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Gene expression analysis in circulating tumour cells to determine resistance to CDK4/6 inhibitors plus endocrine therapy in HR+/HER2- metastatic breast cancer patients

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Abstract

Background Metastatic breast cancer (BC) is the main cause of cancer-related mortality in women worldwide. HR+/HER2- BC patients are treated with endocrine therapy (ET), but therapeutic resistance is common. The combination of cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) with ET was approved for metastatic BC patients and extended the median progression-free survival to 24 months. This therapy is not always effective, and in every patient, resistance ultimately occurs, but the underlying resistance mechanisms remain unclear. To address this gap, we explored circulating tumour cells (CTCs) as biomarkers to assess treatment response and resistance in metastatic HR+/HER2- BC patients receiving CDK4/6i plus ET.

Methods In total, 53 HR+/HER2- metastatic BC patients who received a CDK4/6i plus ET as first-line treatment were analysed, including samples from internal and external validation cohorts. CTCs were isolated using the negative enrichment approach RosetteSep (STEMCELL Technologies) or positive immunomagnetic selection targeting EpCAM, EGFR, and HER2 (AdnaTest EMT-2/StemCell Select™, QIAGEN). RNA was extracted from CTCs and PBMCs for nCounter analysis (Pancancer pathways panel) in a discovery phase. Subsequent validation was performed by RT-qPCR.

Results CTC gene expression analysis revealed that non responder patients (those who experienced disease progression before 180 days) exhibited elevated *PRKCB* (p-value: 0.011), *MAPK3* (*p-value*: 0.006) and *STAT3* (*p-value*: 0.008) expression, while responders showed increased *CDK6* (*p-value*: 0.011) and *CCND1* (*p-value*: 0.035) expression at baseline. CTC transcriptional characterization revealed a gene expression signature ($STAT3^{high}PRKCB^{high}CDK6^{low}$) that accurately classified HR+/HER2- metastatic BC patients who responded to CDK4/6i plus ET, regardless of the CTC isolation method (AUC > 0.8). CTC characterization at progression also identified biomarkers linked to therapy resistance, including the epigenetic regulators *EZH2* and *HDAC6* and the cell cycle regulator *CDC7,* which could guide the selection of subsequent therapy lines. The expression of the *CDK4* and *STAT3* genes in CTCs was associated

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with progression-free survival and overall survival, respectively. Likewise, the presence of ≥ one CTC after one cycle of therapy predicts a worse prognosis.

Conclusions CTC gene expression provides information about treatment outcomes in HR + /HER2- metastatic BC patients receiving CDK4/6i plus ET and could guide personalized strategies and improve prognosis.

Keywords Breast cancer, CDK4/6 inhibitors, Circulating Tumour cells, STAT3, Biomarker

Background

Breast cancer (BC) is the leading cancer among women, and metastatic disease is the main cause of cancer-related deaths [1, 2]. Metastatic BC caused 670,000 deaths in 2022[3]. To date, BC patients are stratified considering the expression of hormonal receptors (HR), such as estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Approximately 70% of BC patients are classified as having the HR + subtype, the most prevalent subtype with the best prognosis. This subtype is traditionally treated with endocrine therapy (ET), but in metastatic stages, endocrine resistance is commonly acquired [4, 5]. To delay progression, the EMA and FDA approved the combination of cyclin-dependent kinase 4/6 inhibitors (CDK4/6i) and ET as a standard of care for HR + /HER2- metastatic BC patients as first-line therapy. The commercially available CDK4/6i are palbociclib, ribociclib and abemaciclib. This therapeutic approach enhances patients' progression-free survival (PFS) by up to 2 years with manageable secondary adverse events, as demonstrated in the PALOMA, MONALEESA and MONARCH trials [6–9]. Nevertheless, a total of 20% of patients do not respond to CDK4/6i plus ET due to intrinsic resistance, and those who initially respond ultimately develop acquired resistance [10]. Thus, one of the current clinical oncology challenges is the management of HR + /HER2- metastatic BC patients resistant to CDK4/6i plus ET. Although several CDK4/6i resistance mechanisms have been proposed, none have been clinically validated thus far [11, 12]. In this regard, clinical guidelines recommend testing for *PIK3CA* and *ESR1* mutations in tissue or ctDNA in this emergent group of patients to guide subsequent therapy selection after CDK4/6i plus ET resistance [13]. Importantly, circulating tumour cells (CTCs) can provide insights into tumour biology and potential CDK4/6i resistance mechanisms, although they have not been studied in detail by many groups in this specific clinical context. To address this issue, a transcriptome analysis of CTCs from HR + /HER2- metastatic BC patients was performed to identify cancer-associated canonical pathways involved in CDK4/6i plus ET response and resistance.

M&M

HR + /HER2- metastatic breast cancer patient cohorts

The inclusion criteria for patients were histologically confirmed metastatic BC, hormone receptor-positive, Her2 negative, a combination of CDK4/6i plus ET as first-line therapy, an age > 18 years, a disease-free interval ≥ 12 months and the absence of another malignant tumour at present or in the last five years (except for cutaneous carcinoma of basal cells or squamous cells or carcinoma in situ of the cervix that was adequately treated). Considering the time of disease progression, patients were classified as non responders if radiological disease progression occurred within 180 days after CDK4/6i plus ET initiation or responders if progression occurred after at least 181 days after CDK4/6i plus ET initiation. The response was considered following RECIST criteria. The responders were divided into initial responders if disease progression occurred between 181 and 730 days and long responders if disease progression occurred after 730 days (Figure S1). Three cohorts of patients were involved in the study. All procedures followed the Helsinki Declaration guidelines and were approved by two separate ethics committees. Cohorts 1 and 2 were approved by the Ethics Committee of Galicia under approval reference number 2015/772. Cohort 3 was approved by the Ethics Commission of the University Duisburg-Essen under approval reference number 12–5265-BO. All patients provided written informed consent.

Cohort 1 Twenty-five patients diagnosed with HR+/HER2- metastatic BC at the Clinical Hospital of Santiago de Compostela (Spain) were recruited from February 2018 to December 2022 (Figure S1). The disease follow-up and clinical information were collected 59 months after the inclusion of the first patient (the median follow-up time was 32.8 months). The clinical data are summarized in Table 1. A total of 18 patients progressed during the follow-up (mean time: 18.4 months). The median progression time for non responders (n=5) was 3.5 months, while for *responders* (n=20), it was 19.7 months. When the latter group was divided, the median progression time for initial responders (n=13) was 16.4 months, while for *long responders* (n=7), it was 52.9 months. A total of 10 patients died during follow-up, one unrelated to disease progression.

Table 1 Clinical data of HR + /HER2- metastatic BC patients treated with CDK4/6i plus Endocrine Therapy (ET) from cohorts 1 and 2 (Clinical Hospital of Santiago de Compostela, Spain) and cohort 3 (University Hospital Essen, Germany) were obtained

Category	Cohort 1		Cohort 2		Cohort 3	
	n	%	n	%	n	%
Age at V1						
≤ 57	12	57.14	5	41.67	8	50.00
> 57	9	42.86	7	58.33	8	50.00
Subtype PT						
Luminal A	10	40,00	5	41.67	3	18.75
Luminal B	14	52.00	6	50.00	9	56.25
*Luminal**	1	8.00	1	8.33	4	25.00
Metastasis de novo						
Yes	6	28.57	4	33.33	8	50.00
No	15	71.43	8	66.67	8	50.00
Metastatic location						
Bone	8	38.1	2	16.67	8	50.00
Visceral	5	23.81	3	25.00	6	37.5
Bone & Visceral	12	38.1	7	58.33	1	6.25
Others					1	6.25
Therapy (CDK4/6i)						
Abemaciclib	5	20.00	2	16.67	0	0.00
Palbociclib	17	68.00	9	75.00	6	37.5
Ribociclib	3	12.00	1	8.33	10	62.5
Therapy (CDK4/6i + ET)						
CDK4/6i + Letrozole	16	64.00	9	75.00	8	50.00
CDK4/6i + Fulvestrant	8	32.00	3	25.00	7	43.75
CDK4/6i + Anastrozole	1	4.00	0	0.00	1	6.25
Sample-CDK4/6i delay	Mean	SD	Mean	SD	Mean	SD
Days	7.49	13.64	3.9	15.30	12.18	22.92

PT: Primary tumour

* Patient could not be classified as luminal A or B by pathology analysis

Cohort 2 Twelve patients diagnosed with HR + /HER2- metastatic BC at the Clinical Hospital of Santiago de Compostela (Spain) were recruited from January 2020 to December 2022 (Figure S1). Patient follow-up data and clinical information were collected 36 months after the inclusion of the first patient (the median follow-up time was 21.8 months). The clinical data are summarized in Table 1. A total of six patients progressed during the follow-up (mean time: 11.3 months). The median progression time for non responders (n = 2) was 2.7 months, while for initial responders (n = 4), it was 15.5 months. Six patients did not progress during follow-up. Four patients died during follow-up.

