JNCI Cancer Spectrum, 2023, **7(2),** pkad014
https://doi.org/10.1093/jncics/pkad014
Advance Access Publication Date: February 20, 2023
Brief Communications

$p27^{Kip1}$ V109G as a biomarker for CDK4/6 inhibitors indication in hormone receptor–positive breast cancer

Silvana Mouron, PhD,[1,†] Maria J Bueno, PhD,[1,†] Manuel Muñoz, AS,[1] Raul Torres, PhD,[2] Sandra Rodríguez, PhD,[2] Juan V. Apala, MD,[1] Jorge Silva, MD,[1] Rodrigo Sánchez-Bayona, MD, PhD,[3] Luis Manso, MD, PhD,[3] Juan Guerra, MD, PhD,[4] Laura Rodriguez-Lajusticia, MD,[4] Diego Malon, MD,[4] Marcos Malumbres, PhD,[5,6,7] Miguel Quintela-Fandino, PhD, MD[1,4,8,*]

[1]Breast Cancer Clinical Research Unit, Centro Nacional de Investigaciones Oncológicas—CNIO, Madrid, Spain
[2]Molecular Cytogenetics Unit, Centro Nacional de Investigaciones Oncológicas—CNIO, Madrid, Spain
[3]Medical Oncology Department, Hospital Universitario, 12 de Octubre, Madrid, Spain
[4]Medical Oncology Department, Hospital Universitario de Fuenlabrada, Madrid, Spain
[5]Cell Division & Cancer Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain
[6]Cancer Cell Cycle group, Vall d'Hebron Institute of Oncology (VHIO), Barcelona, Spain
[7]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain
[8]Endowed Chair of Personalized Precision Medicine, Universidad Autonoma de Madrid (UAM) – Fundacion Instituto Roche, Madrid, Spain

*Correspondence to: Miguel Quintela-Fandino, MD, PhD, Breast Cancer Clinical Research Unit, CNIO—Spanish National Cancer Research Center, Melchor Fernandez Almagro, 3, 28029 Madrid, Spain (e-mail: mquintela@cnio.es).
†These authors contributed equally to this work.

Abstract

CDK4/6 inhibitors benefit a minority of patients who receive them in the breast cancer adjuvant setting. $p27^{Kip1}$ is a protein that inhibits CDK/Cyclin complexes. We hypothesized that single-nucleotide polymorphisms that impaired $p27^{Kip1}$ function could render patients refractory to endocrine therapy but responsive to CDK4/6 inhibitors, narrowing the patient subpopulation that requires CDK4/6 inhibitors. We found that the $p27^{Kip1}$ V109G single-nucleotide polymorphism is homozygous in approximately 15% of hormone-positive breast cancer patients. Polymorphic patients experience rapid failure in response to endocrine monotherapy compared with wild-type or heterozygous patients in the first-line metastatic setting (progression-free survival: 92 vs 485 days, $P < .001$); when CDK4/6 inhibitors are added, the differences disappear (progression-free survival: 658 vs 761 days, $P = .92$). As opposed to wild-type $p27^{Kip1}$, $p27^{Kip1}$ V109G is unable to suppress the kinase activity of CDK4 in the presence of endocrine inhibitors; however, palbociclib blocks CDK4 kinase activity regardless of the $p27^{Kip1}$ status. $p27^{Kip1}$ genotyping could constitute a tool for treatment selection.

As opposed to the advanced disease setting (1-7), current results suggest only limited benefit from blocking CDK4/6 in early hormone receptor–positive breast cancer (HRPBC) (8-11). To our knowledge, no predictive factors have been defined to date (10,11). This point is relevant because a large percentage of patients seem to be adequately managed with endocrine monotherapy, and abemaciclib rescues from metastatic relapse only a limited number of patients (10,11). Differentiating the patients who are adequately treated with endocrine monotherapy from those who require combination with CDK4/6 inhibitors to avoid relapse would save considerable economic resources and avoid toxicity.

