA extraction. DNA concentration was estimated using a droplet digital PCR (ddPCR) assay directed at *RPPH1* on the BioRad QX200.

Sequencing and digital PCR

Mutations were assessed in baseline plasma DNA using a previously reported targeted error-corrected sequencing approach, utilizing a bespoke bioinformatic pipeline incorporating integrated digital error suppression (iDES)[19, 25]. The targeted panel included 17 genes, with all coding exons of *CDK4*, *CDK6*, *CDKN1A*, *CDKN1B*, *RB1* and *NF1*, exons 5-8 of *TP53* and mutation hotspots in *AKT1*, *ERBB2*, *ESR1*, *PIK3CA*, *FGFR1*, *FGFR2*, *FGFR3*, *KRAS*, *HRAS* and *NRAS*. Of the baseline plasma DNA sequencing, 195 patients were previously sequenced to compare mutational profile with end-of-treatment progression plasma[19], with an additional previously unreported 136 patients' baseline plasma DNA sequenced for the comprehensive baseline analysis presented here. Digital PCR had been previously performed on the baseline plasma DNA samples for *PIK3CA* and *ESR1* mutation [26].

Circulating tumor fraction was assessed using a previously reported bespoke targeted amplicon panel including prevalent heterozygous SNPs in 8 regions commonly lost in breast cancer, additionally with amplicons assessing for loss or loss of heterozygosity of *RB1*, *PTEN* and *CDKN2A*, and for gain of *ERBB2*, *EGFR*, *PIK3CA*, *ESR1*, *CDK4*, *FGFR1*, *FGFR2*, *MYC*, *MCL1*, *CCND1* and *CCNE1*[19]. Comparison with tumor fraction estimated from low pass whole genome sequencing was performed in 19 samples sequenced with tumor fraction estimated using ichorCNA[23].

Statistical analysis

The primary outcome of this study was to identify potential prognostic and predictive factors for progression free survival within both treatment arms. PALOMA-3 was designed and powered for a clinical endpoint, and as such was not specifically powered for a translational analysis. Survival analyses to associate progression-free survival (PFS) with genomic aberrations were performed with Cox proportional hazards models, with calculation

of hazard ratios, 95% confidence intervals and logrank p values. Proportionality was assessed using the method described by Grambsch and Thernau[27]. For circulating tumor fraction analysis a 10% cut-off was pre-specified for association with PFS as previously used in the literature [23, 24]. To explore the potential statistical significance of genomic alterations an initial univariable analysis in each treatment arm was planned, to be followed by a multivariable analysis incorporating treatment as a variable to test for interaction. Associations of clinical and pathological characteristics with genomic aberrations were tested with χ^2 tests or Cochran Armitage tests for trend. P values were considered statistically significant for values <.05. The Benjamini-Hochberg approach was used to adjust for multiple comparisons. All statistical tests were two-sided.

Results

Circulating tumor fraction and progression free survival

Of the enrolled patients with available plasma, 401/459 (87.4%) patients had sufficient material and subsequent library quality for circulating tumor fraction and copy number analysis, a group with outcomes representative of the overall trial population (Figure 1A, Supplementary figure 1). Circulating tumor fraction assessment was found to agree well with orthogonal assessment in a sample of n = 19 plasma samples assessed for tumor fraction using low depth whole genome sequencing (Pearson's r = 0.86, p < .001, Supplementary figure 2), and tumor fraction correlated with *PIK3CA* allele fraction (Pearson's r = 0.71, p < .001, Supplementary figure 3) and *TP53* allele fraction (Pearson's r = 0.79, p < .001, Supplementary figure 4).

