Observational Retrospective-Prospective Study Protocol

Study title:

Predictive impact of Peripheral blood lymphocytes on clinicAL outcoMes during first-line CDK4/6 inhibitors plus endocrine therapy in patients with Advanced hormone REceptor-poSitive HER2-negative breast cancer: the retrospective-prospective multicenter Italian PALMARES-2 study

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Background and Rationale of the Study

CDK4/6 inhibitors as the standard of care for the treatment of HR+/HER2- aBC

Breast cancer (BC) is the most common malignancy in women worldwide, with a critical impact on public health due to its incidence, prevalence and global disease burden.[1–6] Historically, BC is classified into three major histopathological categories based on the expression of Hormone Receptors (HR) and Human Epidermal growth factor Receptor 2 (HER2), with the HR+/HER2- subgroup accounting for the majority of cases (~65-70%), and it is responsible for the majority of BC-related deaths. Despite huge recent improvements in the treatment of this subtype of BC, it remains an almost invariably deadly disease.[7,8]

The cyclin-dependent kinase (CDK) 4/6 inhibitors palbociclib, ribociclib and abemaciclib are the mainstay of treatment in combination with endocrine therapies (ET) for patients with advanced (unresectable locally advanced or metastatic) hormone receptor-positive human epidermal growth factor receptor 2 (HER2)-negative breast cancer (HR+/HER2- aBC).[9,10] CDK 4/6 inhibitors (CDK4/6i) induce cell cycle arrest in tumor cells by preventing the phosphorylation of retinoblastoma protein (RB). In its unphosphorylated form,

RB cannot dissociate from transcriptional factor E2F, which is consequently inhibited and unable to promote cell cycle progression.[11]

In the last decade, the introduction of CDK4/6i, namely palbociclib, ribociclib and abemaciclib, in combination with endocrine therapies (ETs) remarkably improved patient progression-free survival (PFS) and, in some cases, also overall survival (OS).[12–15] In particular, multicenter, international, phase III clinical trials, namely PALOMA-2, PALOMA-3, MONALEESA-2, MONALEESA-3, MONALEESA-7, MONARCH 2 and MONARCH 3, demonstrated that adding palbociclib, ribociclib or abemaciclib to standard ET is associated with a significant benefit, in terms of PFS, when compared to ET alone, in both endocrine sensitive and resistant patients.[16–22] Based on these results, CDK4/6i plus ET are currently recommended by national and national and international guidelines as the treatment of first choice for patients with HR+/HER2- aBC who have not received previous lines of treatment for metastatic disease.[9,10,23,24]

Determinants of CDK4/6i benefit as first-line treatment for HR+/HER2- aBC

Subgroup analyses from pivotal trials failed to demonstrate a differential PFS benefit from the addition of CDK4/6i based on a specific clinical biomarker, since all the subgroups achieved a better PFS compared to standard arm. In particular, to investigate the efficacy of CDK4/6i in combination with ET in different clinical-pathologic subgroups of MBC, Gao et al performed a pooled analysis of seven pivotal registration trials submitted to the FDA in support of marketing applications for palbociclib, ribociclib, and abemaciclib. Again, they found that the addition of CDK4/6i to ET benefitted all the analysed subgroups. These results remarked CDK4/6i in combination with ET as first-line treatment for HR+/HER2- aBC in all subgroups of patients.[25]

Therefore, the only predictive biomarker of response to CDK4/6i currently used is the presence of estrogen receptor on tumor cell membranes, as determined by immunohistochemistry (ER ≥1%).[9] However, other biomarkers derived from tumor tissue emerged as potential predictors of response in this context.[26–28] One of the most investigated biomarkers is the retinoblastoma (Rb) protein, which is a direct target of CDK4/6 activity.[29] Functional RB is essential for CDK4/6i to exert their effect, highlighting the loss of RB, as assessed by genomic loss or absence of the protein evaluated on immunohistochemistry, as potential biomarker of CDK4/6i resistance. However, this molecular alteration accounts for only a small percentage of cases (3-9%). Additionally, other cell-cycle alteration can affect the efficacy of this class of drugs. In particular, cyclin D1/E1 amplification, c-myc overexpression, as well as dysregulation of members of the CDKN2/INK family, which are endogenous inhibitors of CDK4/6 (p16, p15, p18, p19 protein, encoded by *CDKN2A/ CDKN2A/CDKN2C/CDKN2D* genes, respectively), are being explored as potential predictive biomarkers, with conflicting results.[26,29] Similarly, other genomic alterations in other genes not primarily

implicated in cell-cycle regulation could influence response to CDK4/6i, e.g. indirectly modulating CDK6 expression such as in the case of *FAT1* loss of function mutation.[30] Furthermore, other cell-cycle independent, relatively common, genomic alterations, such as those regarding the PI3K pathway (*AKT1/2/3* mutations, *PIK3CA* mutation and *PTEN* loss) and the amplification of fibroblast growth factor receptor (*FGFR1-3*) genes, have demonstrated conflicting results in influencing the efficacy of CDK4/6 inhibitors. In particular, the co-inhibition of the cell-cycle through CDK4/6 inhibition and the blockade of PI3K pathway have already been demonstrated to synergize in preclinical models and in phase II studies. Conversely, *FGFR1/2/3*, as well as other receptor t yrosine kinase (RTK) alterations like *EGFR* and *MET*, which are altered in less than 10% of cases, emerged as potential biomarkers of resistance to CDK4/6i, indicating that patients with these alterations might require alternative therapeutic strategies or combinations.[26,31]

Furthermore, breast cancer intrinsic subtypes, as evaluated by PAM50 transcriptomic profiles, were observed to differently benefit from CDK4/6i in combination with ET. In particular, basal-like tumors are associated with poor prognosis and refractoriness to the addition of CDK4/6i to ET alone, as observed in a retrospective pooled analysis across the MONALEESA phase III studies (mPFS 3.7 with ribociclib vs 3.6 with placebo).[32,33]

Prognostic biomarkers in HR+/HER2- aBC patients treated with first-line CDK4/6i plus ET combinations

Despite the tremendous improvement in the prognosis of HR+/HER2- aBC patients, a minority (~10-15%) of patients treated with first-line ET+CDK4/6i still undergo early disease progression.[34,35] This subgroup of patients could therefore benefit from treatment intensification, such as through the addition of a target therapy (e.g., PI3Ki, AKTi, mTORi, PARPi for selected populations), hormonal backbone enhancement (e.g., oral SERD), or even from the use of a completely different treatment strategy such as chemotherapy or novel antibody drug conjugates (ADCs). Conversely, another subgroup of patients demonstrated to derive long-term benefit from ET+CDK4/6i in pivotal trials, with 23.3% of patients enrolled in the MONARCH 3 trial still on treatment after a median follow up of 72 months.[36] These patients could potentially benefit from tailoring treatment in terms of de-escalation, sparing unnecessary toxicities without negatively impacting on survival outcomes.

However, there are no studies conducted to date with the specific aim of identifying the subset of patients at high risk of early progression or, conversely, long-term benefit. Several clinical and biological factors demonstrated in clinical trials to have relevant impact on prognosis, and the addition of specific CDK4/6i differently improved prognosis across pivotal trials. Among these, endocrine resistance, presence of liver metastasis, tumor biology intended as luminal A- or B-like disease, menopausal status, de novo metastatic presentation, patient age and comorbidity.[37–40] Mason et al conducted an individual-level analysis using

patient data from the 7 randomized controlled trials (RCTs) that lead CDK4/6i FDA approval, including a total of 2633 patients treated with CDK4/6i+ET in first- or second-line for metastatic disease. The prediction models for PFS and OS in the ET+CDK4/6i group had overall accuracies of 62% and 63%, respectively. Variables that were most likely to positively influence the model included disease burden, line of therapy, menopausal status, performance status and tumor grading.[41]

Finding new biomarkers to be added to current models to improve prediction ability is crucial to allow their applicability in the clinical practice. These biomarkers should ideally be informative, not-redundant, reproducible, easy-to-access, low-cost and easily interpretable by clinicians. For these reasons, an optimal source of biomarkers could be represented by data extrapolated from diagnostic exams performed during patient clinical care, but often underreported in clinical trials.