Single-nucleotide polymorphisms (SNPs) are variations in the germline genetic code that can result in functional changes. *CDKN1B* encodes $p27^{Kip1}$, a protein involved in cell cycle control. $p27^{Kip1}$ binds and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, slowing down the cell cycle (12,13). We hypothesized that SNPs that impair the function of $p27^{Kip1}$ could render cells insensitive to hormonal blockade because CDK/cyclin complexes would be active regardless of upstream signals. This lack of response, however, could be reverted by CDK4/6 kinase inhibitors, which would block the cell cycle despite the functional impairment of $p27^{Kip1}$. Patients harboring dysfunctional $p27^{Kip1}$ variants could be those who are not adequately treated with endocrine monotherapy and would require CDK4/6 inhibitors for disease control, as opposed to patients with the wild type.

The *CDKN1B* T329G SNP (RS2066827, encoding for $p27^{Kip1}$ V109G) has been previously related to the incidence and prognosis of different cancers (14-23). We genotyped 10 hormone-positive breast cancer cell lines (Supplementary Table 1, available online). T47-D was the only one that was endocrine sensitive and homozygous for the wild-type allele. Taking advantage of CRISPR-Cas9, we generated isogenic $p27^{Kip1}$ V109G G/G variants (homozygous for the variant allele) of the T47-D parental V/V line (homozygous for the wild-type allele; Figure 1, A; Supplementary Figure 1, available online). To account for potential off-target effects of CRISPR-Cas9, 3 independent G/G clones (C1, E1, and F5) were generated. $p27^{Kip1}$ levels did not vary statistically significantly according to the genotype (Figure 1, B).

Compared with V/V cells, colony (Figure 1, C) and BRDU (Bromodesoxiuridine) incorporation assays (Figure 1, D) showed

Received: December 30, 2022. **Revised:** February 03, 2023. **Accepted:** February 13, 2023