A high circulating tumor fraction (>10% fraction, pre-specified) was observed in 38.9% (156/401), patients (Figure 1B). In the palbociclib plus fulvestrant group median PFS in patients with circulating tumor fraction of >10% was 9.2 months (95% CI 5.8-11.1) and for

those with circulating tumor fraction ≤10% was 13.6 months (95% CI 11.3-16.6, HR = 1.62, 95% CI 1.17 – 2.24, logrank p = .004, Figure 1C). In the placebo plus fulvestrant group median progression free survival in patients with circulating tumor fraction of >10% was 2.8 months (95% CI 1.9-3.9) and with circulating tumor fraction ≤10% was 5.5 months (95% CI 3.7-9.1, HR = 1.77, 95%CI 1.21 – 2.59, logrank p = .004, Figure 1D). In an exploratory analysis using discrete cut offs, circulating tumor fractions of >20% were associated with increasingly worse PFS (Supplementary Figure 5).

Genomic analysis in baseline ctDNA and association with clinical characteristics

Of the 521 patients enrolled in the study, 331/521 (63.5%) had sufficient material and subsequent library quality for mutation analysis by sequencing, with this population also representative of the overall trial (Figure 1A, Supplementary figure 6). The most commonly mutated gene was *ESR1* (72/331, 21.8%, Figure 2A and Figure 2B), with a comparable prevalence of *PIK3CA* mutation (55/331, 16.6%) and *TP53* (52/331, 15.7%). Smaller proportions of patients had mutations in *NF1* (21/331, 6.3%), *ERBB2* (12/331, 3.6%), and *AKT1* (10/331, 3.0%). Mutations in *ESR1* were polyclonal in a subset of patients (16/72, 22.2%, Figure 2A).

Detection of copy number aberrations (CNAs) is technically challenging in samples with low circulating tumor fraction, and we assessed the prevalence of CNAs only in the group with >10% circulating tumor fraction. The most frequently observed gains from the genes included in the panel were *MCL1*, *CCND1*, *MYC* and *FGFR1* (Figure 2C) and there was evidence of copy number loss and/or loss of heterozygosity (LOH) in similar proportions of *RB1* (27/156, 17.3%), *PTEN* (30/156, 19.2%) and *CDKN2A* (34/156, 22.0%).

Having established the landscape of genomic aberrations in ctDNA at baseline, we assessed associations with clinical characteristics (Figure 2D). A positive association was observed between *ESR1* mutation and previous endocrine sensitivity (Chi-square p = .015),

previous aromatase inhibitor exposure (Chi-square p = .002), bone metastases (Chi-square p = .005) and number of all previous lines of treatment for metastatic disease (Cochran Armitage p = .019), associations similar to those previously reported using digital PCR analysis (Supplementary Figure 7)[22]. Prior aromatase inhibitor therapy (Chi-square q = .021) and bone metastases (Chi-square q = .028) remained statistically significant after correction for multiple testing using the Benjamini Hochberg method. *TP53* mutations were positively associated with visceral metastases (Chi-square q = .046), soft tissue/lymph node metastases (Chi-square q = .042) and number of disease sites (Cochran Armitage q = .009). No other mutations or copy number changes were statistically significantly associated with a particular clinical characteristic after correction for multiple testing. There was no a detectable association of between circulating tumor fraction >10% and clinical and pathological features, after correcting for multiple comparisons (Figure 2D). Mutations in specific genes associated with higher circulating tumor fractions in patients - this was statistically significant for the most prevalent mutations, in *PIK3CA*, *TP53* and *ESR1*, most likely simply demonstrating that higher circulating tumor fraction means mutation detection in ctDNA is more likely (Supplementary figure 8).

Genomic analysis in baseline ctDNA and progression free survival

We next analyzed associations between mutations and copy number changes and PFS, initially with both treatment groups separately in a univariable analysis. In the group of patients treated with palbociclib plus fulvestrant (n = 223 for mutations n = 259 for copy number, Figure 3A) *TP53* mutations were associated with worse PFS (HR = 2.00, 95% CI 1.28 – 3.12, logrank p = .002). Multiple copy number gains were associated with poorer PFS including *MCL1* gain (HR = 2.29, 95% CI 1.24 – 4.26, logrank q = .014), *FGFR1* gain (HR 3.40, 95% CI 1.91 – 6.04, logrank q < .001), *MYC* gain (HR = 2.97, 95% CI 1.67 – 5.26, logrank q < .001), *CDK4* gain (HR = 4.22, 95% CI 1.33 – 13.41, logrank q = .021) and *CCNE1* gain (HR = 5.71, 95% CI 2.30 – 14.21, logrank q < .001). Loss and/or LOH of *RB1*,

PTEN and *CDKN2A* were also associated with worse prognosis (Supplementary figure 9 and 10).