Based on this hypothesis, we already investigated the prognostic significance of peripheral blood immune cell counts in HR+/HER2- aBC patients treated with ET+CDK4/6i.[42] In fact, although the main mechanism of CDK4/6i-mediated anticancer activity consists in cell autonomous anticancer effects, preclinical evidence indicates the CDK4/6i ability to stimulate antitumor immunity. To preliminary investigate if peripheral blood immune cell counts or composite immune parameters, as evaluated at baseline or during CDK4/6i therapy, are associated with efficacy of any-line ET+CDK4/6i, we performed an analysis in 638 HR+/HER2- aBC patients treated in six Italian centers. In this study (PALMARES study), we investigated if baseline or on-treatment peripheral blood immune cells, such as lymphocytes, neutrophils, platelets or monocytes, or the composite scores neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), monocyte-to-lymphocyte ratio (MLR) or the Pan-Immune Inflammatory Value (PIV), are associated with patient PFS. Peripheral blood immune cell values were collected at baseline (T0), 2 weeks after treatment initiation (T1) and before fourth cycle initiation, approximately 12 weeks after treatment initiation (T2). We used Random Forest models to select clinically relevant covariates (line of therapy for advanced disease, ECOG PS, presence of liver metastases, number of metastatic sites, patient age, disease-free interval (DFI), percentage of Ki-67-positive cells in primary tumor specimens and presence of ER expression in primary tumor specimens) to adjust the role of peripheral blood immune cells in multivariable cox models. Median patient age in this cohort of patients was 61 years (IQR: 52-70 years). The majority of patients were postmenopausal (n=530; 83.1%), had an ECOG PS of 0 (n=464, 72.8%) and were treated with palbociclib (n=529; 82.9%). Approximately half of enrolled patients received CDK4/6i in the first-line setting (n=335; 52.5%) and had visceral disease (n=334; 52.4%). Among all the considered peripheral blood immune variables (neutrophils, platelets, lymphocytes and monocytes, the NLR, the PLR, the MLR and also the PIV), only baseline lymphocyte values and their precocious modulation (i.e. after 2 weeks of treatment), were independently associated with patient PFS.[42]

Interestingly, we found that patients with higher baseline lymphocyte counts and undergoing a lower on-treatment reduction of lymphocytes had significantly longer PFS when compared to patients with lower baseline lymphocytes and undergoing a higher decrease of lymphocyte counts [median PFS 21.4 vs. 11 months, respectively; adjusted hazard ratio (aHR): 0.82; 95% CI 0.73-0.93; p=0.0037]. For the first time, these results underlie the prognostic significance of blood lymphocytes in HR+/HER2- aBC patients treated with CDK4/6 inhibitors. [42]

Since the majority of HR+/HER2- aBC patients nowadays receive CDK4/6i in the first-line treatment setting, we performed a sub-analysis, in which we evaluated the association of baseline lymphocyte count (L_t0) and its precocious modulation (L_r1) with the PFS of patients treated with CDK4/6i in the first-line setting (n=335; 52.5%). Multivariable analysis revealed an independent and statistically significant association between higher L_r1 and reduced risk of disease progression; on the other hand, L_t0 was not associated with patient PFS. In this model, a higher DFI was associated with better PFS, while a higher number of metastatic sites, as well as higher ECOG PS, were associated with significantly worse PFS. We also adjusted the prognostic role of L_r1 for NLR_t0 in this patient subset. Of note, L_r1 was independently associated with patient PFS (HR: 0.83; 95% CI 0.71-0.99; p=0.0325), while the NLR_t0 was not (HR: 1.08; 95% CI 0.86-1.35 (p=0.5085). Together, these data confirm an independent prognostic role of the precocious modulation of peripheral blood lymphocytes also in the first-line setting.[42]

The differential expression of HER2 protein in HR+/HER2- tumors recently emerged as a predictive biomarker of response to novel anti-HER2 anticancer drugs, in particular the antibody-drug conjugates trastuzumab deruxtecan (T-DXd). Data from the phase III RCT DESTINY-Breast04 trial showed that, in patients with HR+/- HER2-low, as defined by an IHC score for HER2 of 1 or 2 in the absence of HER2 gene amplification by ISH analysis, aBC that is refractory to ETs (if HR+), and who received 1-2 prior lines of chemotherapy for metastatic disease, T-DXd is superior to several standard cytotoxic treatments in terms of PFS and OS.[43] In addition, data from the phase III RCT DESTINY-Breast06 recently presented at the international congress ASCO 2024 demonstrated T-DXd to be superior in terms of PFS compared to treatment of physician choice (TPC) for patients with HR+/HER2- aBC and HER2-low status progressing on first-line ET+CDK4/6i therapy (mPFS 13.2 vs 8.1 months).[44]

These new therapeutic opportunities defined two distinct subgroups among HR+/HER2- aBC tumors, namely HER2-low and HER2-0. However, it remains unclear if HER2-low status is also associated with different clinical outcomes (PFS, OS) as compared to HER2-0 status in patients receiving standard first-line ET plus CDK4/6i therapy. Several studies tried to investigate the prognostic role of HER2 status (low vs. 0) in patients with aBC. While studies suggested worse survival in HER2-low patients compared to HER2-0, the statistical significance was reached only in some of these studies.[45–48] In addition, other studies did not show any prognostic difference between HER2-low and HER2-0 tumors. To address this clinical question,

we conducted a subanalysis on the PALMARES study cohort treated with first-line CDK4/6i plus ET. HER2 status was available for 428 patients in the study cohort. We found that patients with HR+/HER2-low aBC had significantly worse PFS (mPFS 23.6 months vs. 32.3 months; p=0.014) and OS (mOS: 48.7 vs 58.3 months; p=0.029) when compared to patients with HER2-0 aBC. Multivariable analysis confirmed an independent association between HER2-low status and worse PFS (aHR 1.42; 95% CI:1.07-1.89; p=0.016) and OS (aHR: 1.64; 95% CI: 1.08-2.48; p=0.02).[48] However, another large study conducted by Mouabbi J. et al investigated the prognostic role of HER2 status in 1649 HR+/HER2- aBC patients treated with ET in combination with different targeted therapies (CDK4/6i, everolimus or alpelisib). In the sub-cohort of 919 patients treated with CDK4/6i plus ET, there were no statistically significant differences between patients with HER2-low and HER2-0 aBC in terms of PFS (13.0 vs 11.6 months, p=0.273) and OS (32.4 vs 31.2 months, p=0.222).[49] The reported discrepancies among published studies, in addition to the increased value of the HER2-low status in the treatment of HR+/HER2- aBC, laid the foundations for further investigations on this potentially useful and easy-to-access biomarker.

To summarize, our previous preliminary results, together with available literature, demonstrated that some biomarkers already available in clinical practice could be potentially used to distinguish different prognostic classes of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i. However, larger studies are necessary to confirm available results and finally implement in clinical practice novel treatment biomarkers.

Differences between the three CDK4/6i drugs in combination with ET and their impact on efficacy

The three CDK4/6i molecules are characterized by minor differences in terms of chemical composition, but with significant consequences in clinical terms. In particular, ribociclib and abemaciclib, unlike palbociclib, have a higher affinity for CDK4 than CDK6. Palbociclib and ribociclib, on the other hand, have much bulkier substituents at the ATP-binding pocket than abemaciclib, resulting in the latter's lower selectivity for CDK4 and CDK6 and higher pleiotropic affinity towards other members of the cyclin-dependent kinase family. This justifies the higher off-target activity, as well as toxicity, of abemaciclib compared to the other two drugs.[50] From a pharmacokinetic point of view, palbociclib and ribociclib have a higher affinity for glycoprotein P and the efflux transporter Breast Cancer Resistance Protein (BCRP), resulting in lower central nervous system (CNS) permeability than abemaciclib.[51] Due to different pharmacokinetic properties, the three CDK4/6i are approved for administration in different fashion, with abemaciclib prescribed in a continuous schedule, that provides sustained inhibition of CDK4 and CDK6, while palbociclib and ribociclib given in intermittent dosing with a 3-weeks-on, 1-week-off schedule.[50]

As a consequence of different pharmacokinetic and pharmacodynamics properties, palbociclib, ribociclib and abemaciclib are characterized by a different toxicity profile. In particular, while palbociclib and ribociclib

are characterized by a more frequent bone marrow toxicity, ribociclib treatment is associated with a higher rate of liver toxicity and prolongation of the corrected QT (QTc) tract on electrocardiographic tracing, while abemaciclib is associated with an increased rate of gastrointestinal disorders, particularly in terms of diarrhea.[52,53]