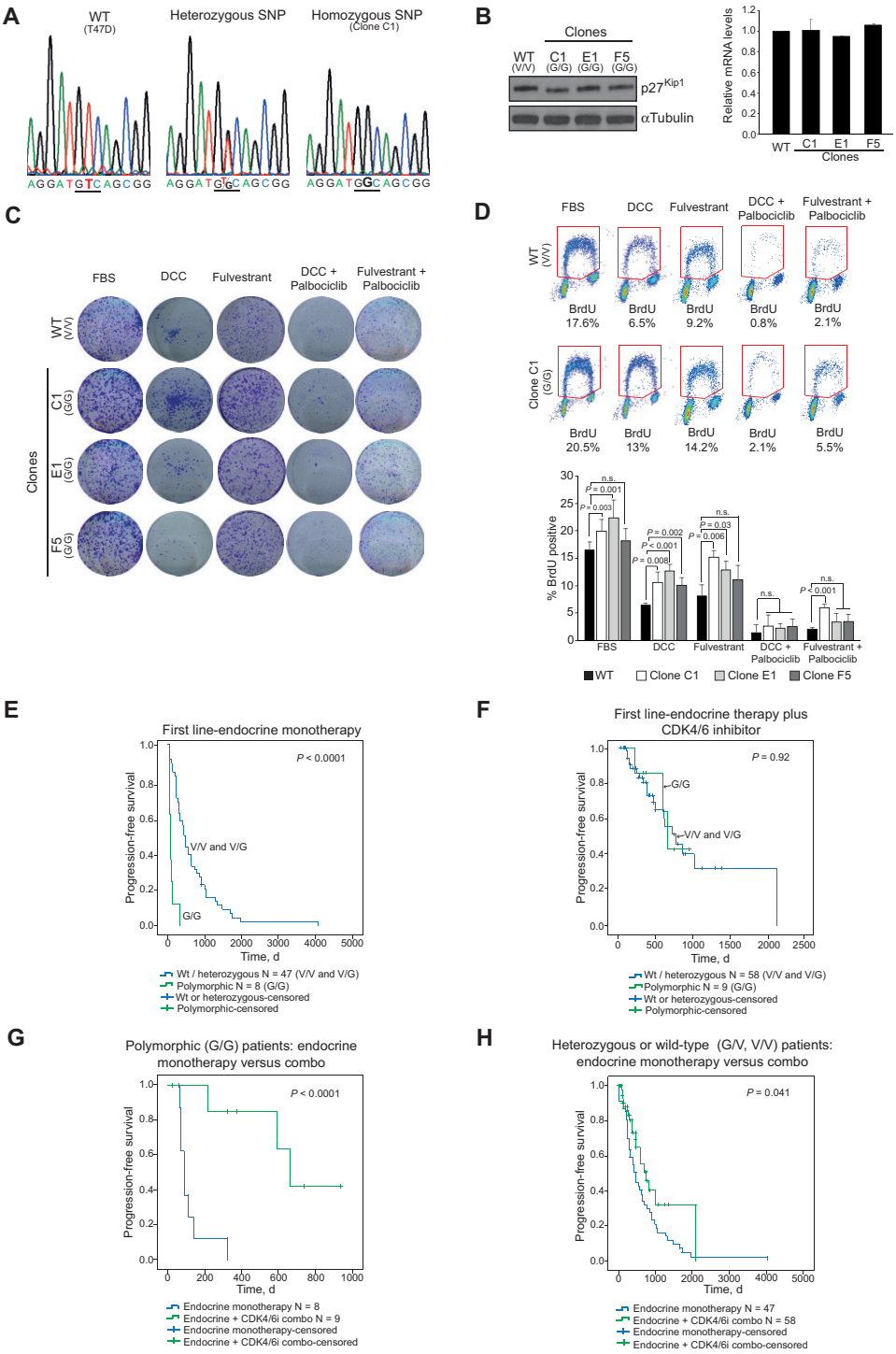

Figure 1. p27^{Kip1} V109G single-nucleotide polymorphism (SNP) impairs endocrine sensitivity, but it is rescued with CDK4/6 inhibitors in preclinical models and patients. **A**) Electropherogram showing the 3 possible sequences in position 329 of P27^{Kip1}, generated from the parental T47-D cells: T/T (**left**, wild type [WT]), T/G (**middle**, heterozygous), and G/G (**right**, homozygous for the polymorphism, clone C1). The results obtained with the heterozygous variants are not shown because they behaved like the wild type. **B**) P27^{Kip1} protein (**left**) and mRNA (**right**) levels in wild-type T47-D cells and 3 polymorphic T47-D clones. **C**) Representative colony assays and relative plating efficiency chart comparing the survival of wild-type and polymorphic T47-D clones in DCC (Dextran-Coated Charcoal) medium (tissue culture medium deprived from estrogens), in FBS (Fetal Bovine Serum) medium (full medium) plus 0.5 nM fulvestrant, or the same conditions plus 25 nM palbociclib. **D**) Representative BRDU-uptake charts of wild-type T47-D cells (**upper panels**) and polymorphic variants (Clone C1, **lower panels**), in full medium, DCC medium, fulvestrant, DCC plus palbociclib or fulvestrant plus palbociclib, showing the relative resistance to cell cycle arrest in response to hormonal deprivation but sensitivity to palbociclib combos in the polymorphic clone. The accompanying chart shows the comparison between the BRDU fraction among the different conditions in all clones. **E**) Kaplan-Meier progression-free survival (PFS) curves for patients treated with endocrine monotherapy in the first-line setting, according to their P27^{Kip1} genotype. **F**) Kaplan-Meier PFS curves for patients treated with CDK4/6 inhibitor plus endocrine therapy according to their P27^{Kip1} genotype. **G**) Kaplan-Meier PFS curves for polymorphic and wild-type or heterozygous patients **H**) comparing the PFS when receiving endocrine monotherapy or combination with CDK4/6 inhibitors. **Error bars**: standard error. The log-rank test performed for comparing the PFS curves shown in **E-H** PFS functions were computed using the Kaplan-Meier estimator. Cell cycle assays (D) were compared with 2-sided unpaired *t* tests and considered statistically significant when $P < .05$. All *P* values are 2-sided.