In the group of patients treated with placebo plus fulvestrant (n = 108 for mutations 142 for copy number, Figure 3B), *TP53* mutations (HR = 2.26, 95% CI 1.30 – 3.93, logrank q = .026) and *ESR1* mutations (HR = 1.85, 95% CI 1.13 – 3.02, logrank q = .047) were associated with worse PFS after correction for multiple testing using the Benjamini Hochberg method. Copy number gain (amplification) of *FGFR1* (HR = 3.61, 95% CI 1.31 – 9.97, logrank q = .047) and *MCL1* (HR = 2.40, 95% CI 1.15 – 4.99, logrank q = .05) were associated with worse PFS. With a 2:1 randomization, the placebo plus fulvestrant group was relatively small, making direct comparisons between treatment groups challenging, and there were no individual aberrations that had a statistically significant interaction p value with treatment.

With increased circulating tumor fraction is required to detect copy number changes in plasma, and higher circulating tumor fraction associates with worse PFS, we performed a multivariable survival analysis (Methods and Table 1). Circulating tumor fraction remained statistically significant in the model (HR = 1.20 per 10% increase in tumor fraction, 95%CI 1.09–1.32, logrank p<.001), along with *TP53* mutation (HR = 1.84, 95%CI 1.27– 2.65, logrank p=.001) and *FGFR1* gain (HR = 2.91, 95%CI 1.61–5.25, logrank p<.001). Patients with *TP53* mutations and *FGFR1* amplifications had a very poor median PFS on palbociclib and fulvestrant of 3.7 months and 3.9 months respectively (Figure 4A and 4B). There was no statistically significant interaction for any genomic aberration with treatment randomization. For the analyzed cohort ctDNA analysis identified at least one of the three poor prognosis factors, circulating tumor fraction>10%, *TP53* mutation or *FGFR1* gain in 42.3% (131/310) patients (Figure 4C).

Discussion

Combinations of CDK4/6i and endocrine therapy are standard of care in advanced ER+ breast cancer. There are few molecular markers available to identify patients at risk of early progression, where increased monitoring to detect progression may be appropriate and for whom research efforts might be focused to improve outcomes. We have previously published work from the PALOMA-3 trial examining the evolution of resistance[19]. Here we build on this by using a multi-modal ctDNA sequencing analysis of all the baseline plasma samples to assess for predictive and prognostic genomic features, greatly expanding the range of baseline genomic alterations from our previous work on *ESR1* and *PIK3CA* using digital PCR[22]. We did not identify any predictive genomic alterations, but circulating tumor fraction, *TP53* mutation and *FGFR1* gain were each independently associated with risk of early relapse for both fulvestrant alone and fulvestrant plus palbociclib treatments. Approximately half of patients with *TP53* mutation or *FGFR1* gain detected in plasma DNA had progressed by 2 months, despite the addition of a CDK4/6 inhibitor. Combined, these genomic markers identify a significant subset of the patients (42.3%), a group who may benefit from augmented treatment strategies.