Regarding differential clinical activity among the three CDK4/6i drugs, in pivotal studies not all molecules demonstrated a statistically significant benefit in overall survival (OS), which endpoint was only achieved for studies with ribociclib (MONALEESA-2, MONALEESA-3, MONALEESA-7 trials), while abemaciclib demonstrated this benefit only in the context of disease endocrine-resistant disease (MONARCH 2).[13,14,19] However, the efficacy of palbociclib, ribociclib and abemaciclib has never been directly compared in large clinical studies. In fact, the available published literature consists of small case series lacking sufficient power for efficacy comparisons, and available studies did not properly adjust for confounders to homogenize treatment cohorts.[54–58] Only three large real-world comparisons of the three CDK4/6i were available in literature, two of these was recently presented at international congresses; however, none of these two studies were published in full to date. Pantano F. et al conducted a comparison among the three CDK4/6 on a cohort of 1184 patients using propensity score weighting for covariate adjustment, showing abemaciclib and ribociclib to be associated with better PFS when compared to palbociclib in the endocrine-sensitive setting, while abemaciclib was associated with better PFS when compared to both ribociclib and palbociclib in patients with endocrine-resistant disease. However, this analysis was limited by the relatively low number of enrolled patients, especially in the group of patients treated with abemaciclib (n=158).[59] Another study presented at ASCO 2024 annual meeting included 1511 HR+/HER2- aBC patients treated with ET+CDK4/6i as first- or second-line of treatment, showing that fulvestrant plus ribociclib was associated with better PFS and OS when compared to both fulvestrant plus palbociclib and fulvestrant plus abemaciclib; no adjustment method was applied when comparing PFS among the three drug cohorts, despite significant imbalance in the three groups.[60] Finally, Gehrchen et al conducted a real-world comparison between the three molecules on data extracted from a national Danish dataset. In the context of first-line subgroup of patients (N = 1554), they found better outcomes in terms of rwPFS of ribociclib and abemaciclib compared to palbociclib, also when adjusting with some clinical co-variates.[61] However, the lack of availability of an appropriate amount of patients' characteristics, such as biological ones, did not allow a proper adjustment between groups and potentially not correcting the confounding bias related to drug attribution by oncologists. This kind of problem underlines the need of deeply annotated, comprehensive datasets to conduct proper and trustworthy statistical analyses in the real-world setting.

Another relevant issue is represented by the potential differential activity of the three molecules across clinical scenarios. In fact, results of published phase III RCTs reported a different magnitude of benefit among clinical settings with three drugs. As an example, in patients with endocrine-sensitive disease, all the three CDK4/6i in combination with ET improve PFS when compared to ET alone. However, only ribociclib

in MONALEESA-2/3/7 trials also improved patient OS in the endocrine sensitive setting, while abemaciclib resulted in not statistically significant OS prolongation in the MONARCH 3 trial, neither OS improvement was observed with palbociclib in the PALOMA-2 trial. In premenopausal women, data from the MONALEESA-7 trial indicate that ribociclib specifically improves PFS and OS in combination with ET in this setting, whereas abemaciclib prolonged PFS and OS in premenopausal women enrolled in the MONARCH 2 trial.[14,18,62] On the other hand, palbociclib resulted in PFS but not OS gain in premenopausal patients form the PALOMA-3 trial. Regarding tumor biology, exploratory analyses from RCTs showed that palbociclib and ribociclib improve PFS in patients with both luminal-A and luminal-B disease, while ribociclib also provided OS benefit in patients with luminal-B disease.[32,33,63] In sub-analyses of the MONARCH 2/3 trials, abemaciclib improved PFS in patients with tumors lacking PgR expression, a feature that contributes to the definition of luminal B-like disease.[20,64] These data suggest a differential activity of the three CDK4/6i drugs in settings that are often encountered during daily clinical practice.

Efficacy of systemic therapies administered on progression to first-line ET+CDK4/6i

While CDK4/6i in combination with endocrine therapy is established as the standard of care treatment for first-line treatment for virtually all HR+/HER2- aBC patients, both in endocrine-sensitive and endocrine-resistant setting, the treatment algorithm for second and subsequent lines for HR+/HER2- aBC is an area of uncertainty.[9,65] To add a further layer of complexity, the aBC scenario after CDK4/6i is extremely heterogeneous from a biological, genomic and clinical point of view, raising the necessity to find the best treatment pattern at the individual level, based on the combination of patient-specific characteristics.

This urgent clinical unmet need is significantly relevant in the actual oncologic era, when a plethora of new treatment options demonstrated activity in HR+/HER2- aBC progressed after endocrine-based treatment. In detail, numerous trials demonstrated the efficacy of several agents to improve survival outcomes in second or subsequent lines after ET-alone first-line treatment. In particular, endocrine (e.g. fulvestrant), biological (e.g. everolimus in combination with exemestane, alpelisib in combination with fulvestrant) and chemotherapy agents (eribuline) demonstrated to prolong PFS upon ET-alone treatment progression in phase III RCTs.[66–68] However, with the advent of CDK4/6i as upfront treatment for HR+/HER2- aBC patients, further trials are needed to confirm the efficacy of these agents as second and/or subsequent lines of therapy. In fact, reduced efficacy of these agents has been observed whenCDK4/6i was added to the first-line treatment (e.g. fulvestrant monotherapy, everolimus in combination with exemestane).[69,70]

Indeed, some therapeutic agents have already demonstrated their efficacy in CDK4/6i pre-treated population. In particular, oral SERDs showed ability to significantly prolong mPFS, as elacestrant already received US and European regulatory approval based on the EMERALD trial results showing PFS survival advantage

over standard second-line endocrine therapy.[71] The inhibition of PTEN-AKT1- PI3K pathway also provided benefit when added to endocrine treatment alone as second-line therapy, leading to marketing authorization of the AKT-inhibitor capivasertib based on CAPITELLO-291 phase III trial results.[72] While the phase II BYLieve trial substantially confirmed the activity of the combination of the PI3K-inhibitor alpelisib and fulvestrant in the post-CDK4/6i setting, results from the phase III randomized EPIK-B5 trial are awaited for alpelisib approval in this setting.[73]

Antibody-Drug Conjugates (ADCs) recently emerged as a novel treatment strategy for solid tumors. Trastuzumab deruxtecan (T-DXd), an ADC composed by trastuzumab linked to the topoisomerase I inhibitor deruxtecan, is associated with a strong bystander effect, thus leading to clinically-meaningful antitumor activity also in malignancies bearing heterogeneous and/or weak HER2 expression such as in case of HER2-low aBC. Indeed, DESTINY-Breast04 was a phase III RCT comparing T-DXd with chemotherapy of physician's choice among 557 patients with HER2-low aBC who had already received one or two previous lines of chemotherapy. The majority of patients were HR+ (88.7%). The study met both the primary and secondary endpoints, proving that T-DXd prolongs both PFS and OS in pre-treated patients affected by HER2-low aBC.[43] As already highlighted above, data from the phase III RCT DESTINY-Breast06 recently showed improved PFS for HR+/HER2-low aBC patients receiving T-DXd compared to TPC (capecitabine = 59.8%), after progression on first-line ET+CDK4/6i therapy. Interestingly, a subsequent amendment also allowed the inclusion of patients with HR+/HER2ultralow disease, defined as weak and incomplete membrane expression of HER2 in <10% of neoplastic cells. PFS in this patient group was thus introduced as a secondary endpoint. The analysis in this subgroup, although smaller in size than that of the HR+/HER2-low patients' group (153 vs 753, respectively), confirmed the same results, with an mPFS of 13.2 vs 8.3 versus TPC.[44]

Patritumab deruxtecan (U3-1402; HER3-DXd) is a novel, first-in-class, HER3-directed ADC composed of a human monoclonal antibody to HER3 (patritumab) covalently bonded to an exatecan-derived topoisomerase I inhibitor payload via a cleavable linker. U31402-A-J101 trial (NCT02980341) was a global, open-label, phase I/II trial assessing the safety and the activity of HER3-DXd in HER3-expressing heavily pretreated aBC. In 113 patients with HER3-overexpressed or HER3-low HR+/HER2– aBC, the confirmed ORR was 30.1% and DCR 80.5%, with a median PFS of 7.4 months. Of the 97 patients with HR+/HER2– disease, the confirmed ORR was 36.2% in patients with HR+/HER2-low and 28.2% in patients with HR+/HER2-0 disease.[74]