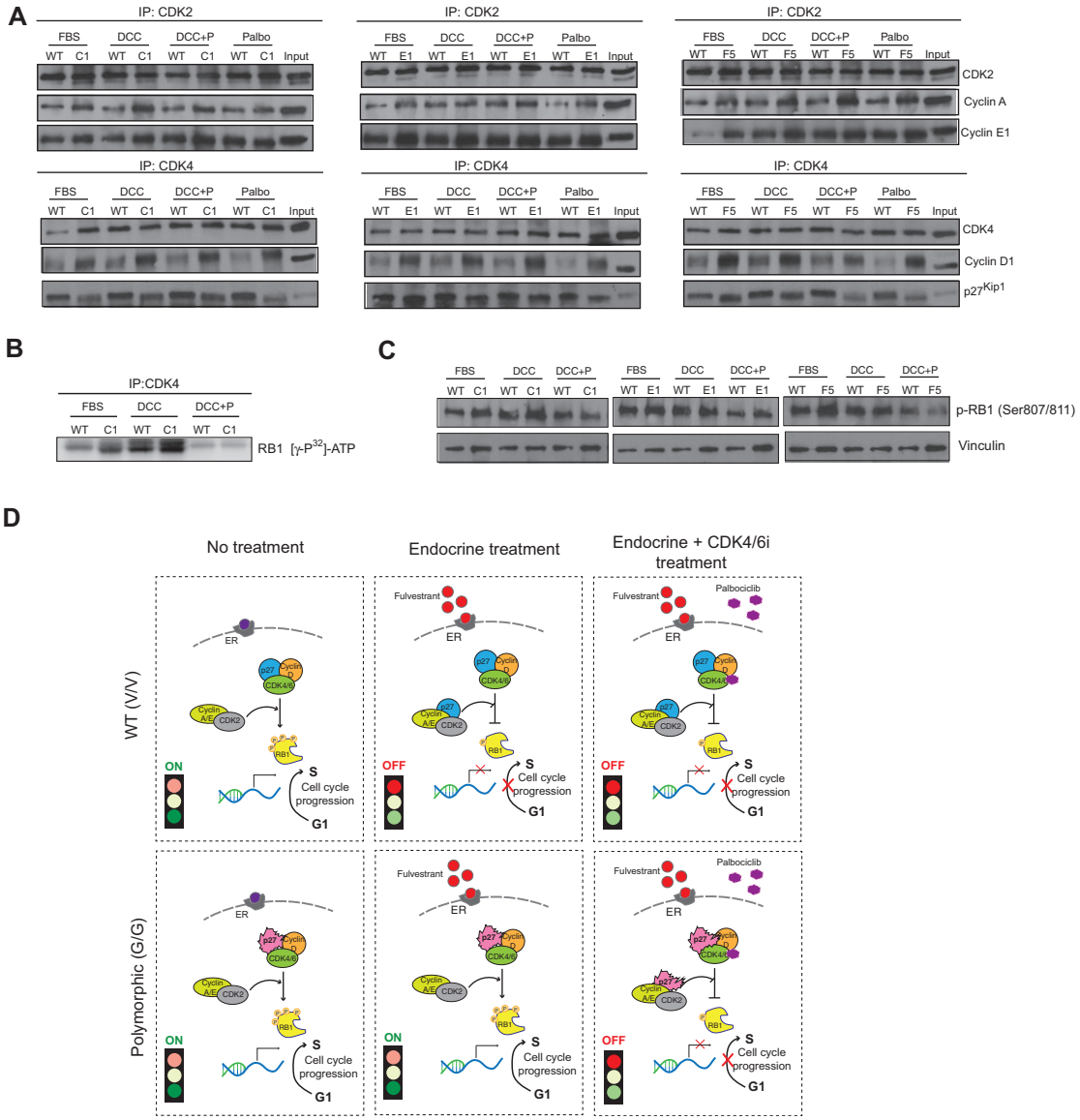

Figure 2. p27^{Kip1} V109G single-nucleotide polymorphism (SNP)-induced increments in CDK4 activity, but not in CDK/Cyclins complex formation, are reversible by palbociclib. **A**) Western blots of CDK2 (**upper panels**) and CDK4 (**lower panels**) pull-downs from V/V and G/G clones, in untreated (FBS [Fetal Bovine Serum]) or treated (DCC [Dextran-Coated Charcoal], DCC plus palbociclib or palbociclib) conditions, showing an increased amount of CDK2/Cyclin A, CDK2/Cyclin E, and CDK4/Cyclin D1 complex formation in both. **B**) CDK4 kinase in vitro kinase assays performed with lysates from polymorphic clone C1 and wild-type cells, obtained from a CDK4 pull-down, showing how palbociclib is able to fully suppress CDK4 kinase activity regardless of the p27^{Kip1} status. **C**) Western blot of phosphorylated RB1 and loading control showing that the cell cycle repressor is always phosphorylated at higher levels in the polymorphic clones compared with the parental cells, except from when palbociclib is added. **D**) Cartoon depicting the proposed implications of the p27^{Kip1} V109G SNP: wild-type cells have functional p27^{Kip1}, which is able to exert its inhibiting control over CDK/Cyclin complexes. In cancer cells, the cell cycle is already unrestrained; in non-treated conditions, both wild-type and polymorphic cells continuously cycle. In the presence of an endocrine inhibitor, the function of wild-type p27^{Kip1} is sufficient to inhibit the activity of the CDK/Cyclin complexes; thus, RB1 no longer gets phosphorylated and degraded. Conversely, in polymorphic cells, abnormal p27^{Kip1} is unable to shut down CDK kinase activity, and the cell cycle continues despite endocrine inhibition. However, if the kinase activity of CDKs is directly blocked (by a CDK4/6 inhibitor), the disfunction of p27^{Kip1} is no longer relevant and the cell cycle is suppressed as well in polymorphic clones.

that the G/G clones were resistant to estrogen deprivation (an in vitro model for aromatase inhibitors resistance) (24,25) and fulvestrant. Adding palbociclib reverted therapeutic resistance: the combos achieved similar efficacy in G/G or V/V clones (Figure 1, C and D).