Broadly, there was strong agreement between the estimated circulating tumor fraction and those mutations expected to be commonly clonal, such as in *PIK3CA* and *TP53*, though the association was weaker at lower mutation allele fractions, likely reflecting subclonal mutations and stochastic effects (Supplementary figures 2, 3 and 4). Circulating tumor fraction was strongly associated with adverse PFS in both treatment groups in the PALOMA-3 study (Figure 1), and emerged as an independent prognostic factor in the multivariable analysis - the first demonstration of this association in ER+ breast cancer. Although levels of ctDNA are associated with stage and tumor burden[28], they are not simply a surrogate for tumor volume and are associated with proliferation[26, 29-32], and it is likely that circulating tumor fraction is an independent prognostic marker due to the collective

effect of all these elements. Consistent with prior reports, we found no association of ctDNA fraction with the number of disease sites. Our findings are also consistent with observations in triple negative breast cancer[24], suggesting such analysis could become a general tool in stratifying risk for breast cancer patients. In addition, given that circulating tumor fraction is associated with the ability to detect genomic aberrations in ctDNA analysis, our analysis highlights the importance of considering circulating tumor fraction when validating associations between ctDNA detected mutations/copy number changes and clinical outcomes.

We did not identify any genomic alterations that were predictive for outcome on palbociclib. In the univariable analysis some alterations were observed to have a consistent association with PFS in both arms, notably *TP53* and *FGFR1* gain (Figure 3) with others appearing in only one, such as *CCNE1* and *CDK4* gain in the palbociclib arm and *ESR1* mutation in the fulvestrant alone arm. However, no statistically significant treatment interaction effect was observed with any alteration. Some of these alterations, notably *CCNE1* gain, which was associated with marked poor prognosis in the palbociclib plus fulvestrant group (Figure 3), remain plausible palbociclib resistance markers with prediction analysis underpowered due to low prevalence. For prognosis, only *TP53* mutation and *FGFR1* gain remained statistically significantly associated with worse outcome once treatment and circulating tumor fraction were taken into account.

TP53 is one of the most commonly mutated genes in breast cancer[33], observed at a higher prevalence in luminal B cancers as compared to luminal A cancers[33]. In this analysis *TP53* mutations were associated with a distinct clinical phenotype characterized by more sites of metastases and more prevalent visceral and soft tissue/LN metastases. *TP53* mutations associate with poorer clinical outcome in ER positive primary breast cancer [10, 11], and endocrine resistance[34]. Our work suggests that the aggressive biology for *TP53* mutant ER+ breast cancer continues in the advanced setting, with the association between

TP53 mutation and poor outcome in both treatment arms of the PALOMA-3 trial demonstrating consistency of this finding across two different treatments, and raising the question of considering this subset of breast cancer a distinct clinical entity.

FGFR1 amplification emerged as independently associated with early progression. *FGFR1* amplification is associated with endocrine resistance[13], and with no observed interaction effect with treatment, this finding predominantly suggests resistance to the fulvestrant backbone element of the combination. As with *TP53* mutation a similar effect was observed in the separate treatment arms. Nevertheless, recent data has highlighted a potential role for FGFR signaling in resistance to CDK4/6i[35]. This suggests the potential of FGFR inhibitors, in particular in cancers with high-level *FGFR1* amplification[36] that would be more readily detectable in ctDNA, to enhance treatment efficacy. However, the *FGFR1* 8p11/12 amplicon is often broad, with *FGFR1* signaling likely a driver only in a subset of cancers[37].

This report has a number of important limitations. Although we were able to assess and account for circulating tumor fraction accurately above 10%, robust assessment of tumor fractions below 10% was not possible, and we are unable to ascertain the potential impact of lower cut-offs. Calling copy-number is challenging in plasma DNA sequencing, and the number of tumors with copy number changes has been under-called; amplifications are only detectable in tumors with high tumor fraction or in cancers with lower tumor fractions when high levels of copy number are present in the tumor. Genomic loss is even harder to assess in plasma DNA, restricted to cancers with the highest tumor purity. Lastly, *TP53* mutations are also found in clonal hematopoiesis[38], and without direct analysis of matching buffy coat for the plasma samples we are unable to exclude the effect of this. Prior to application in clinical trials, independent validation of these findings will be important.