TROP-2 is a carcinoma-associated antigen overexpressed in different solid tumors, which plays a role in tumor progression by actively interacting with several key molecular signalling pathways. The randomized phase 3 trial TROPiCS-02 sacituzumab govitecan, a first in class ADC directed against TROP-2, demonstrated a survival advantage in patients with pre-treated HR+/HER2- aBC compared to chemotherapy

of physician choice in terms of PFS (5.5 vs 4.0 mo) and OS (14.4 vs 11.2 months). Interestingly, survival benefit was consistent across TROP-2 expression-level subgroups, suggesting that protein expression assessed as IHC cannot be a reliable biomarker to assess treatment efficacy.[75] Datopotamab deruxtecan (Dato-DXd), another TROP-2 directed monoclonal antibody attached to a topoisomerase I inhibitor payload, was tested in a phase 3 randomized trial, the TROPION-Breast01 in 732 patients with HR+/HER2- aBC who have received one or two prior lines of chemotherapy in the advanced setting. Patients receiving Dato-DXd had significantly improved PFS versus chemotherapy (HR 0.63, 95% CI 0.52–0.76; p<0.0001), with a favourable toxicity profile compared to standard chemotherapy. While OS data were not mature, a trend for improved OS favouring Dato-DXd was observed.[76,77]

BRCA1 and *BRCA2* germline mutations occur in HR+/HER2- aBC, with a prevalence up to 5%. PARP inhibitors (PARPi) are drugs specifically designed to exploit the vulnerability of tumor cells linked to the presence of these mutations. Employing a synthetic lethality mechanism, PARPi demonstrated efficacy in several disease settings including ovarian, breast and pancreatic cancers. Specifically, two studies have investigated the use of PARPi in HR+/HER2- patients progressing to 2 or 3 lines of treatment (OlympiAD trial, evaluating olaparib, and EMBRACA, with talazoparib), demonstrating a similar benefit in terms of PFS, but not in the OS endpoint.[78–80]

Finally, also the use of immunotherapy could represent a potential option for pre-treated HR+/HER2- aBC. The phase 2 PACE trial demonstrated potential activity of the immune-checkpoint inhibitor avelumab plus fulvestrant compared with fulvestrant alone or the combination of fulvestrant and palbociclib, in patients with HR+/HER2- aBC progressing after first-line ET+CDK4/6i, with a statistically significant advantage in terms of PFS (8.1 months for triplet vs 4.6 for doublet vs 4.8 for Fulvestrant alone), opening an opportunity for immunotherapy in selected patients in this setting.[81]

Together, these data indicate that several therapeutic options are available, or may become available soon, for the treatment of HR+/HER2-aBC patients in different lines of therapy. However, few reliable predictive biomarkers actually exist for optimizing the treatment trajectory of individual patients and finally improving overall survival (OS). Since there is no accepted evidence on which is the best choice after CDK4/6i+ET for HR+/HER2-aBC patients, clinicians are left alone during the treatment decision, causing fragmentation in therapeutic practice between cancer centers and even among individual oncologists.

The importance of real world data (RWD) in HR+/HER2- aBC patients treated with ET+CDK4/6i

Recently, there has been a growing interest in observational real-world studies, due to their ability to observe the effects of a given treatment even outside the ideal clinical conditions that characterize RCTs. These types of studies also allow sub-analyses to be carried out in certain subgroups, usually underreported in RCTs, and

to study biomarkers potentially useful for establishing treatment efficacy. This is particularly relevant in the context of established clinical settings, such as HR+/HER2- aBC patients who are candidates for first-line therapy, where it is difficult, in terms of ethics and pharmaco-economics, to conduct comparison prospective randomized trials to address specific clinical questions.

In this context, and in view of the long average duration of patients' exposure to this class of drugs, it is of primary importance to more accurately establish the real-world efficacy and toxicity of the three CDK4/6i.

Different attempts have been made in this context. The largest real-world experience is represented by the real-world collection of data on patients treated with palbociclib in the P-REALITY-X trial. Data were extracted from the Flatiron US Flatiron Health Analytic Database and eventually adjusted with appropriate statistical methods, such as stabilized inverse probability of treatment weighting (sIPTW) to conduct different analyses such as the comparison of CDK4/6i+ET vs ET alone in the real-world setting, with special regard to OS. [82,83] Similar, even though smaller, experiences are reported in literature with the other two CDK4/6i.[84,85]

These studies underlined the feasibility and utility of conducting real-world studies in the context of HR+/HER2- aBC. Specifically, this type of studies are essential to assess the effectiveness and safety of CDK4/6i in a real-world setting, including neglected subset of patients usually excluded from pivotal clinical trials.[86] In addition, they represent a unique opportunity to conduct specific sub-analyses that respond to relevant questions directly emerged from clinical practice and academic research. Finally, RWD are a struggling source of data to discover and validate potential biomarkers, to finally include in predictive models potentially useful to distinguish class of patients associated with diverse phenotypes and/or prognosis.

Unmet clinical needs in the treatment of HR+/HER2- aBC

In absence of direct prospective evidence, it is of crucial importance to conduct large, population-based observational, real-world studies to provide evidences in the complex scenario of HR+/HER2- aBC. Of particular interest at the moment is the development and validation of new biomarkers for patients receiving ET+CDK4/6i in the first-line setting, in order to identify subgroups of patients with different prognoses, for which treatment escalation and de-escalation approaches can be considered. For this purpose, it would be of paramount importance to have a predictive model, able to define prognosis at the individual patient level, taking into account the specific characteristics and complex inter-relationships existing between the clinical and biological variables. Another critical issue is represented by the scenario of HR+/HER2- aBC progressing after first-line treatment with ET+CDK4/6i. Since several therapies are currently available, including novel promising anticancer drugs such ADCs, and in consideration of the large heterogeneity of these patients in terms of tumor biology and clinical behaviour, the development of new powerful, easy-to-

access biomarkers and the integration of existing ones with novel signatures represents nowadays and urgent clinical need.

Novel methodological approaches, including random forest, gradient boosting models and neural networks, thanks to their ability to extract significant and quantitative features potentially associated with different clinical outcomes from electronic health records (EHRs), medical images and biological data through the use of complex high-level predictive models, represents a promising approach to achieve these results. In this context, these approaches may help in optimizing the treatment sequence in HR+/HER2- aBC patients with the aim of optimizing patient OS. In particular, radiomics and pathomics are promising tools of applications in oncology, as they are capable of identifying relevant features not accessible by raw human eye from radiological images and from pathology slides, respectively. As an example, Khorrami M. et al built a CT scan-based radiomic model able to predict survival benefit in aBC with liver metastases treated with CDK4/6i in combination with endocrine therapy, reaching a notable accuracy (AUC = 0.77).[87]

Master protocol study design

PALMARES-2 is a retrospective/prospective, observational, multicenter, population-based study, aiming at providing real-world evidences on HR+/HER2- aBC patients treated with first-line CDK4/6i plus ET.

The present study has the objective to collect data coming from different sources, i.e. RWD, medical images and biological samples, from patients treated with CDK4/6i as first-line of therapy for HR+/HER2- aBC. In consideration of the complexity of data collected and different objectives of the study, this master protocol foresees different sub-studies, which encompasses different methodologies for data collection, data extraction and analyses. The sub-studies will be described in the following sections.