We compared the outcomes of G/G against G/V or V/V HRPBC patients when treated with endocrine monotherapy or in combination with CDK4/6 inhibitors in the first-line metastatic setting (N = 122; Supplementary Table 2, available online). PFS (Progression Free Survival) favored G/V and V/V patients when treated with endocrine monotherapy (485 vs 92 days; $P < .001$; Figure 1, E). When patients received CDK4/6 inhibitor combinations, no differences were observed (761 vs 658 days, $P = .920$; Figure 1, F). Adding CDK4/6 inhibitors to hormonotherapy improved PFS to a greater extent in G/G patients (92 to 685 days, $P < .0001$; Figure 1, G) than in G/V or V/V (485 to 761 days, $P = .041$; Figure 1, H).

The cell cycle is regulated by the temporal activation of different CDK/Cyclin complexes. p27^{Kip1} can interact with several of them, negatively regulating their activity (26,27). We reasoned

that the $p27^{Kip1}$ V109G SNP could cause the functional defect by 2 main mechanisms: alteration in the formation of CDK/Cyclin complexes or modulation of their kinase activity. Although we observed increased formation of CDK2/Cyclin A, CDK2/Cyclin E, and CDK4/Cyclin D complexes in G/G clones—and this increment was sustained despite hormonal deprivation—the addition of palbociclib was unable to decrease the complexes back to normal levels (Figure 2, A). We then analyzed the CDK4 kinase activity of the $p27^{Kip1}$-CDK4-Cyclin D complexes pulled down from V/V or G/G cells, using recombinant RB1 (Retinoblatoma protein 1) as substrate. We observed that both in the presence of full medium and estrogen-deprived medium, CDK4 kinase activity was higher in the polymorphic clones (Figure 2, B). The addition of palbociclib, however, was able to fully block CDK4 kinase activity both in wild-type and polymorphic cells, bypassing the insufficient inhibitory activity derived from polymorphic $p27^{Kip1}$. Phosphorylated levels of RB1 in V/V and G/G cells were congruent with the kinase assays (Figure 2, C).