In summary, using ctDNA analysis we identify genomic features that associate with a risk of early progression on fulvestrant and palbociclib, with at least one feature present in

42% of patients in PALOMA-3. Validation of these findings will be required before trials assessing clinical utility are conducted [39]. If the observations here can be independently validated, then patients with these features may be suitable for clinical trials of more intensive surveillance on treatment, or of trials examining escalation of therapy to assess these strategies for clinical benefit.

Funding

This work was supported by The Medical Research Council (MR/N002121/1), Breast Cancer Now with support from the Mary-Jean Mitchell Green Foundation and Pfizer. We acknowledge National Institute for Health Research funding to the Royal Marsden and Institute of Cancer Research Biomedical Research Centre. We thank Breast Cancer Now for funding this work as part of Program Funding to the Breast Cancer Now Toby Robins Research Centre.

Notes

Role of the sponsor: The sponsor collaborated with the senior academic authors to design the clinical study, and assisted with data collection, analysis and interpretation. All authors had responsibility for the decision to submit the manuscript for publication.

Conflict of interest disclosures: BO'L – Research funding (Inst): Pfizer. YL, XH, CHB – Ex-Employment: Pfizer, Stock ownership: Pfizer. FA – Travel, Accommodation, Expenses: Novartis, Roche, GlaxoSmithKline, AstraZeneca, Research Funding (Inst): AstraZeneca, Novartis, Pfizer, Lilly, Roche. SLoibl - Consulting or Advisory Role (Inst): Pfizer, Roche, Novartis, Seattle Genetics, Honoraria: Pfizer, Roche, Research Funding (Inst): Pfizer, Roche, Celgene, Amgene, Novartis, Abbvie, AstraZeneca, Seattle Genetics, Teva, Vifor Pharma. SLoi – Consulting or Advisory Role (Inst): AstraZeneca/MedImmune, Seattle

Genetics, Bristol-Myers Squibb, Pfizer, Novartis, Roche/Genentech, Merck Sharp & Dohme, Research Funding (Inst): Roche/Genentech, Pfizer, Novartis, Merck, Puma Biotechnology, Bristol-Myers Squib. MC – Consulting or Advisory Role: Dompé Farmaceutici, Newomics, Vortex Biosciences, Honoraria: Dompé Farmaceutici, Pfizer. NC - Consulting or Advisory Role: Roche, Pfizer, Novartis, AstraZeneca Research Funding: Pfizer (Inst), Roche (Inst), AstraZeneca. The other authors have no conflict of interest disclosures to make.

Author contributions

CHB, MC and NT conceived and designed the PALOMA-3 study

BO'L, IGM, YL, CHB and NT - conceived and designed the project

BO'L, RC, SH, YL, IGM, SLoibl, FA, SLoi, CHB, MC and NT – collected and assembled the data

BO'L, RC, XH, SH, YL, IGM, SLoibl, FA, SLoi, CHB, MC and NT - data analysis and interpretation

BO'L, RC, XH, SH, YL, IGM, SLoibl, FA, SLoi, CHB, MC and NT – writing manuscript

Data availability: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgements: We would like to thank the patients, families and carers who participated in the PALOMA-3 trial, and all the investigators and site personnel.