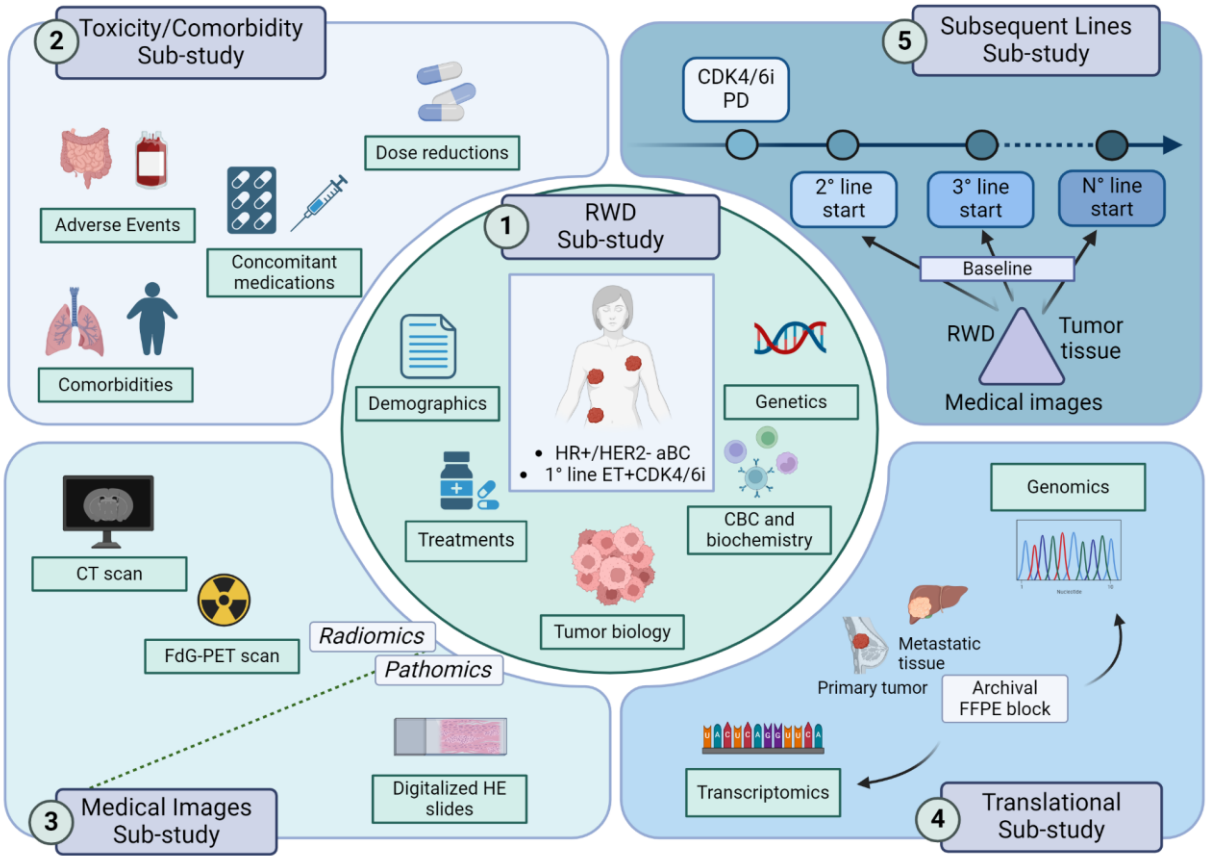

Figure 1. PALMARES-2 graphical abstract. CBC: cell blood count; CT: computed tomography; ET+CDK4/6i: endocrine therapy plus cyclin-dependent kinase 4/6 inhibitor; FdG-PET: fluorodeoxyglucose positron emission tomography; FFPE: formalin-fixed paraffin-embedded; HE: hematoxylin-eosin; HR+/HER2- aBC: hormone receptor-positive, human epidermal growth factor receptor 2-negative advanced breast cancer; PD: progressive disease; RWD: real-world data.

(1) Real world data (RWD) PALMARES-2 sub-study

Objectives and endpoints of the sub-study:

Primary objectives:

- To test the difference in terms of OS between the group of patients receiving ribociclib and palbociclib;
- To test the difference in terms of OS between the group of patients receiving abemaciclib and palbociclib.

Secondary objectives:

- To test the difference in terms of OS between the group of patients receiving abemaciclib and ribociclib.
- To determine the real-world progression-free survival (rwPFS), time to next treatment or death (TTNT-D), time to chemotherapy or death (TTC-D) and OS in the real-world setting;
- To test the difference in terms of rwPFS between the group of patients receiving ribociclib and palbociclib;
- To test the difference in terms of rwPFS between the group of patients receiving abemaciclib and palbociclib;
- To test the difference in terms of rwPFS between the group of patients receiving abemaciclib and ribociclib;
- To test the difference in terms of TTNT-D between the group of patients receiving ribociclib and palbociclib;
- To test the difference in terms of TTNT-D between the group of patients receiving abemaciclib and palbociclib;
- To test the difference in terms of TTNT-D between the group of patients receiving abemaciclib and ribociclib;
- To test the difference in terms of TTC-D between the group of patients receiving ribociclib and palbociclib;
- To test the difference in terms of TTC-D between the group of patients receiving abemaciclib and palbociclib;
- To test the difference in terms of TTC-D between the group of patients receiving abemaciclib and ribociclib;
- To test the difference in terms of rwPFS, TTNT-D and TTC-D among the three CDK4/6i in clinically-relevant subgroups, such as endocrine sensitive and resistant patients, premenopausal women, patients with poorer ECOG PS, patients harboring luminal-B like disease, patients with liver metastases;
- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i in older women (defined as >75 years old);
- To test the effect of histology (non special type vs lobular vs other) on rwPFS, TTNT-D, TTC-D;
- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i in patients with lobular breast cancer histology;
- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i according to disease-free interval (DFI);
- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i according to endocrine resistance status, including primary versus secondary resistance;

- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i according to different ER expression levels;
- To determine the rwPFS, TTNT-D, TTC-D and OS in the subgroup of patients with brain metastases;
- To determine the rwPFS, TTNT-D, TTC-D and OS in the subgroup of patients with oligometastatic disease and in the subgroup of patients with high metastatic burden;
- To test the effect on rwPFS, TTNT-D, TTC-D and OS of available metabolic biomarkers (e.g. body mass index, glycemia);
- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i in patients harboring germline mutations, including *BRCA1*, *BRCA2*, *PALB2*, and in patients with homologous recombination deficiency;
- To test the difference in terms of rwPFS, TTNT-D, TTC-D and OS among the three CDK4/6i in patients harboring genomic somatic alterations, like *PIK3CA* and *ESR1* status;
- To test the association between blood lymphocyte counts at baseline, or their precocious on-treatment modifications (T1: 2 weeks after treatment initiation, and T2: before fourth cycle initiation, approximately 12 weeks after treatment initiation) and rwPFS, TTNT-D, TTC-D and OS of HR+/HER2- patients treated with first-line ET plus CDK4/6i;
- To evaluate the association between peripheral blood crude cell counts, namely whole blood cells, neutrophils, monocytes and platelets, as evaluated at baseline and as precocious on-treatment modifications, and patient rwPFS, TTNT-D, TTC-D and OS;
- To evaluate the association between baseline composite parameters, such as the Neutrophil-to-Lymphocyte ratio (NLR), the platelet-to-lymphocyte ratio (PLR), the monocyte-to-lymphocyte ratio (MLR), or the Pan-Immune Inflammation Value (PIV), and patient PFS;
- To evaluate the association between precocious on-treatment modifications (i.e., 2 weeks and ~12 weeks after treatment initiation) of NLR, PLR, MLR and PIV, and patient PFS;
- To evaluate the association between HER2 status (HER2-low vs. HER2-0; HER2 2+ with negative ISH vs. HER2 1+ vs. HER2-0) in the primary tumor and rwPFS, TTNT-D, TTC-D and OS;
- To evaluate the association between HER2 status (HER2-low vs. HER2-0; HER2 2+ with negative ISH vs. HER2 1+ vs. HER2-0) in one metastatic site (subjected to biopsy before CDK 4/6 inhibitor initiation), and rwPFS, TTNT-D, TTC-D and OS;
- To explore pattern of progression of HR+/HER2- aBC after ET plus CDK4/6i treatment;
- To test the effect on rwPFS, TTNT-D, TTC-D and OS of mastectomy in patients with *de novo* HR+/HER2- aBC.

Study Population

Inclusion criteria:

- Pre- and Post-menopausal patient (woman OR man)
- Age≥18 years
- Diagnosis of aBC. HR+/HER2- aBC, as defined as at least 1% estrogen receptor (ER) and/or progesterone receptor (PgR) positivity at IHC. HER2 negativity is defined on the basis of an IHC score of 0, 1+, or 2+ with absence of gene amplification at in situ hybridization (ISH) analyses.
- Have received or are candidate to receive treatment with palbociclib, ribociclib or abemaciclib in combination with endocrine therapy as first-line treatment for HR+/HER2- aBC.