Cohort 3 Sixteen patients diagnosed with HR + /HER2- metastatic BC at Essen University Hospital (Germany) were recruited from June 2017 to November 2020 (Figure S1). The follow-up of patients was closed, and clinical information was collected 72 months after the inclusion of the first patient (the median follow-up time was 43 months). The clinical data are summarized in Table 1. Twelve patients progressed during the follow-up (mean time: 71.6 months). The median progression time for non responders (n = 7) was 3.3 months, while for responders (n = 9), it was 21.3 months. When the latter group was divided, the median progression time for initial responders (n = 6) was 14 months, while for long responders (n = 3), it was 36.0 months. Six patients died during follow-up.

Blood sample collection

Three 10 mL EDTA-coated vacutainer tubes were collected per patient at different time points for CTC enrichment or PBMC and cfDNA isolation: when metastatic disease was diagnosed before therapy initiation (visit 1 or baseline), after one cycle of therapy (visit 2) and at disease progression (visit 3) whenever it takes place (Figure S1). For cohort 1, 21 metastatic BC patients were included at visit 1 and visit 2, while 20

patients were included at visit 3. Only 16 paired samples were among visits (1, 2 and 3) per patient. In addition, in twelve cases, one CellSave tube was also collected. For cohorts 2 and 3, a total of 12 and 16 blood samples, respectively, were analysed at visit 1 (Figure S1). The mean time delays between V1 sample collection and the initiation of therapy are summarized in Table 1 for each cohort. In cohort 1, for visit 2, after one cycle of therapy, the median time from sample collection to therapy initiation was 30 days (SD 7.8 days, n = 18), while for 3 cases the median time was 86 days, a higher delay due to the COVID-19 lockdown.

Circulating tumour cell isolation

CTC enrichment

i) One EDTA tube (10 mL) of peripheral blood was used to isolate CTCs from cohorts 1 and 2 using a negative enrichment tetrameric antibody cocktail (RosetteSep, STEMCELL Technologies) following the manufacturer's recommendations with subsequent density gradient centrifugation as previously published [14]. The enriched fraction was kept in RNA Later Solution (Invitrogen, Thermo Fisher Scientific) and stored at −80 °C until downstream analysis.
ii) CTCs from cohort 3 were isolated from 5 mL of whole blood by positive immunomagnetic selection targeting EpCAM, EGFR and HER2 (AdnaTest EMT-2/StemCell Select™, QIAGEN) following the manufacturer's recommendations and as previously described [15]. The lysate of the enriched fraction was stored for a maximum of 14 days at −80 °C.

PBMC isolation

Two EDTA tubes (10 mL) were used to isolate PBMCs and plasma by density gradient centrifugation using Lymphoprep medium in SepMate tubes (STEMCELL Technologies) following the manufacturer's recommendations. The PBMC fraction was kept in RNA Later Solution (Invitrogen, Thermo Fisher Scientific) and stored at −80 °C until downstream analysis.

CTC enumeration

One CellSave preservative tube (Menarini-Silicon Biosystems) was used for CTC enumeration by the CellSearch® System utilizing antibodies against EpCAM, CKs (−8, −18 and −19), CD45 and DAPI (4,6-diamino-2-phenylindole) staining. A CellTracks Analyser (Menarini-Silicon Biosystems) was used to acquire digital images of the three fluorescent dyes to determine the number of CTCs [16].

Plasma and cfDNA isolation

Two EDTA tubes (7.5 mL) of fresh blood, used for PBMC isolation, were centrifuged twice to precipitate the debris, and the plasma was aliquoted. Subsequently, 100 µl of cfDNA was extracted from 3 to 5 ml of plasma with a QIAamp Circulating Nucleic Acid Kit and a QIAvac 24 Plus vacuum system (QIAGEN) following the manufacturer's protocol. Finally, cfDNA was quantified using a Qubit 1 × dsDNA HS assay kit (Thermo Fisher). cfDNA was stored at −20 °C until further analysis.

RNA extraction and cDNA synthesis

The RNA from negatively enriched CTCs and PBMCs was extracted using an AllPrep DNA/RNA kit (QIAGEN) following the manufacturer's protocol in a final eluate of 30 µl. A total of 11 µL of RNA was reverse transcribed into cDNA using SuperScript III (Thermo Fisher Scientific) following the manufacturer's protocol.

The mRNA from the entire lysed AdnaTest-enriched fraction (samples from cohort 3) was isolated and reverse transcribed in a final reaction volume of 40 µl (AdnaTest EMT-2/StemCell DetectTM, QIAGEN), and the cDNA was stored at −20 °C until subsequent analysis.

cDNA preamplification

Due to the scarcity of CTCs in circulation, 20 µl of cDNA from the CTCs and PBMCs of cohorts 1 and 2 and 1 µl of cDNA with beads from the CTCs of cohort 3 were preamplified for 14 cycles using Taqman Preamp Master Mix (Thermo Fisher Scientific), which contained a pool of selected TaqMan probes (Applied Biosystems). Subsequently, cDNA expression was analysed on a LightCycler 480 II (Roche Diagnostics, Indianapolis, IN, USA) with TaqMan Gene Expression Master Mix and TaqMan probes (Applied Biosystems, Carlsbad, CA, USA) for 33 genes (Table S1). The expression of CTCs and PBMCs was subsequently normalized to that of *B2M* using a logarithm base 2 ($2^{-\Delta Ct}$). Finally, the difference between CTCs and paired PBMCs was subtracted to avoid differences in the background of PBMCs in the samples of cohorts 1 and 2. No PBMC samples were available for cohort 3.

nCounter assay

The NanoString nCounter assay was performed on the GENvip platform at the Health Research Institute of Santiago de Compostela using the PanCancer Pathways panel. This panel included 770 genes representing all major cancer pathways, including Wnt, Hedgehog, apoptosis, cell

cycle, RAS, PI3K, STAT, MAPK, Notch, TGF-β, chromatin modification, transcriptional regulation, and DNA damage control pathways. The CTC RNA of six HR + /HER2- metastatic BC patients from cohort 1 was included per panel (two patients per group: non responder, initial and long responder) from visits 1, 2 and 3. A pool of paired PBMCs (from the different visits) was included for expression relativization. In addition, normalization analysis was performed considering positive and negative controls. Positive controls were used to calculate a normalization factor to determine the RNA counts per gene and sample. The negative controls define the minimum number of counts considered per sample. The panel included 40 PanCancer reference genes. When CTCs and PBMCs were analysed together, *B2M* was the best reference gene for both. A small sample size was used in this discovery phase to ensure representative data while minimizing batch effects, as larger-scale validation was followed.

Digital droplet multiplex PCR analysis

The quantification of *PIK3CA* mutations from at least 10 ng/μL (mean: 25,9 ng/μL) of cfDNA was performed by ddPCR (QX200 Droplet Digital PCR systems, Bio-Rad). The emulsion of droplets was created using a QD200 Droplet Generator (Bio-Rad). The droplets were transferred to a 96-well PCR plate. PCR amplification was performed using a C1000 Thermal Cycler (Bio-Rad). After amplification, the plate was evaluated on a QX200 Droplet Reader (Bio-Rad). Finally, data analysis was performed with QuantaSoft software (Bio-Rad). Three replicates per sample were analysed. Specific PIK3CA probes (Bio-Rad) were used to detect p.E542K (dHsaMDV2010073), p.E545K (dHsaMDV2010075), p.H1047L (dHsaMDV2010123) and p.H1047R mutations (dHsaMDV2010077). Genomic wild-type DNA was used as a negative control. The positive controls were synthetic double-stranded DNA fragments, called gBlock® Gene Fragments (Bio-Rad), which contained 10 or 50 copies of the selected mutation.

Culture cell line

MCF7 (GeneCopoeia, Inc.) and T47D (ATCC) cells were cultured in DMEM (Sigma Aldrich) and RPMI 1640 (Sigma Aldrich), respectively, supplemented with 10% FBS (Sigma Aldrich) and 1% penicillin–streptomycin solution (Sigma Aldrich) at 37 °C in a humidified atmosphere with 5% CO_2. Both cell lines were cultured with CDK4/6i (palbociclib, ribociclib), obtained from Selleckchem to generate resistant cell lines (Supplementary material).

To induce STAT3 expression, MCF7 cells were also treated with 20 ng/mL IL-6 (PreProTech) in DMEM for 8 days (renewal every 2 days), generating the MCF7$^{\Delta STAT3}$ cell line. Additionally, a media change was performed in the MCF7 wt line in parallel to avoid biases due to cell manipulation.

Immunohistochemistry

The primary tumour samples were routinely immersed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Four-micron-thick sections were mounted on FLEX IHC microscope slides (Agilent, Santa Clara, CA). After deparaffinization, immunohistochemistry was automatically performed using a Dako Omnis immunostainer (Agilent). Briefly, the slides were incubated at room temperature in (1) EnVision™ FLEX target retrieval solution at high pH; (2) ready-to-use E-cadherin mouse monoclonal antibody (clone NHC-38, Agilent) for 25 min; (3) EnVision FLEX + mouse (linker) for 10 min; (4) EnVision + dual-link system-HRP (Agilent) for 20 min; (5) DAB + substrate-chromogen solution (Agilent) for 10 min; and (6) EnVision FLEX hematoxylin (Agilent) for 15 min. Samples and data from patients included in this study were provided by the Biobank of C.H.U.S.(SERGAS)-registration code: B0000807-and they were processed following standard operating procedures with the appropriate approval of the Ethical and Scientific Committees.