References

1. Turner NC, Neven P, Loibl S, *et al.* Advances in the treatment of advanced oestrogen-receptor-positive breast cancer. The Lancet 2016;389(10087):2403-2414.
2. Turner NC, Ro J, Andre F, *et al.* Palbociclib in Hormone-Receptor-Positive Advanced Breast Cancer. N Engl J Med 2015; 10.1056/NEJMoa1505270.
3. Finn RS, Martin M, Rugo HS, *et al.* Palbociclib and Letrozole in Advanced Breast Cancer. New England Journal of Medicine 2016;375(20):1925-1936.
4. Hortobagyi GN, Stemmer SM, Burris HA, *et al.* Ribociclib as First-Line Therapy for HR-Positive, Advanced Breast Cancer. New England Journal of Medicine 2016;0(0):null.
5. Slamon DJ, Neven P, Chia S, *et al.* Phase III Randomized Study of Ribociclib and Fulvestrant in Hormone Receptor–Positive, Human Epidermal Growth Factor Receptor 2–Negative Advanced Breast Cancer: MONALEESA-3. Journal of Clinical Oncology 2018;36(24):2465-2472.
6. Tripathy D, Im S-A, Colleoni M, *et al.* Ribociclib plus endocrine therapy for premenopausal women with hormone-receptor-positive, advanced breast cancer (MONALEESA-7): a randomised phase 3 trial. The Lancet Oncology 2018;19(7):904-915.
7. Sledge GW, Toi M, Neven P, *et al.* MONARCH 2: Abemaciclib in Combination With Fulvestrant in Women With HR+/HER2− Advanced Breast Cancer Who Had Progressed While Receiving Endocrine Therapy. Journal of Clinical Oncology 2017; 10.1200/JCO.2017.73.7585:JCO.2017.73.7585.
8. Goetz MP, Toi M, Campone M, *et al.* MONARCH 3: Abemaciclib As Initial Therapy for Advanced Breast Cancer. Journal of Clinical Oncology 2017;35(32):3638-3646.
9. Harris LN, Ismaila N, McShane LM, *et al.* Use of Biomarkers to Guide Decisions on Adjuvant Systemic Therapy for Women With Early-Stage Invasive Breast Cancer: American Society of Clinical Oncology Clinical Practice Guideline. Journal of Clinical Oncology 2016;34(10):1134-1150.
10. Olivier M, Langer√∏d A, Carrieri P, *et al.* The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clinical Cancer Research 2006;12(4):1157.
11. Bonnefoi H, Piccart M, Bogaerts J, *et al.* TP53 status for prediction of sensitivity to taxane versus non-taxane neoadjuvant chemotherapy in breast cancer (EORTC 10994/BIG 1-00): a randomised phase 3 trial. The Lancet Oncology 2011;12(6):527-539.
12. Elbauomy Elsheikh S, Green A, Lambros M, *et al.* FGFR1 amplification in breast carcinomas: a chromogenic in situ hybridisation analysis. Breast Cancer Research 2007;9(2):R23.
13. Turner N, Pearson A, Sharpe R, *et al.* FGFR1 Amplification Drives Endocrine Therapy Resistance and Is a Therapeutic Target in Breast Cancer. Cancer Research 2010;70(5):2085-2094.
14. Deming SL, Nass SJ, Dickson RB, *et al.* C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. British Journal of Cancer 2000;83(12):1688-1695.
15. Herrera-Abreu MT, Palafox M, Asghar U, *et al.* Early Adaptation and Acquired Resistance to CDK4/6 Inhibition in Estrogen Receptor-Positive Breast Cancer. Cancer Res 2016; 10.1158/0008-5472.can-15-0728.
16. Yang C, Li Z, Bhatt T, *et al.* Acquired CDK6 amplification promotes breast cancer resistance to CDK4/6 inhibitors and loss of ER signaling and dependence. Oncogene 2017;36(16):2255-2264.
17. De Angelis C, Fu X, Cataldo ML, *et al.* Abstract GS2-01: High levels of interferon-response gene signatures are associated with de novo and acquired resistance to CDK4/6 inhibitors in ER+ breast cancer. Cancer Research 2020;80(4 Supplement):GS2-01.
18. Wander SA, Cohen O, Gong X, *et al.* Abstract PD2-09: The genomic landscape of intrinsic and acquired resistance to cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) in patients with hormone receptor-positive (HR+)/HER2- metastatic breast cancer (MBC). Cancer Research 2020;80(4 Supplement):PD2-09.
19. O'Leary B, Cutts RJ, Liu Y, *et al.* The Genetic Landscape and Clonal Evolution of Breast Cancer Resistance to Palbociclib plus Fulvestrant in the PALOMA-3 Trial. Cancer Discovery 2018;8(11):1390.
20. Condorelli R, Spring L, O'Shaughnessy J, *et al.* Polyclonal RB1 mutations and acquired resistance to CDK 4/6 inhibitors in patients with metastatic breast cancer. Annals of Oncology 2017; 10.1093/annonc/mdx784:mdx784-mdx784.
21. Li Z, Razavi P, Li Q, *et al.* Loss of the FAT1 Tumor Suppressor Promotes Resistance to CDK4/6 Inhibitors via the Hippo Pathway. Cancer Cell 2018;34(6):893-905.e8.
22. Fribbens C, O'Leary B, Kilburn L, *et al.* Plasma ESR1 Mutations and the Treatment of Estrogen Receptor–Positive Advanced Breast Cancer. Journal of Clinical Oncology 2016; 10.1200/jco.2016.67.3061.
23. Adalsteinsson VA, Ha G, Freeman SS, *et al.* Scalable whole-exome sequencing of cell-free DNA reveals high concordance with metastatic tumors. Nature Communications 2017;8(1):1324.
24. Stover DG, Parsons HA, Ha G, *et al.* Association of Cell-Free DNA Tumor Fraction and Somatic Copy Number Alterations With Survival in Metastatic Triple-Negative Breast Cancer. Journal of Clinical Oncology 2018;0(0):JCO.2017.76.0033.