Exclusion criteria:

- Less than 3 months of follow up from the CDK4/6i start to the date of data cut-off;
- Have received CDK4/6i as monotherapy;
- Have received CDK4/6i as adjuvant treatment for localized disease.

Data collection

The study involves the collection of RWD, i.e. obtained directly from the medical records, electronic or paper, of the patients enrolled in the study. Therefore, no additional patient assessment will be required specifically for study purposes. Data will be collected locally within the center participating in the study, subject to patient consent, and then reported on a platform in .xlsx format.

The types of data collected within this sub-study include:

- Demographic data: date of birth, gender, menopausal status;
- Neoplasm history data: de novo vs metachronous presentation; date of diagnosis of localised disease; prior treatment for localised disease, including type and duration of prior hormonal, chemotherapy and CDK4/6i treatment; number and type of metastatic sites;
- CDK4/6i treatment data: drug used; backbone of hormone therapy and date of initiation of hormone therapy versus initiation of CDK4/6i; use of LHRH analogue;
- Tumor biology data: hormone receptor status, grading, proliferation index at biopsy site of metastasis and, if not available, relative to primary tumor; OncotypeDX;
- Data on haematochemical parameters: complete bloodcounts and some biochemical parameters such as LDH, blood glucose and CA15.3, collected at baseline, 15 days after starting CDK4/6i, after 30 days and at treatment progression;
- Genomic data: genomic alterations available to the patient, performed according to clinical practice; this section includes both somatic mutations and germline DNA alterations.

(2) Toxicity – Comorbidity sub-study

The "toxicity – comorbidity" sub-study aims to add a key piece to the real-world evidence of the three CDK4/6i, allowing for the first time a systematic comparison of the real-world toxicities of palbociclib, ribociclib and abemaciclib.

Objectives and endpoints of the sub-study

Primary objective:

- To compare the real-world incidence of severe toxicity, defined as a grade 3 or higher adverse event reported by the clinician, occurring during treatment, among the three CDK4/6i.

Secondary objectives:

- To compare the real-world incidence of overall toxicity, defined as any-grade adverse event reported by the clinician, occurring during treatment, among the three CDK4/6i;
- To assess the incidence of dose reduction conditioning toxicity;
- To assess the incidence of toxicity requiring discontinuation of treatment or switch to another molecule of the CDK4/6i class;
- To test the impact of dose reduction, drug switch and/or CDK4/6i discontinuation on survival outcomes (rwPFS, TTNT-D, TTC-D and OS);
- To assess the incidence of HIV infection among HR+/HER2- aBC patients treated with first-line CDK4/6i+ET;
- To assess the impact of HIV infection on survival outcomes (rwPFS, TTNT-D, TTC-D and OS) among HR+/HER2- aBC patients treated with first-line CDK4/6i+ET;
- To assess the incidence of alterations in liver or kidney function parameters such as aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin, lactic dehydrogenase (LDH), leukocyte alkaline phosphatase (ALP), creatininemia;
- To assess the incidence of alterations in electrocardiographic tracing definedas an increase in the corrected QT (according to the Fridericia criterion) greater than 480 msec;
- To assess the incidence of adverse events in groups of patients with comorbidities;
- To assess the incidence of adverse events in groups of patients taking concomitant therapies at the start of CDK4/6i treatment.

Study Population

The same inclusion and exclusion criteria adopted for the RWD sub-study will be adopted for the present sub-study, for the only exception of the following inclusion criteria:

- Availability of safety data during >50% of the time of study treatment;

- Availability of baseline patient information about comorbidities and concomitant medications.

Data collection

The data collection of this sub-study will take place in a similar manner to that of the RWD sub-study. In particular, the planned information will be collected directly from the patients' EHRs and collected in a separate .xlsx file, adopting the same pseudonymized code used for the RWD cohort. In detail, this sub-study will involve the collection of:

- All adverse events occurring during treatment, indicating the maximum degree reached;
- Dose reduction, indicating the level of reduction and the cycle at which this dose reduction occurred;
- Any interruption of treatment due to toxicity or switch to another CDK4/6i;
- Specific haematochemical parameters useful for assessing the progress of hepato-renal function, in particular AST, ALT, total bilirubin, LDH, ALP, creatininemia; these parameters will be collected at treatment baseline, after 15 days and 3 months after starting CDK4/6i (i.e. before starting the fourth cycle of therapy);
- Electrocardiographic parameters, in particular specific conduction alterations or arrhythmias and QTc value according to Fridericia, collected at baseline, after 15 and after 30 days from start;
- Patient comorbidity, understood as concomitant disease at the start of treatment with CDK4/6i ascertained by the physician and classified in macro-areas (e.g. cardiological, respiratory, metabolic, infectious, chronic diseases such as diabetes mellitus type 2);
- Concomitant therapies, initiated and ongoing before the start of treatment with CDK4/6i, reported as drug classes (e.g. steroid therapy, antibiotic therapy, proton pump inhibitors);
- Blood glucose and body mass index at baseline of CDK4/6i treatment.

(3) Medical imaging sub-study

Objectives and endpoints of the sub-study

Primary objective:

- To build a model able to predict prognosis of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using radiomics features extracted from baseline radiological images;
- To build a model able to predict prognosis of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using radiomics features extracted from baseline radiological images;
- To combine radiomics and pathomics features with clinical data to build a model able to predict prognosis of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i;

Secondary objectives:

- To combine radiomics features with clinical data, pathomics, genomics and transcriptomics features to build a model able to predict prognosis of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i;
- To build a model able to classificate HR+/HER2- aBC patients treated with first-line ET+CDK4/6i in early and late progressors using radiomics features extracted from baseline radiological images;
- To build a model able to predict prognosis of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using baseline radiomics features and early radiomics modifications detected from first on-treatment radiological evaluation;
- To build a model able to predict molecular characteristics, such as *PIK3CA* and *ESR1* mutational status, of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using radiomics features extracted from baseline radiological images;
- To build a model able to predict molecular characteristics, such as *PIK3CA* and *ESR1* mutational status, of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using radiomics features extracted from digitalized HE slides;
- To build a model able to predict intrinsic BC subtypes, defined by PAM50 panel on transcriptomic analyses, of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using radiomics features extracted from baseline radiological images;
- To build a model able to predict intrinsic BC subtypes, defined by PAM50 panel on transcriptomic analyses, of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i using radiomics features extracted from digitalized HE slides.

Study Population

The same inclusion and exclusion criteria adopted for the RWD sub-study will be adopted for the present sub-study, for the only exception of the following inclusion criterion:

- Availability of at least one of the following two data:
    - A baseline Computerized Tomography (CT) scan or 18F-Fluorodeoxyglucose Positron Emission Tomography (FdG-PET)
    - A digitalized pathology slides derived from biopsy of metastatic sites and/or from primary tumor biopsy and/or from surgery specimen.

Data collection

This sub-study encompasses the collection of medical images of patients enrolled in the PALMARES-2 RWD sub-study. Two type of images will be collected:

- Radiological images: CT scan, or FdG-PET scan, performed at the baseline of CDK4/6i, i.e. up to three months before the treatment start until one week after the start; the CT scan should include at

least the thorax and abdomen scanning; both pre- and post-contrast acquisition will be collected. CT scan, or FdG-PET, performed as the first radiological evaluation will be collected as well to assess early radiomics modifications and their effect on survival outcomes. For the purposes of the "Subsequent lines" sub-study (as detailed below, in the (5) section), CT scan, or FdG-PET scan, performed at the baseline of each of the subsequent line of therapy will be collected as well.

- Digitalized pathology slide: hematoxylin and eosin (HE) slides coming from diagnostic biopsy or surgical specimens will be digitalized and collected in a pre-defined folder. Both slides from primary tumor and from metastatic sites that underwent biopsy assessment will be collected, if available. If more than one metastatic biopsy has been performed during the course of patient's disease, all the HE slides should be collected, if available. If a patient did not undergo neoadjuvant treatment, diagnostic biopsy of the breast or surgical tumor slide could be collected equivalently; otherwise, the diagnostic biopsy performed before treatment initiation should be taken.

(4) Translational sub-study

Objectives and endpoints of the sub-study

Primary objective:

- To find the mutational status of a gene, or a subset of genes, associated with differential survival outcomes (rwPFS, TTNT-D, TTC-D and OS) of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i;
- To find transcriptomic differential gene expression, or differential expressed pathways, associated with differential survival outcomes (rwPFS, TTNT-D, TTC-D and OS) of HR+/HER2- aBC treated with first-line ET+CDK4/6i.