Cell viability post-treatment using MTT assay

MCF7 and MCF7$^{\Delta STAT3}$ cells (after 5 days of induction with IL6) were seeded in a p96 plate (8000 cells/well). In MCF7$^{\Delta STAT-3}$ cells, IL6 exposure was continued for 2 additional days. Next, cells were treated with Palbociclib 1 μM, Letrozole (20 μM) or combined therapy (Palbociclib plus Letrozole) for 48 h, keeping a group of wells as control (without treatment). Throughout the experiment, the treatment with 20 ng/mL IL-6 for the MCF7$^{\Delta STAT3}$ cell line was maintained, ensuring the induction of STAT3 at any time. After this period, the medium from each well was aspirated, and MTT (Sigma Aldrich) was performed following manufacture instructions [17]. The percentage of viability relative to the untreated control was obtained with the adjusted absorbance. Five technical replicates per condition were performed in each assay (n = 4). The treatment dosage and timing were established based on a literature review and previous MTT assay results assessing cell viability.

Statistical analysis

Statistical analysis was performed using R Studio (version 4.3.0.) and GraphPad Prism 10.0.2 software (GraphPad Software, Inc.). The Wilcoxon signed-rank test (for matched pairs) or Wilcoxon rank-sum test was used to compare CTC gene expression among patient groups or visits and between cell line conditions. Fisher's exact test

was used to assess associations between gene expression and clinical data and gene expression among different genes. PFS and OS were visualized using Kaplan–Meier plots and tested by the log-rank test. Receiver operating characteristic (ROC) analysis was conducted to assess the ability of individual gene expression to predict progression [18] using the pROC package, which provides the best threshold value for CTC expression. A logistic regression model was used to test the AUC of the combination of different genes. The prediction function [19] was employed to evaluate the precision of utilizing a signature as a progression predictor. For nCounter analysis, the optimal number and the most stable reference genes were selected using the geNorm package [20]. The genes differentially expressed among the groups were determined using the DESEq2 package. The long responder group at visit 1 was used as a reference group for the expression analysis. Gene Ontology enrichment analysis was performed using the STRING tool. Only p values < 0.05 were considered to indicate statistical significance.

Results

CTC gene expression analysis: identification of predictive biomarkers to determine the response to CDK4/6i plus ET

To identify biomarkers related to the CDK4/6i plus ET response, a transcriptomic CTC analysis at different time points was performed. We started with a gene discovery study performed with NanoString nCounter technology using the PanCancer Pathways Panel, which included CTC RNA from six patients. The subsequent differential gene expression analysis (DGE) between responder and non responder patients revealed that 31 genes were differentially expressed at the time of diagnosis of metastatic disease (visit 1, Fig. 1A) and 158 genes were differentially expressed after one cycle of therapy (visit 2, Fig. 1C) (Table S2-3). Next, Gene Ontology (GO) analysis was carried out to determine the functional significance of the identified genes. This analysis revealed enrichment in numerous pivotal pathways associated with cancer progression at both visits 1 and 2, such as the FoxO signalling pathway, cell cycle regulation, transcriptional misregulation in cancer, and the MAPK and JAK-STAT signalling pathways. After one cycle of therapy, the PI3K-Akt signalling pathway, Ras signalling pathway, and microRNAs in cancer and EGFR tyrosine kinase inhibitors were also overrepresented, providing valuable insights into the underlying molecular mechanisms of tumour cells (Fig. 1B–D). Genes were selected for RT-qPCR analysis in 21 HR + /HER2- metastatic BC patients (cohort 1), primarily based on their biological functions and published literature, as well as statistical criteria such as p-value and fold change. Additionally, genes related to the CCND1-CDK4/6 axis were included due to their specific relevance to this pathway (Table S1).

At baseline (visit 1), non responder patients showed significantly greater expression of the *PRKCB* (*p-value*: 0.011), *MAPK3* (*p-value*: 0.006) and *STAT3* (*p-value*: 0.008) genes in CTCs than responder patients (Fig. 1E), while these latter patients (n = 16) had greater expression of *CDK6* (*p-value*: 0.011) and *CCND1* (*p-value*: 0.035) (Fig. 1E). Likewise, compared with those from non responder patients, CTCs from long-responder patients had higher *CDK4* gene expression (*p-value*: 0.02) (Figure S2A). Differences in CTC gene expression were also detected when the responder group was stratified according to PFS (initial and long-responder patients). Thus, the initial-responder group had a greater expression of *CCND1* (*p-value*: 0.04) and lower expression of *DUSP5* (*p-value*: 0.01) than did the long-responder group (Figure S2B-C).

CTC expression after one cycle of therapy (visit 2) also showed variations based on the patient´s therapy response. High CTC expression of *CUL1* (*p-value*: 0.03) and *CDKN1C* (*p-value*: 0.03) and low expression of *CDH1* (*p-value*: 0.01) was linked to progression within 180 days (Fig. 1F). No significant differences were found when the responder group was stratified into initial responders and long responders.

To further investigate whether elevated *STAT3* expression, observed in CTCs from non-responder patients, affects sensitivity to CDK4/6i *in vitro*, we used the luminal breast cancer cell line MCF7 as a model. *STAT3* expression was first induced in MCF7 cells using IL-6 (Figure S3), generating the MCF7$^{\Delta STAT-3}$ cell line. Both MCF7 wild type (wt) and MCF7$^{\Delta STAT3}$ cells were then treated with Palbociclib (1 μM), Letrozole (20 μM) or a combination of both (polytherapy, PT) for 48 hours, and the effect of the different therapies on the cell viability of both lines was evaluated. It was observed that the induction of STAT3 in MCF7 cells resulted in reduced sensitivity to the CDK4/6 inhibitor, both alone and in combination with ET, with higher cell viability compared to the wild-type cells (*p-value* = 0.028) (Figure 1G, Figure S3C).

The combined expression of *STAT3, PRKCB* and *CDK6* predicts the response to CDK4/6i plus ET

To test whether CTC gene expression can be used to classify patients according to CDK4/6i plus ET response, a receiver operating characteristic (ROC) analysis was performed considering those genes that were differentially expressed between responder and non responder patients at baseline (visit 1). The individual predictive performance of the differentially expressed genes identified (*STAT3, PRKCB, MAPK3, CDK6, CDK4* or *CCND1*)

was optimal, as the area under the curve (AUC) exceeded 0.8 (except for that of *CDK4,* which was 0.78), indicating its ability to distinguish between responder and non responder patients in cohort 1 (Table S5). After testing different gene combinations, the model that perfectly separated the patient group was based on the *STAT3*^{high}, *PRKCB*^{high} and *CDK6*^{low} *signature*, with an AUC = 1 (Fig. 2A).

This gene expression signature (*STAT3* ^{high} *PRKCB* ^{high}*CDK6* ^{low}) was further tested in two additional validation cohorts of HR + /HER2- metastatic BC patients treated with CDK4/6i plus ET as first-line treatment (see M&M) to assess the ability of the CTC gene expression signature to predict PFS. The predictive model trained on data from cohort 1 was applied to cohort 2, comprising 12 metastatic HR + /HER2- BC patients. Remarkably, 11 out of 12 patients in cohort 2 were accurately categorized as either responders or non responders, yielding a model success rate of 91.67% (Fig. 2B). The model error was in a patient who was classified as a non responder being an initial responder patient. Cohort 3 was an external validation cohort that included 16 metastatic BC patients from the University Hospital of Essen, Germany, whose CTC fraction was isolated using the AdnaTest instead of RosetteSep. In this instance, the logistic regression model utilizing the expression of *STAT3*, *PRKCB*, and *CDK6* in the CTCs of these patients (normalized to B2M) demonstrated a robust discriminative ability, achieving an AUC of 0.841 for stratifying patients as either responders or non responders (Fig. 2C). Thus, 81.25% of the patients analysed were accurately classified, with a positive predictive value (PPV) of 80% and a negative predictive value (NPV) of 83.33% (Fig. 2D). In summary, this transcriptional CTC signature (*STAT3*, *PRKCB* and *CDK6*) classifies HR + /HER2- metastatic BC patients as either responders or non responders to CDK4/6i plus ET regardless of the CTC isolation method used.