The management of early HRPBC requires predictive factors for guiding the indication of CDK4/6 inhibitors. Studies performed before the advent of CDK4/6 inhibitors suggest that low $p27^{Kip1}$ levels are associated with worse prognosis in the absence of endocrine therapy and with relative hormone refractoriness (28-30). Our study design does not allow addressing prognostic implications, but we present how the $p27^{Kip1}$ V109G SNP can split the hormone-positive breast cancer population into 2 main subgroups: one (G/V or V/V patients, approximately 85%) in which endocrine treatment is sufficient to block cell replication and achieve disease control; and a second one (G/G patients, approximately 15%) in which endocrine therapy is insufficient but is rescued by CDK4/6 inhibitors, suggesting a predictive role. Two limitations of our study are its retrospective nature and the relatively low number of patients. The imbalance in metastatic relapse within 12 months of completing adjuvant hormonotherapy between G/G and G/V-V/V patients (Supplementary Table S2, available online) could contribute to the observed differences in the first-line setting (Figure 1, E) while reflecting an inherent resistance of G/G patients to endocrine monotherapy.

The impaired $p27^{Kip1}$ inhibitory activity is evidenced by increased CDK4 kinase activity and phosphorylated RB1 in baseline or hormonal-deprived conditions; however, CDK4/6 inhibitors achieve cell cycle control, akin in the wild types (Figure 2, D). Regardless of the potential off-target effects of CRISPR/Cas9, the homogeneity observed across the 3 tested clones (Figures 1, C and D and 2) suggests that the effects are due to the V109G change.

Taken together, our data suggest that G/V and V/V patients are adequately treated with endocrine monotherapy; G/G $p27^{Kip1}$, however, impairs the ability of endocrine therapy to control the cell cycle, requiring the addition of CDK4/6 inhibitors. This study may be relevant for the adjuvant setting. Validation is required, and the role of the V109G SNP in the PALLAS (8) and monarchE (10) study cohorts currently is being addressed, which should clarify whether this SNP deserves incorporation in the clinical decision algorithm. Whether genetic ancestry modulates the G/G effect should also be clarified, because our study was conducted exclusively in Hispanic White patients.

Data availability

All experimental data and patients' clinical characteristics are incorporated into the article and its online supplementary material.

Author contributions

Silvana Mouron, PhD (conceptualization; formal analysis; investigation; methodology; writing—original draft; writing—review and editing); Maria J. Bueno, PhD (conceptualization; formal analysis; investigation; methodology; writing—original draft; writing—review and editing); Manuel Muñoz, AS (investigation; methodology; writing—review and editing); Raul Torres, PhD (investigation; methodology; writing—review and editing); Sandra Rodriguez, PhD (investigation; methodology; writing—review and editing); Juan V. Apala, MD (resources; writing—review and editing); Jorge Silva, MD (resources; writing—review and editing); Rodrigo Sanchez-Bayona, MD, PhD (resources; writing—review and editing); Luis Manso, MD, PhD (resources; writing—review and editing); Juan Guerra, MD, PhD (resources; writing—review and editing); Laura Rodriguez-Lajusticia, MD (resources; writing—review and editing); Diego Malon, MD (resources; writing—review and editing); Marcos Malumbres, PhD (conceptualization; writing—original draft; writing—review and editing); Miguel Quintela-Fandino, MD, PhD (conceptualization; formal analysis; funding acquisition; resources; writing—original draft; writing—review and editing).