Secondary objectives:

- To test the effect of PAM50 intrinsic subtype on survival outcomes (rwPFS, TTNT-D, TTC-D and OS) of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i;
- To combine genomics and transcriptomics features with clinical data and radiomics and pathomics features, to build a model able to predict prognosis of HR+/HER2- aBC patients treated with first-line ET+CDK4/6i;
- To test the association between agnostic features included in radiomics model and transcriptomic pathways and mutational status.

Study Population

The same inclusion and exclusion criteria adopted for the RWD sub-study will be adopted for the present sub-study, for the only exception of the following inclusion criterion:

- Availability of adequate tumor sample (see Data Collection below) from biopsy of metastatic sites and/or from primary tumor biopsy and/or from surgery specimen.

Data collection

This sub-study encompasses the collection of tumor samples of patients enrolled in the PALMARES-2 RWD sub-study. At least one formalin-fixed paraffin-embedded tumor block will be collected from diagnostic biopsy or surgical specimens, performed for diagnostic purposes as per clinical practice. Both tumor samples from primary tumor and from metastatic sites underwent biopsy will be collected, if available. If more than one metastatic biopsy has been performed during the course of patient's disease, all the non-stained slides and/or tumor blocks should be collected, if available. If a patient did not undergo neoadjuvant treatment, diagnostic biopsy of the breast or surgical tumor slide could be collected equivalently; otherwise, the diagnostic biopsy performed before treatment initiation should be taken.

(5) Second and further lines sub-study

Objectives and endpoints of the sub-study

Primary objective:

- To build a model based on clinical, radiomics and pathomics data to select the best treatment option in terms of OS in HR+/HER2- aBC patients progressing after first-line ET+CDK4/6i.

Secondary objectives:

- To build a model based on clinical data to select the best treatment option in terms of OS in HR+/HER2- aBC patients progressing after first-line ET+CDK4/6i.
- To build a model based on clinical, radiomics, pathomics, genomics and transcriptomics data to select the best treatment option in terms of OS in HR+/HER2- aBC patients progressing after first-line ET+CDK4/6i.
- To assess the efficacy in terms of rwPFS and OS of second and subsequent line of therapy in a real-world setting of HR+/HER2- aBC patients progressing after first-line ET+CDK4/6i.

Study Population

The same inclusion and exclusion criteria adopted for the RWD sub-study will be applied for the present sub-study, for the only exception of the following inclusion criteria:

- Clinical, biochemical or radiological progression on CDK4/6i+ET first-line treatment
- Second-line therapy start after CDK4/6i+ET progression
- Data availability about at least one of the subsequent lines, including at least the treatment line of therapy, treatment start date, treatment progression date or last follow up, type of treatment and drug used.

Data collection

The study involves the collection of RWD of patients enrolled in the PALMARES-2 RWD study, who started a second line therapy after CDK4/6i progression. Data will be collected at the study centers in the same manner of RWD sub-study. Data collected within this sub-study will include the type of treatment performed (i.e. ET-based therapy, chemotherapy, other) and line of therapy for metastatic disease for which the treatment has been performed; treatment start date and progression date. Data derived from medical images and biological samples collected in the context of sub-study (3) and (4) will be used to pursue the objectives of this sub-study.

Study procedures

Informed consent process and patients' enrollment

Patients who are alive at the time of study conduction will be asked to sign the informed consent for the study partecipation and for treatment of personal data for research purposes, during the first clinical visit. Patients with available material to conduct analyses for the translational sub-study will be asked to the use of data generated from available material for the scientific purposes planned for the present study. It will also be asked to consent to receive unexpected genetic-hereditary information, should it emerge from the analysis of tumor samples.

For patients who are not alive at the moment of study initiation and data collection, the study will adhere to the UE-Reg 2016/679 - GDPR rules for the protection of personal data. In accordance with the Declaration of Helsinki and other applicable regulations, a subject, or his/her legal representative, has the right to withdraw from the study at any time and for any reason without prejudice to his/her future medical care.[88]

Data protection procedures

Patients are assigned by the local investigator a unique progressive identifier code, that will be used for all the tasks implied in the study. The pseudonymization procedure will ensure the unique identification of patients still keeping adequate data security. This pseudonymized code will used to save medical images

once shared with the Sponsor center. The same code used to mark biological samples at the moment of the shipment to the centralization for analyses conduction.

Clinical data collection and management

Clinical real world data will be collected by centers in ad hoc document in .xlsx format. At pre-specified data cut-off (see sections below), data collected will be shared with the Sponsor for data analyses. Investigators at study centers could be required to modify data in case of data inconsistencies encountered during data analyses.

All consecutive HR+ HER2- aBC patients treated with first-line ET plus CDK 4/6i as per clinical practice in the time interval between January 2017 and December 2030 will be enrolled retrospectively and prospectively. Data will be collected in a continuous manner and will be shared with the Sponsor center on an annual basis, at pre-specified data cut-offs. Data cut-offs will occur annually, at certain cutoffs falling on 1st January of each year. Taking into account the time interval and the centers involved, approximately 2000 patients will be included at the first data cut-off of 1st January 2024. Those patients who have already concluded the treatment at the time of study inclusion will be evaluated for clinical outcomes (i.e. PFS, OS) and biological parameters of interest will be retrospectively collected. Patients who are still receiving ET plus CDK4/6i at the time of inclusion in the study will be evaluated for the same variables until the end of the study. At the time of data collection and analysis, these patients will be censored for the events of interest (e.g. progression, death) if these events have not occurred yet.

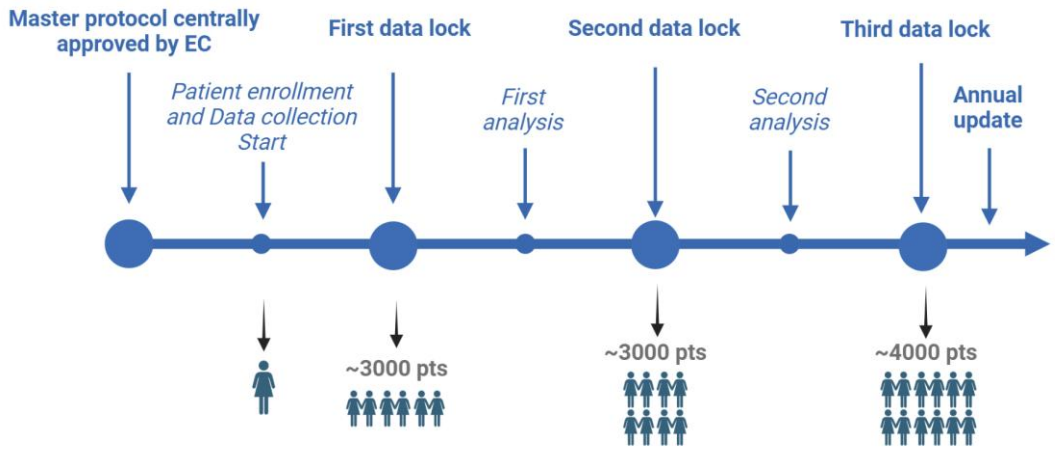

Figure 2. PALMARES-2 study expected timeline and enrollment. EC: ethical committee; pts: patients.

Medical images collection and sharing

The medical images, collected within the timeframe specified in the appropriate section (see section "Medical images sub-study"), will be collected locally in the individual centers, pseudonymised and uploaded to an ad hoc secure sharepoint. A folder will be set up for each individual center participating in this sub-study, so that only the center uploading the images and the Sponsor center itself will be able to access them.

Tumor samples collection, storage and shipment

For the "Translational sub-study" purposes, tumor samples related to patients enrolled in the RWD sub-study will be considered. If multiple tumor samples are available, the ones to be used for the study has to be chosen as specified in the section "Translational sub-study". Tumor materials have to be shipped to the Sponsor center at the moment of the conduction of translational analyses, attributing the same pseudonymized code used for the other sub-study. The remaining unused tumor tissue will be sent back to the pathology laboratory at the end of the study.