Gene expression analysis of CTCs at disease progression reveals key resistance biomarkers

To elucidate the resistance mechanisms against CDK4/6i combined with ET, CTC gene expression analysis at the time of progressive disease, proven by radiological imaging (visit 3), was performed on paired blood samples from patients diagnosed with HR + /HER2- metastatic BC whose sample from baseline (visit 1) was available (n = 16). Consistent with previous methods, a CTC gene expression discovery study was first performed using NanoString nCounter technology (n = 6). DGE analysis revealed that a total of 33 genes were differentially expressed at disease progression compared with the expression in responder patients at visit 1 (Fig. 3A and Table S4). The GO enrichment analysis revealed a greater transcript count of genes associated with multiple pivotal pathways, such as the PI3K-Akt, MAPK, Rap1, Wnt, and mTOR signalling pathways, as well as genes involved in cell cycle regulation, transcriptional misregulation in cancer, or signalling pathways regulating pluripotency of stem cells at visit 3 (Fig. 3B), which underscores the complex interplay regulatory networks implicated in therapy resistance. RT−qPCR analysis was conducted on 16 paired samples from visits 1 and 3 (Table S4). At disease progression, the expression of *BAX* (*p-value*: 0.0091), *EZH2* (*p-value*: 0.0033), *HDAC6* (*p-value*: 0.017), *PLAU* (*p-value*: 0.028), and *CDC7* (*p-value*: 0.0069) was greater than that in the matched samples at visit 1, while a lower expression of *NFKBIα* (*p-value*: 0.00089) (Fig. 3C) was detected at visit 3 compared to the matched visit 1. The later analysis was repeated and included four unpaired samples at visit 3 (n = 20 HR + /HER2- metastatic BC patients). Similar results were obtained when performing non-paired analysis, which considered median values, with increased expression levels of *BAX* (*p-value*: 0.008), *EZH2* (*p-value*: 0.000024), *HDAC6* (*p-value*: 0.00017), *PLAU* (*p-value*: 0.0014), and *CDC7* (*p-value*: 0.00010) and decreased expression of *NFKBIα* (*p-value*: 0.000026) at visit 3 compared to visit 1 (data not shown). Notably, *RELA* (*p-value*: 0.019), *HDAC4* (*p-value*: 0.026) and *SNAIL1* (*p-value*: 0.01) expression in CTCs was significantly altered between visits 1 and 3 (data not shown).

These resistance-related genes were further analysed in two CDK4/6i-resistant luminal breast cancer cell lines, MCF7 resistant to palbociclib and T47D resistant to ribociclib (Supplementary material). As depicted in Fig. 3D, the vast majority of genes whose expression increased during disease progression, such as *BAX, EZH2, HDAC6* and *CDC7,* exhibited increased expression in MCF7-resistant cells compared with the parental cell line. Notably, the expression of *STAT3* and *MAPK3* and other genes related to the *CCND1-CDK4/6* axis, INK family inhibitors or other cyclins that can overcome *CCND1*-CDK4/6 dependence (*CDKN2C, CCND1 and CCNE2)* and *PALB2* was enhanced in this tumour-resistant cell line. Contrary to the CTC data, *NFKBIA* expression was also greater in the resistant cell line (Fig. 3D). In the T47D-resistant cell line, a slight increase in *STAT3* and *CDK6* was observed compared with those in the wild-type cells (Figure S4), but no other differences were found.

Longitudinal CTC analysis to identify prognostic biomarkers

CTC gene expression data were used for Kaplan–Meier analysis to assess PFS and OS in cohort 1. Eighteen out of 21 patients progressed, while 8 out of 21 died during the follow-up period. In the survival analysis, CTC

gene expression was categorized as high or low according to the median CTC gene expression value.

Before starting therapy (visit 1), high expression of *CDK4* in CTCs was associated with longer PFS (830 days vs 206 days, *p-value*: 0.0005 according to the log-rank test) (Fig. 4A). Moreover, the potential for discriminating patients with worse PFS was enhanced when CDK4 was combined with *STAT3 (CDK4^{low}STAT3^{high})* (141 days vs 739 days, *p-value*: < 0.0001 by log-rank test). Similarly, the genomic signature identified previously (*STAT3*^{high}*PRKCB*^{high}*CDK6*^{low}) also demonstrated prognostic value (123 days vs 719 days, *p-value*: 0.02 by log-rank test) (Figure S5) in these patients. High expression of *DUSP5* was associated with longer PFS (788 days vs 243 days, *p-value*: 0.01 according to the log-rank test) (Fig. 4B). Likewise, high levels of *PALB2* were associated with longer OS (undefined vs 899 days, *p-value*: 0.03 by log-rank test) (Figure S5). After one cycle of therapy (visit 2), low *CDKN1C* expression was associated with longer PFS (166 days vs 720 days, *p-value*: 0.02 according to the log-rank test) (Fig. 4C). At the time of disease progression, high expression of *STAT3* in CTCs was associated with shorter OS (296 vs 788 days, *p-value*: 0.02 by log-rank test) (Fig. 4D). The significance of various CTC biomarkers in outcome prediction is outlined in Table S6, highlighting the notable involvement of cell cycle regulators.

The patient´s outcome was not dependent on clinical parameters. Thus, the patient's age at diagnosis of stage IV disease, subtype, tumour type (ductal or lobular), and the type of CDK4/6i or endocrine therapy were not significant for PFS or OS ($p > 0.05$). Additionally, the presence of de novo metastasis, as well as the number and location of metastases did not show a significant impact on PFS or OS. Having metastasis in both visceral and bone locations showed a trend towards a shorter OS compared with having only one location affected (*p-value* = 0.08, log-rank test). Hence, clinical data were not considered for subsequent Cox regression analysis. Neither association was found between CTC gene expression and clinical data.

CTC presence predicts patient outcomes after one cycle of therapy

CTCs were counted with the CellSearch® system at three-time points in 12 patients from cohort 1. At visit 1, nine patients (75%) had ≥ 1 CTC, and 5 patients (41, 67%) had ≥ 5 CTCs, respectively. After one cycle of therapy, six patients (50%) had ≥ 1 CTC and only one patient had ≥ 5 CTCs (8.33%), respectively. At the time of disease progression, five patients (71.43%) had ≥ 1 CTC, and three patients (42.86%) had ≥ 5 CTCs.

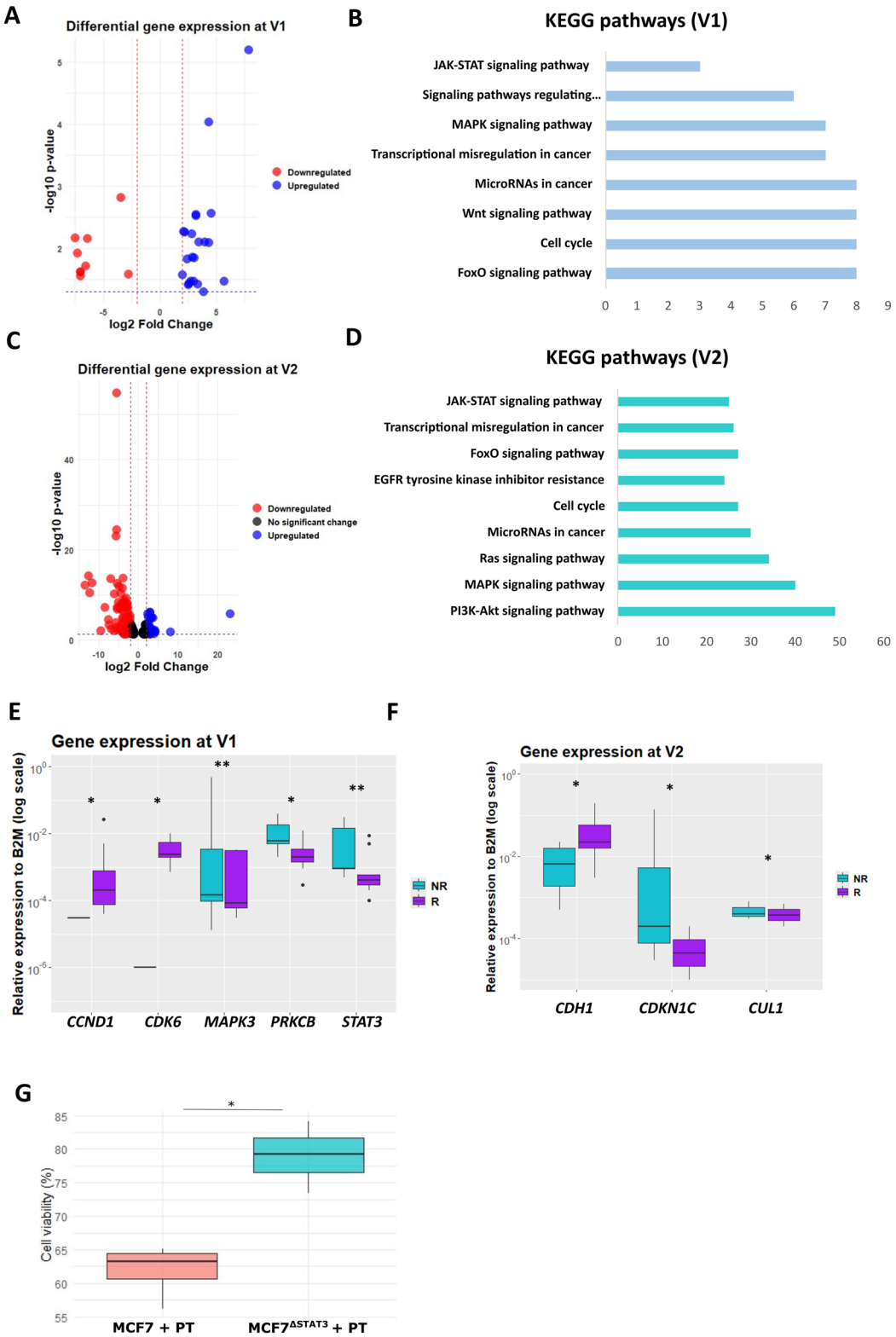
The number of CTCs after one cycle of therapy is associated with patient prognosis. Patients with ≥ 1 CTC had shorter PFS (224 vs 1018 days, *p-value*: 0.01 by log-rank test) (Fig. 5A) and OS (undefined vs 922 days, *p-value*: 0.006 by log-rank test) (Fig. 5B). CTC levels were not associated with clinical parameters or CTC gene expression.