Funding

MM is supported by the Spanish Ministry of Science and Innovation (RTI2018-095582-B-100; PLEC2021-007892 and RED2018-102723-T), AES (DTS21/00132) and Comunidad de Madrid (B2017/BMD-3884 and Y2020/BIO-6519). MQF is a recipient of the following grants: AES—PI 19/00454 funded by the ISCIII and co-funded by the European Regional Development Fund (ERDF), and B2017/BMD3733 (Immunothercan-CM) – Call for Coordinated Research Groups from the Madrid Region—Madrid Regional Government—ERDF funds. This study was also funded by a donation from CRIS Contra El Cancer Foundation.

Conflicts of interest

MQF holds a patent for the predictive role of the SNP $p27^{Kip1}$ V109G as a potential selection factor for treatment with CDK4/6 inhibitors in hormone-positive breast cancer (PCT/EP2022/051700).

MQF received research funds from Bayer and Pfizer to conduct investigator-initiated studies. MQF also received personal payments from Pfizer (advisory board).

Acknowledgements

The funders only supported the studies reported here under competitive call conditions or philanthropic donations but did not intervene in any aspect of the design, experimental work or data interpretation, or manuscript writing.

This study has been presented at San Antonio Breast Cancer Symposium 2022 (Poster presentation).

References

1. Finn RS, Martin M, Rugo HS, et al. Palbociclib and letrozole in advanced breast cancer. *N Engl J Med*. 2016;375(20):1925-1936.
2. Turner NC, Ro J, Andre F, et al.; PALOMA3 Study Group. Palbociclib in hormone-receptor-positive advanced breast cancer. *N Engl J Med*. 2015;373(3):209-219.