Statistical considerations

Statistical design

Based on pivotal trials showing a statistically significant OS advantage for ribociclib and abemaciclib, but not for palbociclib in the endocrine resistant setting, and clinically meaningful OS advantage for ribociclib and abemaciclib, but not for palbociclib in the endocrine sensitive setting, we will hypothesize a difference of 20% in terms of OS between ribociclib versus palbociclib and between abemaciclib and palbociclib. To detect this difference with 80% power with a two-sided alpha error of 0.05, a sample size of 3258 patients reaching 977 OS events will be required. The prognostic role of CDK4/6i drug will be weighed for clinically and biologically relevant characteristics.[89]

Since the number of patients enrolled in the other sub-studies depends on the sample size of the RWD sub-study, no formal statistical calculation has been performed for 2 to 5 PALMARES-2 sub-studies.

Statistical methods

Standard descriptive statistics will be used to describe clinical and biological patients' characteristics. The median and interquartile range (IQR) were reported for continuous variables. Categorical variables were summarized as percentages of available data. To evaluate differences, in terms of clinic-pathological characteristics, among different CDK4/6i groups, we will use the Kruskal-Wallis Rank Sum test for

continuous variables and the Chi-square and Fisher exact tests for categorical variables. Unpaired t-test will be used to compare numeric variables, at baseline and different timepoints. Classic survival analysis methods will be used to evaluate patient survival outcomes and survival curves will be obtained through the Kaplan–Meier method. Follow-up times will be estimated using the reverse Kaplan–Meier method. Multivariable analysis will be performed through Cox regression modeling to estimate the effects of covariates. To homogenize patients' characteristics among treatment groups, additional adjustment techniques will be eventually used, such as propensity score matching (PSM) and inverse probability of treatment weighting (IPTW). Estimated weights will be then incorporated into a weighted Cox regression model to estimate rwPFS. Area Under the ROC Curve (AUC) values of the models will be calculated using the Caret and pROC R package and compared using Delong's test.

Patients with missing outcome data will be excluded from the analysis. Covariates whose values will be missing in less than 20% were imputed by using the median or the mode value (for numerical or categorical variables, respectively), while covariates with missing values exceeding 20% will be excluded from the present analysis.[90] All statistical tests will two-tailed, and a p value (*P)* <0.05 was considered as significant. Statistical analyses will be performed using R software and R Studio (version 4.1.2).

Image omics and model generation methodology

Radiomics Methodology

A team of four radiologists with experience on CT scan/PET images segmentation will identify the Volume Of Interest (VOI) through a semi-automated 3D segmentation process performed with Syngo.via software. An ad hoc designed segmentation protocol will be applied to homogenize the choice of CT/PET sequence on which to perform segmentation and the choice of the lesion to be segmented. Peritumoral area will be obtained through automated augmentation techniques (+5 mm, +10 and +15 mm); if the peritumoral area obtained crosses blood vessels, gas, bone, bile duct, or exceeded the organ edge, the contour will be manually modified to exclude this area. After image pre-processing through different techniques (e.g., gray discretization, intensity normalization, and voxel resampling), radiomic features will be extracted from VOIs and peritumoral areas using the PyRadiomics library in Python. In order to prevent signature overfitting, dimensionality of features is reduced before signature construction, firstly excluding features with high intraclass correlation coefficient and significantly different between the two outcome groups as assessed by one-way analysis of variance (ANOVA). Least absolute shrinkage and selection operator (LASSO) regression and/or Maximum Relevance Minimum Redundancy (MRMR) will be then used for the selection of features that will be included in the final model. The cohort will be split in a training (80%) and testing cohort (20%). Different standard classifiers, such as Random Forest, Multilayer perceptron, Logistic

Regression, Support Vector Machine, CatBoost, AdaBoost, XGBoost, will be trained and evaluated for this task.[91,92]

Pathomics Methodology

Pathology slides will be digitalized with Leica Scanscope AT2 and stored as ".svs" files to obtain Whole Slide Images (WSIs). Hematoxilin and Eosin (HE) slides will be digitalized for pathomics purposes. WSIs will be analysed through Slideflow, a Python package that provides a unified Application Programming Interface for building and testing models for histopathology. After color normalization procedures, quantitative pathomics features will be extracted. Model creation and performance evaluation will follow the same methodology discussed in the "Radiomics" workflow.[93]

Multimodal integration

We will use several architectures, including logistic regression, classification and regression trees, random forest, multi-layer perceptron, gradient boosted trees (XGBoost), gradient boosting machines (LightGBM) and attentive tabular networks TabNet and we will compare their performance to heuristically decide on the best model choice for follow-up experiments. Our modelling experiments will include different combinations of input embeddings, with concatenation of each data stream permutation to produce fusion embeddings and train models using single-modality or multi-modality of input combinations. All models will be trained multiple times with multiple different data splits to repeat the experiments and compute average metrics and SDs. All defined models will be trained and tested to evaluate the advantage of multimodal predictive systems, as compared to single modality ones for the pre-established classification prediction. To assess model performance, different metrics will be used, including AUC, Sensitivity and Specificity, which will be calculated on the training set through 10-fold cross-validation and on the test set, which will be obtained by randomly splitting the entire cohort in a 80:20 way. The hyperparameter combinations of individual models, such as maximum depth of the trees, number of estimators and learning rate, will be selected within each training loop using a fivefold cross-validated grid search on the training set.[94,95]

Since these complex models are considered as "black boxes", since they make decisions without clear reasoning, explainability techniques will be adopted to provide insights into how models make predictions, guaranteeing human interpretability. In particular, SHapley Additive exPlanations (SHAP) techniques will be used for both global and local explanations.[96]

Biological analyses methodology

Genomic analyses

A next generation sequencing (NGS) panel (Oncomine Comprehensive Assay Plus) will be used to perform analysis of various single-gene variants, such as single nucleotide variations (SNVs), indels, fusions, splice

variants, and copy number variations (CNVs), including both copy number gains and losses, across ~500 genes. The panel will include, but will not be limited to, genes involved in DNA repair systems (e.g. *BRCA1, BRCA2, PALB2, CHEK2, ATM*), PIK3CA pathway (e.g. *PIK3CA, AKT1, PTEN*), estrogen receptor (e.g. *ESR1*). It also allows the quantitative evaluation of tumor mutational burden (TMB) and the analysis of the microsatellite instability (MSI) status.[97]

Transcriptomic analyses

After cellular RNA extraction, RNA quality check and preparation of libraries, RNA will be sequenced with NovaSeq 6000 System. Counts will be analyzed to dissect differential gene expression both at single gene level – through differential gene expression analysis (i.e, Deseq2 method) – and at pathway level – through Gene Set Enrichment Analysis (GSEA), Over Representation Analyses (ORA) and Gene Set Variation Analysis (GSVA).(30,31) The pathway-level analysis will be carried out using the Bioconductor packages fgsea, gsva, and clusterprofiler, taking advantage of HALLMARK, KEGG, and REACTOME gene sets available from GSEA Molecular Signatures Database (http://www.gsea-msigdb.org/gsea/msigdb/collections.jsp). Intrinsic subtypes will be assessed through Absolute Intrinsic Molecular Subtyping (AIMS) algorithm.[98–100]

Study duration

Patient enrollment and data collection will start upon approval of the study protocol by the competent ethics committee. All consecutive HR+/HER2- aBC patients treated with first-line ET plus CDK 4/6 inhibitors as per clinical practice in the time interval between January 2016 and December 2030 will be enrolled. The collection and the update of clinical data, medical images and biological samples will continue for an expected duration of 10 years from the enrollment of the last patient. The clinical data, medical images and data obtained from the biological samples will be retained for further 10 years after the end of the study, while the biological samples will be returned to the pathology departments of the Center of origin at the end of the analyses foreseen in the protocol.

Ethical considerations

This study will be conducted according to the Declaration of Helsinki and in compliance with ethical principles regarding human research.

Based on its observational nature, and in accordance with the Italian Ministerial Decree of 30th November 2021, the present study was approved by the Ethics Committee of the Sponsor Institution (Fondazione IRCCS Istituto Nazionale dei Tumori), and it was subsequently adopted by all the participating centers. Based on

Article 110bis of the Italian Privacy Code (Legislative Decree of 30 June 2003, n.196 updated 01 May 2024), the privacy impact assessment of the study is performed at the level of the Sponsor center and published on Institutional website, therefore no additional assessment by the other centers participating in the study is required.

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