CTC gene expression patterns and longitudinal associations

RT–qPCR analysis of genes related to epithelial (*CDH1*), mesenchymal (*SNAIL1, VIM, PLS3*), stem cell (*ALDH1A1, PROM1*), and tumour markers (*PALB2*) was conducted in 16 patients from cohort 1 longitudinally. CTCs displayed mixed phenotypes with epithelial–mesenchymal and stemness features. Notably, PLS3 expression increased significantly at visit 3 (*p-value*: 0.03). These genes were not associated with a therapeutic response except for *CDH1* at visit 2, as previously mentioned. Notably, *CDH1* showed stable expression across visits and was present in almost all patients and visits (except for five out of 58 samples). Similarly, the protein expression of E-CAD, which is encoded by *CDH1*, was detected in 17 out of 19 tumour tissue samples. The two E-CAD-negative patients identified through immunohistochemical analysis exhibited lobular features. In addition, the longitudinal analysis of the genes analysed in the previous sections revealed significantly lower expression of *CASP8* (*p-value*: 0.014), *CDKN1C* (*p-value*: 0.001), *CUL1* (*p-value*: 0.01), *NFKBIA* (*p-value*: 0.026), and *Snail1* (*p-value*: 0.003) at V2 than at V1.

(See figure on next page.)

Fig. 1 CTC gene expression analysis. **A**, **C** Volcano plot of significantly differentially expressed genes based on nCounter analysis between non responder and responder patients at baseline (visit 1) (**A**) and after one-cycle therapy (visit 2) (**C**). Responder patients composed the reference group. Downregulated genes are depicted in red, while upregulated genes are depicted in blue. **B**, **D** GO analysis: altered KEGG pathways after Differential Gene Expression (DGE) analysis at visit 1 (**B**) and visit 2 (**D**). **E** Normalized gene expression in CTCs of the depicted genes from non responder (NR, n = 5) and responder (R, n = 16) patients analysed by RT–qPCR at visit 1. Normalization was performed with the reference gene *B2M* and with the matched PBMC sample. **F** Normalized gene expression in CTCs of the depicted genes from non responder (NR, n = 5) and responder (R, n = 16) patients analysed by RT–qPCR at visit 2. CTC gene expression was normalized to that of *B2M* and matched PBMCs. Black dots represent outlier values. **G** Effect of the Polytherapy (PT: Palbociclib plus Letrozole) on the cell viability of MCF7 and MCF7$^{\Delta STAT-3}$ cells (induced for 8 days with IL-6 at 20 ng/mL) (n = 4). Statistics were performed by the Wilcoxon rank sum test (*p-value* < 0.05 (*), < 0.01 (**) and < 0.001 (***))



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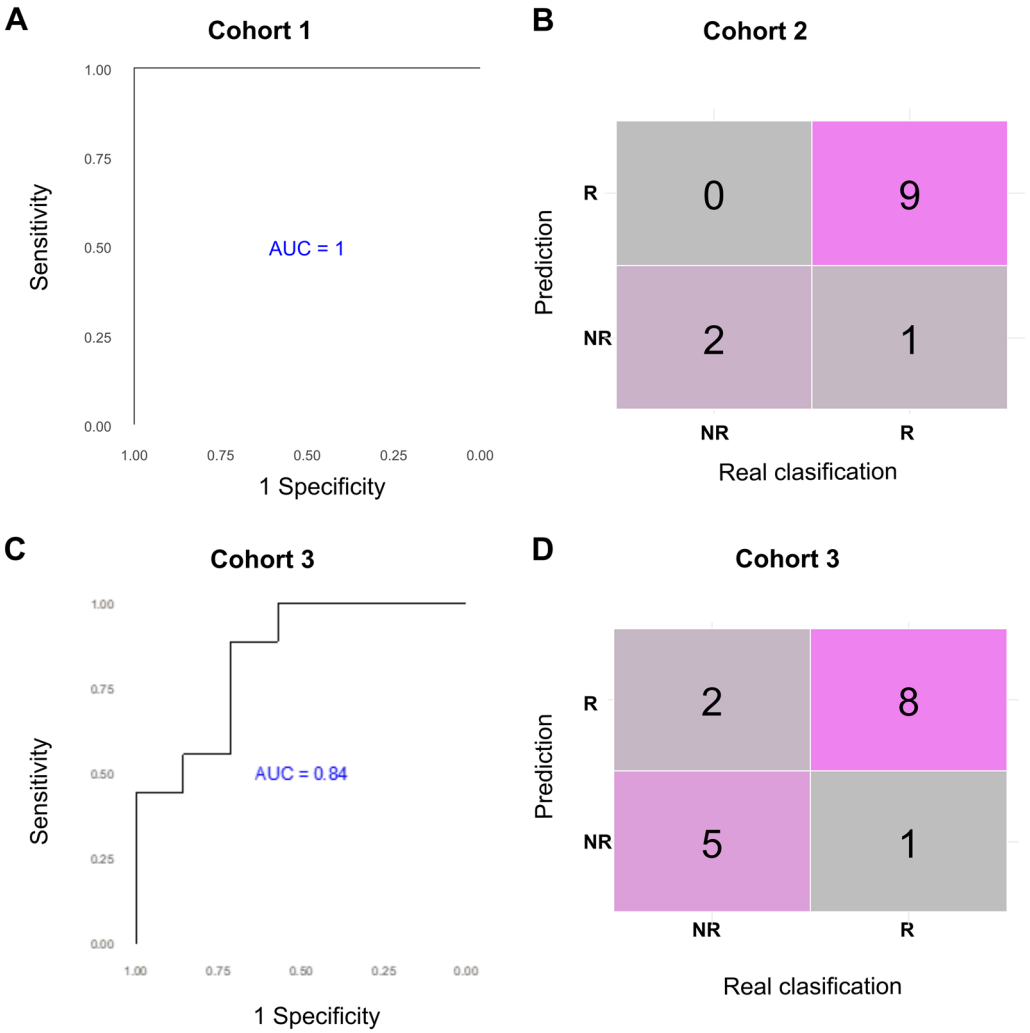

Fig. 2 CTC gene expression signature, including *STAT3, PRKCB,* and *CDK6,* effectively stratify patients treated with CDK4/6 inhibitors plus endocrine therapy into responders and non responders. **A** Receiver operating characteristic (ROC) curve for the signature including the indicated genes at visit 1 to classify patients' therapy response, achieving an AUC of one for cohort 1 (n = 21, 5 non responders vs 16 responders). **B** Confusion matrix showing the accuracy of the prediction model for cohort 2 (n = 12, one non responder vs 11 responders). **C** ROC curve for the same signature, which successfully categorized patients according to their therapeutic response at visit 1 for cohort 3 with an AUC of 0.84 (n = 16, seven non responders vs nine responders). **D** Confusion matrix showing the accuracy of the prediction model for cohort 3 (n = 16, seven non responders vs nine responders)

Contingency analysis, considering the median value as a cut-off to define high/low expression levels, revealed that high *SNAIL1* was associated with high *VIM* at visits 1 and 3 (*p-value*: 0.008 and *p-value*: 0.00025, respectively) (Table 2). Significant associations were found between *PI3KCG* and *JAK2* (*p-value*: 0.0003), between *PI3KCG* and *CUL1* (*p-value*: 0.004), and between *STAT3* and *PRKCB* (*p-value*: 0.008), with a strong correlation between *STAT3* and *PRKCB* (*p-value*: $5.7 \times 10–6$, $R^2 = 0.97$). Positive associations were also detected between *ALDH1A1* and *PLS3* (*p-value*: 0.02), between *CDK4* and *CDK6* (*p-value*: 0.008), between *CDK4* and low *CDKN1C* after therapy (*p-value*: 0.03), and between *CCND1* and *CDKN2C* (p-value: 0.04). During disease progression, *STAT3* correlated with *PRKCB*, *JAK2*, and *ALDH1A1* (all, *p-values*: 0.005), and *BAX* correlated with *CASP8* (*p-value*: 0.003). Additionally, *CDK4* and *CDK6* showed a near-significant association (p value: 0.06).