25. Newman AM, Lovejoy AF, Klass DM, *et al.* Integrated digital error suppression for improved detection of circulating tumor DNA. Nat Biotech 2016;advance online publication.
26. O'Leary B, Hrebien S, Morden JP, *et al.* Early circulating tumor DNA dynamics and clonal selection with palbociclib and fulvestrant for breast cancer. Nature Communications 2018;9(1):896.
27. Grambsch PM, Therneau TM. Proportional hazards tests and diagnostics based on weighted residuals. Biometrika 1994;81(3):515-526.
28. Bettegowda C, Sausen M, Leary RJ, *et al.* Detection of Circulating Tumor DNA in Early- and Late-Stage Human Malignancies. Science Translational Medicine 2014;6(224):224ra24.
29. Abbosh C, Birkbak NJ, Wilson GA, *et al.* Phylogenetic ctDNA analysis depicts early stage lung cancer evolution. Nature 2017;advance online publication.
30. Parkinson CA, Gale D, Piskorz AM, *et al.* Exploratory Analysis of TP53 Mutations in Circulating Tumour DNA as Biomarkers of Treatment Response for Patients with Relapsed High-Grade Serous Ovarian Carcinoma: A Retrospective Study. PLOS Medicine 2016;13(12):e1002198.
31. Garlan F, Laurent-Puig P, Sefrioui D, *et al.* Early evaluation of circulating tumor DNA as marker of therapeutic efficacy in metastatic colorectal cancer patients (PLACOL study). Clinical Cancer Research 2017; 10.1158/1078-0432.ccr-16-3155.
32. Tie J, Kinde I, Wang Y, *et al.* Circulating tumor DNA as an early marker of therapeutic response in patients with metastatic colorectal cancer. Annals of Oncology 2015;26(8):1715-1722.
33. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature 2012;490(7418):61-70.
34. Griffith OL, Spies NC, Anurag M, *et al.* The prognostic effects of somatic mutations in ER-positive breast cancer. Nature Communications 2018;9(1):3476.
35. Formisano L, Lu Y, Servetto A, *et al.* Aberrant FGFR signaling mediates resistance to CDK4/6 inhibitors in ER+ breast cancer. Nature Communications 2019;10(1):1373.
36. Pearson A, Smyth E, Babina IS, *et al.* High-Level Clonal FGFR Amplification and Response to FGFR Inhibition in a Translational Clinical Trial. Cancer Discovery 2016;6(8):838.
37. Babina IS, Turner NC. Advances and challenges in targeting FGFR signalling in cancer. Nature Reviews Cancer 2017;17:318.
38. Xie M, Lu C, Wang J, *et al.* Age-related mutations associated with clonal hematopoietic expansion and malignancies. Nature Medicine 2014;20(12):1472-1478.
39. Merker JD, Oxnard GR, Compton C, *et al.* Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. Journal of Clinical Oncology 2018;36(16):1631-1641.