3. Hortobagyi GN, Stemmer SM, Burris HA, et al. Ribociclib as first-line therapy for HR-positive, advanced breast cancer. *N Engl J Med*. 2016;375(18):1738-1748.
4. Slamon DJ, Neven P, Chia S, et al. Overall survival with ribociclib plus fulvestrant in advanced breast cancer. *N Engl J Med*. 2020; 382(6):514-524.
5. Sledge GW Jr, 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. *J Clin Oncol*. 2017;35(25):2875-2884.
6. Goetz MP, Toi M, Campone M, et al. MONARCH 3: abemaciclib as initial therapy for advanced breast cancer. *J Clin Oncol*. 2017; 35(32):3638-3646.
7. Sledge GW Jr, Toi M, Neven P, et al. The effect of abemaciclib plus fulvestrant on overall survival in hormone receptor-positive, ERBB2-negative breast cancer that progressed on endocrine therapy-MONARCH 2: a randomized clinical trial. *JAMA Oncol*. 2020;6(1):116-124.
8. Gnant M, Dueck AC, Frantal S, et al.; PALLAS Groups and Investigators. Adjuvant palbociclib for early breast cancer: the PALLAS trial results (ABCSG-42/AFT-05/BIG-14-03). *J Clin Oncol*. 2022;40(3):282-293.
9. Loibl S, Marme F, Martin M, et al. Palbociclib for residual high-risk invasive HR-positive and HER2-negative early breast cancer-the penelope-B trial. *J Clin Oncol*. 2021;39(14):1518-1530.
10. Harbeck N, Rastogi P, Martin M, et al.; monarchE Committee Members. Adjuvant abemaciclib combined with endocrine therapy for high-risk early breast cancer: updated efficacy and Ki-67 analysis from the monarchE study. *Ann Oncol*. 2021;32(12): 1571-1581.
11. Johnston SRD, Harbeck N, Hegg R, et al.; monarchE Committee Members and Investigators. Abemaciclib combined with endocrine therapy for the adjuvant treatment of HR+, HER2-, node-positive, high-risk, early breast cancer (monarchE). *J Clin Oncol*. 2020;38(34):3987-3998.
12. Macri E, Loda M. Role of p27 in prostate carcinogenesis. *Cancer Metastasis Rev*. 1998;17(4):337-344.
13. Fernandez PL, Jares P, Rey MJ, et al. Cell cycle regulators and their abnormalities in breast cancer. *Mol Pathol*. 1998;51(6): 305-309.
14. Chang BL, Zheng SL, Isaacs SD, et al. A polymorphism in the CDKN1B gene is associated with increased risk of hereditary prostate cancer. *Cancer Res*. 2004;64(6):1997-1999.
15. Li G, Sturgis EM, Wang LE, et al. Association between the V109G polymorphism of the p27 gene and the risk and progression of oral squamous cell carcinoma. *Clin Cancer Res*. 2004;10(12 Pt 1): 3996-4002.
16. Schondorf T, Eisele L, Gohring UJ, et al. The V109G polymorphism of the p27 gene CDKN1B indicates a worse outcome in node-negative breast cancer patients. *Tumour Biol*. 2004;25(5-6): 306-312.
17. Naidu R, Har YC, Taib NA. P27 V109G Polymorphism is associated with lymph node metastases but not with increased risk of breast cancer. *J Exp Clin Cancer Res*. 2007;26(1):133-140.
18. Figueiredo JC, Knight JA, Cho S, et al. Polymorphisms cMyc-N11S and p27-V109G and breast cancer risk and prognosis. *BMC Cancer*. 2007;7:99.
19. Pasquali D, Circelli L, Faggiano A, et al. CDKN1B V109G polymorphism a new prognostic factor in sporadic medullary thyroid carcinoma. *Eur J Endocrinol*. 2011;164(3):397-404.
20. Wei F, Xu J, Tang L, et al. p27(Kip1) V109G polymorphism and cancer risk: a systematic review and meta-analysis. *Cancer Biother Radiopharm*. 2012;27(10):665-671.
21. Sekiya T, Bronstein MD, Benfini K, et al. p27 variant and corticotropinoma susceptibility: a genetic and in vitro study. *Endocr Relat Cancer*. 2014;21(3):395-404.
22. Longuini VC, Lourenco DM Jr, Sekiya T, et al. Association between the p27 rs2066827 variant and tumor multiplicity in patients harboring MEN1 germline mutations. *Eur J Endocrinol*. 2014;171(3):335-342.
23. Lima G, Santos E, Angelo H, et al. Association between p21 Ser31Arg polymorphism and the development of cervical lesion in women infected with high risk HPV. *Tumour Biol*. 2016;37(8): 10935-10941.
24. Martin LA, Farmer I, Johnston SR, et al. Enhanced estrogen receptor (ER) alpha, ERBB2, and MAPK signal transduction pathways operate during the adaptation of MCF-7 cells to long term estrogen deprivation. *J Biol Chem*. 2003;278(33):30458-30468.
25. Mouron S, Manso L, Caleiras E, et al. FGFR1 amplification or overexpression and hormonal resistance in luminal breast cancer: rationale for a triple blockade of ER, CDK4/6, and FGFR1. *Breast Cancer Res*. 2021;23(1):21.
26. Malorni L, Piazza S, Ciani Y, et al. A gene expression signature of retinoblastoma loss-of-function is a predictive biomarker of resistance to palbociclib in breast cancer cell lines and is prognostic in patients with ER positive early breast cancer. *Oncotarget*. 2016;7(42):68012-68022.
27. Jansen VM, Bhola NE, Bauer JA, et al. Kinome-wide RNA interference screen reveals a role for PDK1 in acquired resistance to CDK4/6 inhibition in ER-positive breast cancer. *Cancer Res*. 2017; 77(9):2488-2499.
28. Guan X, Wang Y, Xie R, et al. p27(Kip1) as a prognostic factor in breast cancer: a systematic review and meta-analysis. *J Cell Mol Med*. 2010;14(4):944-953.
29. Filipits M, Rudas M, Heinzl H, et al.; Austrian Breast and Colorectal Cancer Study Group. Low p27 expression predicts early relapse and death in postmenopausal hormone receptor-positive breast cancer patients receiving adjuvant tamoxifen therapy. *Clin Cancer Res*. 2009;15(18):5888-5894.
30. Stendahl M, Nilsson S, Wigerup C, et al. p27Kip1 is a predictive factor for tamoxifen treatment response but not a prognostic marker in premenopausal breast cancer patients. *Int J Cancer*. 2010;127(12):2851-2858.