Correlation between CTC gene expression and *PIK3CA* mutation status in ctDNA

Clinical guidelines recommend testing HR + /HER2- metastatic BC patients for *PIK3CA* mutations in

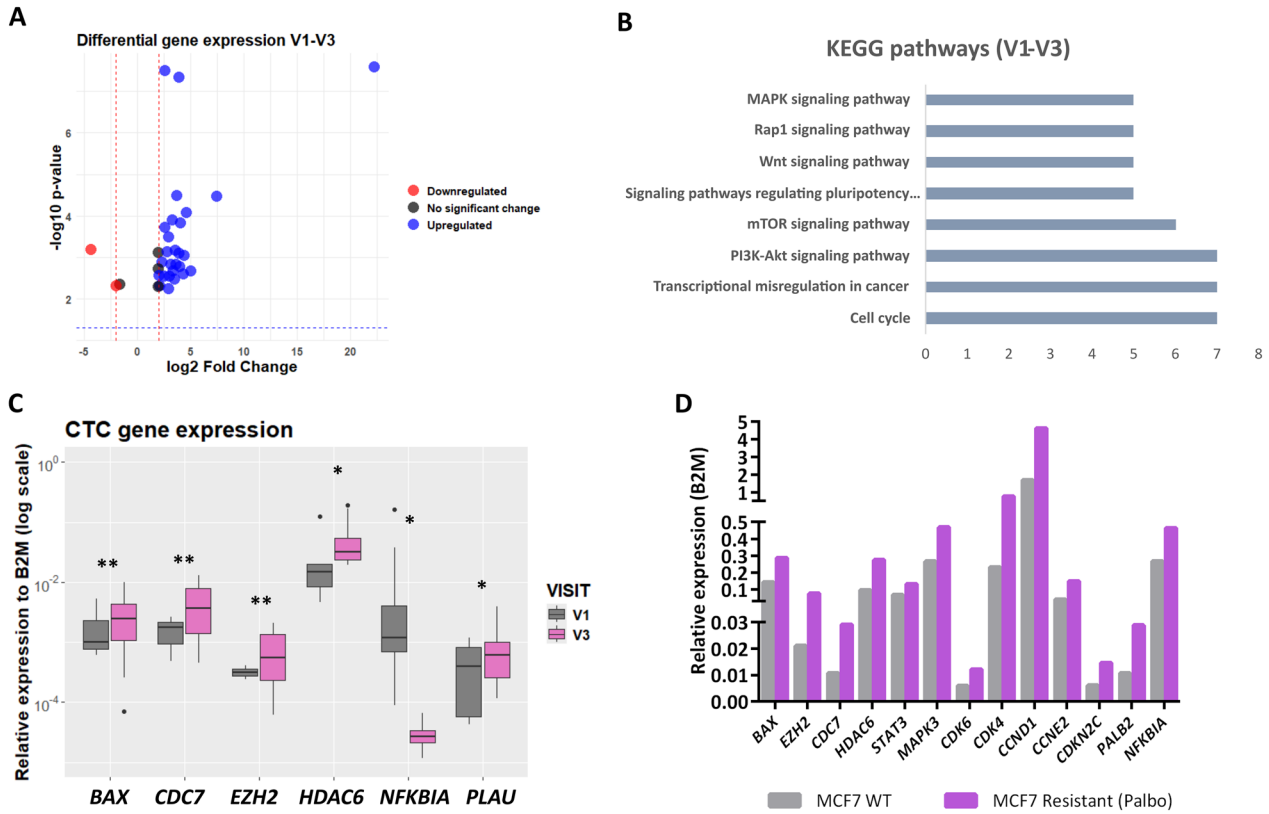

Fig. 3 CTC gene expression analysis. **A** Volcano plot of DEGs at visit 3 after comparison of gene expression with that in matched samples at visit 1. Downregulated genes are depicted in red, while upregulated genes are marked in blue. **B** GO analysis: altered KEGG pathways according to Differential Gene Expression (DGE) analysis comparing visit 3 and visit 1. **C** CTC gene expression of the depicted genes at visit 1 and visit 3 in matched samples of 16 patients analysed by RT–qPCR. CTC gene expression was normalized to that of *B2M* and matched PBMCs. **D** Gene expression of the depicted genes in the MCF7 luminal parental (grey) and palbociclib-resistant (purple) cell lines. Statistics were performed by the Wilcoxon rank-sum or signed-rank test (p value < 0.05 (*), < 0.01 (**) and < 0.001 (***))

tumour tissue or ctDNA upon disease progression under CDK4/6 inhibitors. This recommendation is based on the availability of approved targeted therapies for this population, including alpelisib, inavolisib, and capivasertib. Thus, the presence of the most common *PIK3CA* mutations (p.E542K, p.E545K, p.H1047L and p.H1047R) was studied in the ctDNA of patients from cohort 1 (n = 21). *PIK3CA* mutations were detected in 6/21 patients at visit 1 and 10/21 patients at visit 3, representing 47.62% of patients. However, *PIK3CA* mutational status was not associated with patient outcomes.

Subsequently, the associations between the presence of *PIK3CA* mutations in ctDNA and gene expression in CTCs were investigated. No association was found in samples collected at visit 1; however, patients with *PIK3CA* mutations at visit 3 had lower expression of *BAX* (*p-value*: 0.04), *CASP8* (*p-value*: 0.029), *CUL1* (*p-value*: 0.0095) and *RAC2* (*p-value*: 0.017) at the same time point in their CTCs (Figure S6).

Discussion

The approval of CDK4/6i plus ET as a first-line treatment for HR + /HER2- metastatic BC patients led to increased PFS with manageable adverse events [21]. Nevertheless, 20% of patients have intrinsic resistance, and therapy resistance eventually occurs in every patient [10]. To date, no biomarkers for CDK4/6i therapeutic response have been identified, which is one of the current challenges in BC oncology [22]. Most metastatic breast cancer patients have gone through surgery to remove the primary tumour and to biopsy the metastasis is not always feasible. Since liquid biopsy analysis is an attractive alternative for therapy monitoring in the metastatic setting, this study aimed to longitudinally analyse CTCs to identify biomarkers to tailor HR + /HER2- metastatic BC patient therapy, which has scarcely been studied in this clinical context.

Our investigation revealed for the first time that the nCounter assay is useful for assessing the gene expression profile of CTCs from BC samples, like previously

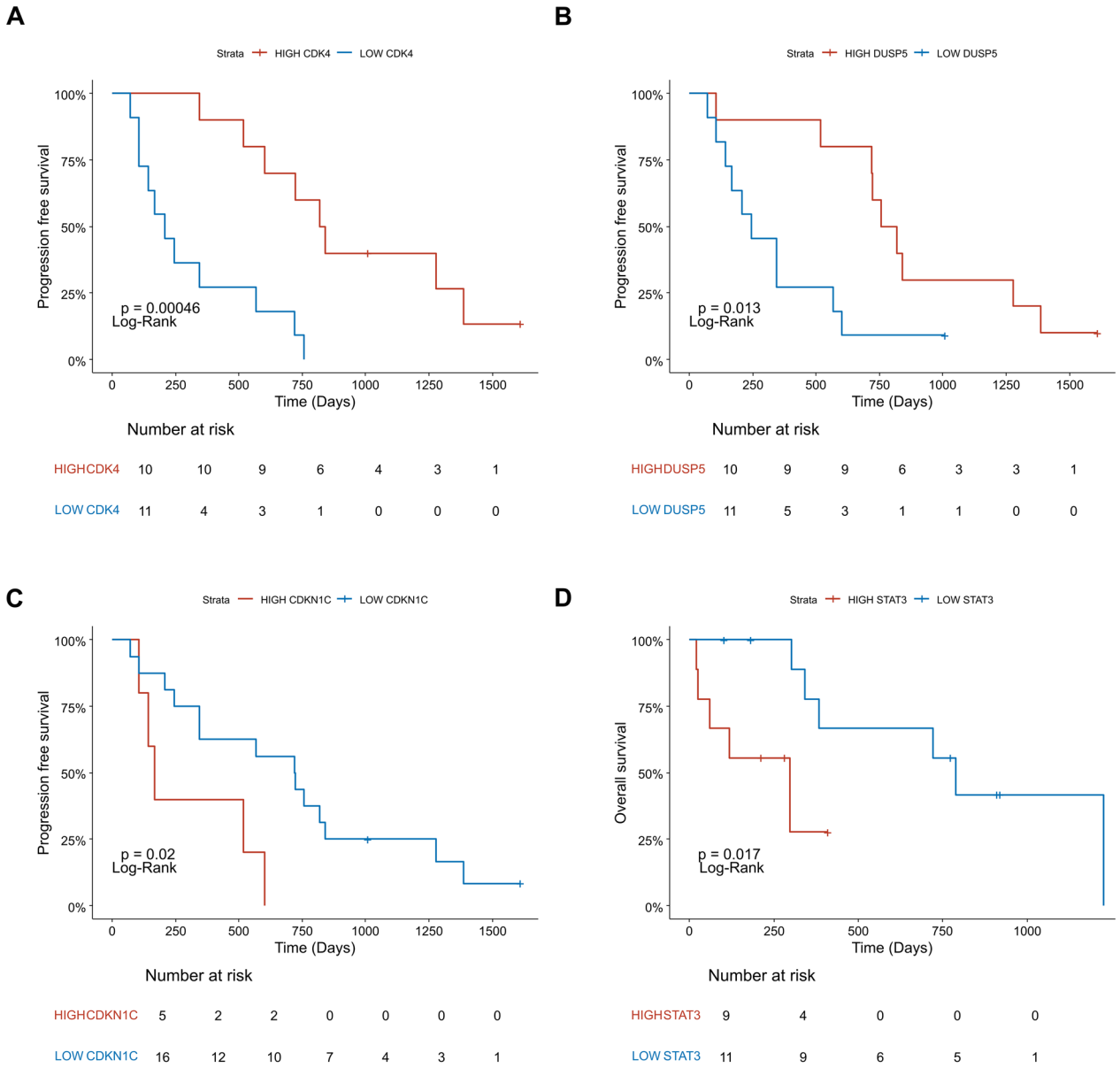

Fig. 4 CTC gene expression was associated with patient outcomes in cohort 1 (n = 21). Kaplan–Meier plot for progression-free survival according to **A** CTC *CDK4* expression at visit 1, **B** CTC *DUSP5* expression at visit 1, and **C** CTC *CDKN1C* expression at visit 2. **D** Kaplan–Meier plot representing overall survival based on *STAT3* expression at visit 3. Statistics were performed by the log-rank test. Stratification was performed considering the median value of expression for each gene to define high/low expression levels

reported observations of CTCs from hepatocellular carcinoma and prostate cancer [23][24]. This discovery step led to the validation of a set of DEGs between non responders and responders patients. At the time of diagnosis of metastatic disease and before therapy initiation, compared with responder patients, non responder patients had higher gene expression of *STAT3, PRKCB* and *MAPK3* and lower expression of *CDK6* and/or *CCND1*. The expression of these individual markers can discriminate patients according to their therapeutic response, but the $STAT3^{high}PRKCB^{high}CDK6^{low}$ signature can classify HR + /HER2- metastatic BC patients according to their therapeutic response, regardless of the CTC isolation method used.