Tables

Table 1. Multivariable analysis of the association between circulating tumor DNA genomic features and progression free survival*

Variable	Hazard ratio (95% CI)	P value†
FGFR1 gain	2.91 (1.61 – 5.25)	<.001
TP53 mutation	1.84 (1.27 – 2.65)	.001
ctDNA tumor fraction	1.20 (1.09 – 1.32)	<.001
Palbociclib	0.43 (0.32 - 0.57)	<.001

*Treatment and circulating tumor fraction are included as variables in the model, the latter as a continuous variable calculated per unit 10% increase. P values are logrank. The table includes only those factors remaining statistically significant with a p value < .05. The model was constructed using forward stepwise selection including all genomic alterations that were statistically significant with logrank p < .05 in either of both of the treatment arms (Figure 3), specifically, gain of *FGFR1*, *CCNE1*, *MCL1*, *MYC*, *CDK4*, loss of *RB1*, *CDKN2A*, *PTEN* and mutation in *TP53*, and mutation in *ESR1*.

† [Two-sided logrank]

Figure legends

1. Write “Figure 1” in the figure file, “Figure 2”, “Figure 3”, etc. Otherwise the reader won’t know what figure they are looking at in the accepted version.
2. Report all P values using journal style: delete the leading zero; follow the rules on the RMC for rounding P values.
3. All HRs and 95%CIs should have “=” in them (do not use parentheses) and should be given to two decimal places.

Figure 1. Circulating tumor fraction and progression free survival in PALOMA3

A - CONSORT and Venn diagram showing analysis of plasma samples from the PALOMA-3 trial. B – Distribution of detected circulating tumor fraction at baseline (n = 401). C – Progression free survival for the palbociclib plus fulvestrant group (n = 259) split by circulating tumor fraction above or below 10%.D - Progression free survival for the placebo plus fulvestrant group (n = 142) split by circulating tumor fraction above or below 10%. P values logrank. CI – confidence interval. PFS – progression free survival.

Figure 2. Genomic landscape of endocrine resistant breast cancer in circulating tumor DNA analysis.

A –Distribution and number of mutations by patient at baseline. B – Prevalence of mutated genes observed in the baseline plasma samples (n = 331). C – Prevalence of copy number gain from the subset of patients with >10% circulating tumor fraction. D - Associations between clinic-pathological characteristics and ctDNA genomic features. P values calculated with chi squared if categorical or Cochran Armitage tests if ordinal, corrected using Benjamini-Hochberg method. ER – estrogen receptor, LN – lymph node, LOH – loss of heterozygosity.

Figure 3. Association between ctDNA genomic features and progression free survival

A – Univariable analyses of progression free survival by detected genomic aberrations for the palbociclib plus fulvestrant group. B - Univariable analyses of progression free survival by detected genomic aberrations for the placebo plus fulvestrant group. The size of bubble indicates the prevalence within the treatment group. P values are logrank. Correction is with the Benjamini-Hochberg method.

Figure 4. *TP53* mutation, *FGFR1* amplification, and progression free survival.

A - Progression free survival by detected *TP53* mutation for the palbociclib plus fulvestrant and placebo plus fulvestrant groups. B - Progression free survival by detected *FGFR1* gain for the palbociclib plus fulvestrant and placebo plus fulvestrant arms. C – Per patient distribution of detected *TP53* mutation, *FGFR1* gain and circulating tumor fraction from the n = 310 patients with all of mutations, copy number and circulating tumor fraction data available. Hazard ratio univariable analysis, P values logrank. CI – confidence interval. NE – not estimable. PFS – progression free survival.