STAT3, a powerful proto-oncogene involved in BC development [25][26], is the gene expressed in CTCs that has the greatest AUC for discriminating responders from non responders patients. In in vivo BC models,

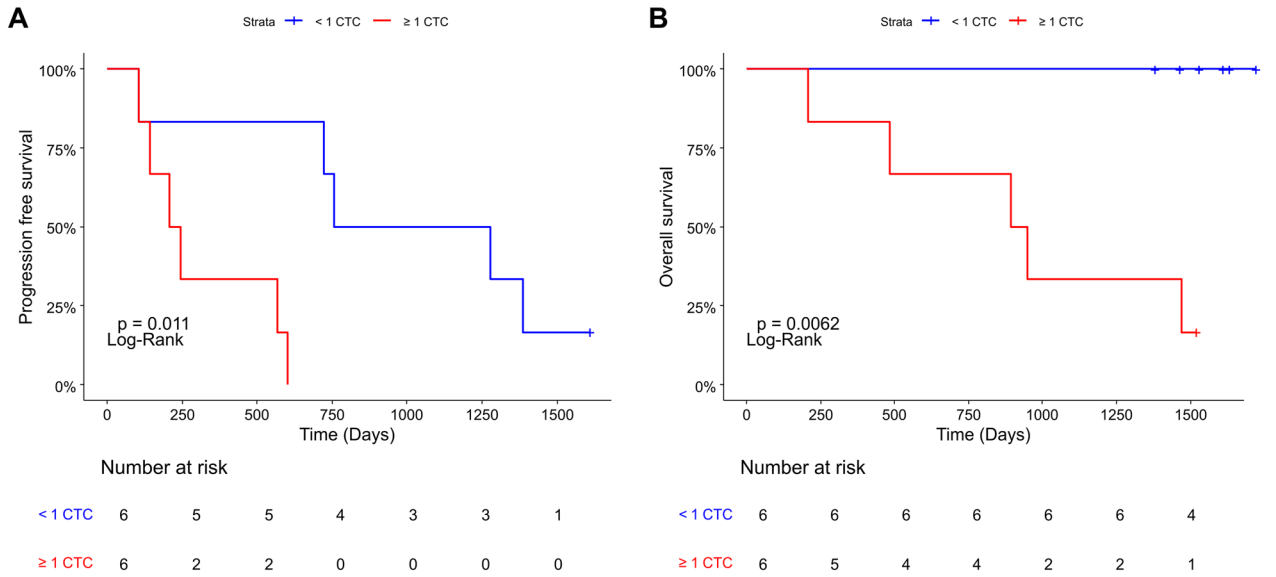

Fig. 5 CTC enumeration by CellSearch was associated with patient outcomes. Kaplan–Meier plot for PFS (**A**) and OS (**B**) stratified by the presence of ≥ 1 CTC after one cycle of therapy (n = 12). Statistics were performed by the log-rank test

Table 2 Contingency analysis for gene expression association (Fisher exact test)

Gene 1	Gene 2	Visit	Association type	*p-value*
SNAIL1	*VIM*	V1	Positive	0.008
PI3KCG	*JAK2*	V1	Positive	0.000
PI3KCG	*CUL1*	V1	Positive	0.004
STAT3	*PRKCB*	V1	Positive	0.008
ALDH1A1	*PLS3*	V1	Positive	0.020
CDK4	*CDK6*	V1	Positive	0.008
CDK4	*CDKN1C*	V2	Negative	0.030
STAT3	*MAPK3*	V2	Positive	0.003
STAT3	*PRKCB*	V3	Positive	0.049
STAT3	*JAK2*	V3	Positive	0.005
STAT3	*ALDH1A1*	V3	Positive	0.005
BAX	*CASP8*	V3	Positive	0.003
CDK4	*CDK6*	V3	Positive	0.060
SNAIL1	*VIM*	V3	Positive	0.000

STAT3 expression in the primary tumour correlated with the cfDNA and CTC levels in the peripheral blood [27]. Due to the lack of paired samples from primary tissue, we cannot compare our data. Nevertheless, *STAT3* expression in CTCs has a potential prognostic role in this patient cohort. Besides, we observed that the induction of STAT3 in a luminal cell line diminished the effect of Palbociclib, regardless of whether it was administered as part of combination therapy with anti-estrogen treatment. This supports the findings observed in patient-derived CTCs. It has also been reported that the IL6/STAT3 signalling pathway is activated upon resistance to CDK4/6is [28, 29]. Currently, research is underway to explore the efficacy of STAT3 inhibitors in reversing resistance to CDK4/6is and/or ET in metastatic HR + / HER2- BC patients [30–32]. If clinical trial outcomes prove favourable, *STAT3* expression in CTCs for therapy selection in these patients should be extended to the time point before starting therapy. Furthermore, additional in vitro studies exploring how differential expression of markers such as *PRKCB* and *CDK6* may modulate sensitivity to CDK4/6i therapy may be of great interest.

In agreement with our findings, *MAPK3* activation not only generates endocrine resistance but also CDK4/6i resistance [33]. Similarly, its inhibitor, *DUSP5*, is associated with a longer response to CDK4/6i plus ET. A lack of *DUSP5* expression can cause *MAPK3* activation, which is especially prevalent in non-responder patients. Our contingency analysis revealed a contrasting expression pattern between these two genes. Consequently, the expression of both genes should be considered when determining therapy sensitivity and cancer progression. The combination of CDK4/6i with MAPK inhibitors regulates transcription and blocks cell cycle progression in other tumour types [34, 35]. Thus, further exploration of therapeutic interventions targeting *DUSP5* and *MAPK* is required to identify potential alternatives to prevent CDK4/6i resistance in BC.

In our study, the increased expression of genes involved in the CCND1-CDK4/6 axis, such as *CDK4* and *CDK6*, in CTCs was linked to increased PFS and is a potential biomarker for guiding therapy selection. In

the PALOMA-3 trial, the presence of mutations in *CDK4* and *CDK6* kinases in the ctDNA was not informative of the therapeutic response of HR + metastatic BC patients [36], but in the PALOMA-3 trial, no CTC information nor gene expression was considered. Although our work cannot directly compare the data with those of previous studies due to the lack of similar research, our results align with findings from published primary tumours or cell lines [37], suggesting that the efficacy of CDK4/6i therapy depends on the availability of drug targets. Our findings may initially seem contradictory to prior studies that have associated elevated CDK6 expression with resistance to CDK4/6i, particularly in the context of FAT1 loss, as reported by Li et al. [38]. However, it is important to note that the mechanism underlying CDK6 overexpression in our study is likely different since the tumor context differs (CTCs vs. primary tumors or cell lines), suggesting a context dependence. While elevated CDK6 may indicate resistance in the presence of specific mutations, in other settings, it may reflect the availability of the drug target. Notably, high gene expression of the tumour suppressor gene *CDKN1C* in CTCs is associated with a lack of response to CDK4/6i plus ET and shorter PFS after one cycle of therapy. Overexpression of INK genes prevents the binding of CDK4/6i to *CDK4/6* [37], which could also explain our observation in *CDKN1C*.

In contrast to published findings, we found that high levels of *PALB2* transcripts in CTCs were associated with longer OS. High *PALB2* expression in tumour tissue as well as in CTCs from metastatic BC patients correlated with worse outcomes [39][14], but these studies did not consider CDK4/6i therapy. Thus, the role of *PALB2* in determining BC prognosis is dependent on the therapeutic regimen, and its expression could correlate with CDK4/6i efficacy, which has not been described thus far. Given the above, despite the potential clinical relevance of CTC expression data, more research is needed on the protein activation of these pathways.

In this study, the percentage of patients with ≥ 5 CTCs detected by CellSearch (over 40%) was lower than that described in the literature (50–70%) [40]. This discrepancy could be explained by the small patient cohort analysed, which was composed of 12 individuals. Despite the latter, after one cycle of therapy, the presence of ≥ 1 CTC predicted shorter PFS and OS, while having ≥ 5 CTCs had no significant impact. Similarly, Galardi et al. reported the value of CTC enumeration for monitoring CDK4/6i therapy [41].

CTC gene expression analysis at the time of radiological progression also revealed resistance-related genes that were mainly involved in the NF-kappa B signalling pathway (*PLAU, RELA* and *NFKBIA*), apoptosis (*BAX, RELA and NFKBIA*), cell division (*CDC7)* or epigenetic regulation (*EZH2, HDAC6* and *HDAC4).* The expression of these genes was also analysed in two CDK4/6i-resistant in vitro models. MCF7-resistant cells better reflect our patient cohort due to their concordance with the ER status, mostly ER + , and the therapy received, mainly palbociclib. The lack of consistency in results between MCF7 and T47D cells could be attributed to inherent biological differences between the two lines, such as variations in receptor expression, genetic mutations, and their distinct molecular profiles. These differences may influence their response to different CDK4/6 inhibitors in vitro. In this study, cell cycle inactivation by CDK4/6i was overcome through the expression of *CDC7* in patients with acquired resistance. *CDC7* is a gene that has a redundant function in DNA replication [42], and targeted therapies have been developed [43]. *CDC7* inhibition could be a promising clinical strategy when CDK4/6i resistance appears.

Regarding other differentially expressed genes, we observed that *EZH2* and *HDACs*, two epigenetic regulators, had increased expression during disease progression. *EZH2* is a histone methyltransferase that has been linked to BC progression and metastasis [44–47]. Notably, Abu-Khalaf et al. reported that a greater percentage of patients with mutations in EZH2 (cfDNA) at the post-treatment stage experienced disease progression than patients who started palbociclib therapy [48]. *HDAC6* is a histone deacetylase regulated by estrogen signalling associated with enhanced cell motility in BC [49]. In the latter study, *HDAC6* expression correlated with acquired resistance, mainly when *PI3K* was mutated. Similarly, the efficacy of combining HDAC6 inhibitors with PI3K inhibitors and palbociclib is being studied to explore novel treatment options for HR + /HER2- BC patients [50–52]. Consistent with prior research [53], our findings indicate that epigenetic modifications serve as promising indicators of treatment efficacy for CDK4/6i and potential targets for personalized therapy. It is important to highlight that the molecular biomarkers associated with acquired resistance in this study were identified in the context of combination therapy with an antiestrogen agent and a CDK4/6 inhibitor. Given the complexity of identifying resistance mechanisms in combination therapies, it remains unclear whether these genes are associated with diminished sensitivity to one or both drugs. Further functional studies to model resistance to estrogen deprivation, CDK4/6 inhibition, and their combination are essential to address these questions. Another gene that is induced at the time of disease progression is *PLAU,* which has also been related to enhancing cell proliferation, migration and EMT in different tumour types [54, 55]. *PLAU* gene expression in CTCs was previously

reported in metastatic BC patient samples [56], and CTC clusters were associated with improved CTC survival in circulation [57]. Thus, *PLAU*-positive CTCs observed at the time of disease progression could represent those CTCs with enhanced survival capacities. Given this comprehensive information, further preclinical research is essential to validate inhibitors developed against these genes as alternative or combination therapies for patients who do not respond to CDK4/6 inhibitors or to delay resistance. A key strength of this study is the analysis of gene expression data matched pre- and post-therapy samples, which allowed for an understanding of CDK4/6i resistance, enhancing its translational relevance.

Notably, PI3K alterations represent one of the predominant mechanisms of resistance in breast cancer. Therefore, a commonly accepted follow-up therapy after CDK4/6i resistance is the administration of PI3K inhibitors in patients with PIK3CA mutations [58, 59]. In our study, no significant association between the *PIK3CA* mutational status and OS was detected, which is in line with the findings of the MONALEESA-7 trial, although this association was detected in the PALOMA-3 and MONALEESA-2 trials [7, 60, 61]. However, a larger cohort is needed to further confirm this result because it is biased towards responder patients in our cohort. The prevalence of *PIK3CA* mutations at the time of progression reported here is similar to what has been reported [62, 63]. Interestingly, we found that the presence of *PIK3CA* mutations in cfDNA is associated with the CTC gene expression landscape. Thus, increased expression of genes involved in apoptosis, together with *CUL1* and *RAC2*, was observed in patients with wild-type *PI3K*. In contrast, *HDAC6* gene expression in CTCs from cohort 1 was increased in patients with acquired resistance and with *PI3K* mutations. Further research is required to fully elucidate the underlying molecular interactions among these genes since crosstalk between these pathways may exacerbate tumour aggressiveness and influence treatment response.

We demonstrated that the gene expression of CTCs reflects the underlying tumour biology and can be useful in the management of metastatic HR+/HER2- BC patients. We also found that longitudinal CTC gene expression analysis revealed a mixed CTC phenotype, suggesting that CTCs can undergo partial EMT, promoting cell invasion and metastasis in BC, as previously reported [64]. In our study, the majority (95%) of patients had ductal carcinoma, showing consistent E-cadherin (*CDH1*) expression in CTCs at various time points and in primary tissue samples, in line with our previous findings [14, 65]. Furthermore, we observed an association between the expression of *CDH1* in CTCs at visit 2 and the response to CDK4/6i plus ET. Previous research has indicated that *CDH1* expression in HR+ ductal carcinomas is a favourable prognostic factor, whereas its loss is correlated with poor prognosis, metastasis, and reduced OS in patients with metastatic BC [66–68]. Therefore, targeting E-cadherin may offer a potential therapeutic avenue for these BC patients [69].

To the best of our knowledge, this longitudinal transcriptomic analysis of CTCs is one of the first reports of HR+/HER2- metastatic BC patients treated with CDK4/6i plus ET (Keup et al., submitted). Our findings elucidate which patients will benefit from CDK4/6i plus ET and shed light on acquired resistance mechanisms to CDK4/6i and ET combination therapy, providing valuable insights for further research in this area, such as investigations of target inhibitors or the epigenetic silencing of metastatic suppressor genes, both at baseline and during disease progression. However, the present study has several limitations, including a small cohort size and potential bias towards patients who responded to palbociclib treatment. The study lacked a control group receiving only endocrine therapy, excluded patients treated with CDK4/6i in later lines, and included a limited number of paired samples. Furthermore, methodological concerns, such as the use of preamplification and lack of variance reporting or limits of detection, are noted. Additionally, the study used the median expression as a cut-off, which may affect generalizability, and the detection of informative markers could be hampered because the discovery analyses used a smaller cohort. Thus, additional studies (preclinical and clinical) are necessary to explore whether the identified biomarkers can be alternative targets for tailored therapy after CDK4/6i plus ET resistance. Nonetheless, our findings suggest a potential predictive and prognostic value for CTC gene expression and enumeration in this emergent patient group.

Conclusions

This study highlights the potential of CTC gene expression profiling as a valuable tool for managing patients with HR+/HER2- metastatic breast cancer treated with CDK4/6i plus endocrine therapy. We identified biomarkers associated with the response and resistance to treatment by analysing CTC gene expression. Despite the limitations of this study, the use of CTCs to predict treatment outcomes and understand the mechanisms of resistance is promising. Future research should aim to validate these findings in larger cohorts and explore targeted therapies based on the identified biomarkers to improve personalized treatment strategies in metastatic breast cancer patients.

Abbreviations

AUC Area Under the Curve

BC	Breast Cancer
CDK4/6i	Cyclin-Dependent Kinase 4/6 Inhibitors
cfDNA	Cell-Free DNA
ctDNA	Circulating tumour DNA
CTC	Circulating Tumour Cells
ddPCR	Droplet Digital PCR
DGE	Differential Gene Expression analysis
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylene diamine tetraacetic acid
EMA	European Medicines Agency
EMT	Epithelial-Mesenchymal Transition
ER	Estrogen Receptor
ET	Endocrine Therapy
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
GO	Gene Ontology
HER2	Human Epidermal Growth Factor Receptor 2
HR	Hormonal Receptors
IHC	Immunohistochemistry
KEGG	Kyoto Encyclopedia of Genes and Genomes
NPV	Negative Predictive Value
NR	Non-Responder
OS	Overall Survival
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PFS	Progression-Free Survival
PPV	Positive Predictive Value
PR	Progesterone Receptor
PT	Primary tumour
R	Responder
ROC	Receiver Operating Characteristic
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse Transcription Quantitative Polymerase
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06374-w.

Supplementary material 1

Supplementary material 2

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Author contributions
C.C. and R.L. were responsible for the conceptualization. Methodology was performed by M.G.C., C.Y., C.A., C.K., MA, LP, R.L.L., L.M.R. and C.C. Formal analysis, visualization and writing-original draft preparation were carried out by M.G.C., and C.C. Resources were provided by C.A., C.K., P.P., T.C., J.C., C.R., M.C., and T.G.C. Writing-review & editing were undertaken by M.G.C., C.K., L. M.R., J.C., T.G.C. and C.C. Supervision and funding acquisition were managed by R.L. and C.C.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethics approval and consent to participate
All procedures followed the Helsinki Declaration guidelines and were approved by two separate ethics committees (the Ethics Committee of Galicia under approval reference number 2015/772 and the Ethics Commission of the University Duisburg-Essen under approval reference number 12-5265-BO). All patients provided written informed consent.

Consent for publication
Not applicable. No individual person's data were included.

Competing interests
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