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Review

# Liquid Biopsy: a new tool for overcoming CDKi resistance mechanisms in luminal metastatic breast cancer

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**Abstract:** Breast cancer (BC) is the most common cancer diagnosed in women worldwide. Approximately 70% of BC patients have the luminal subtype, which expresses hormone receptors (HR+). Adjuvant endocrine treatments are the standard of care for HR+/HER2- BC patients. Over time, approximately 30% of those patients develop endocrine resistance and metastatic disease. Cyclin-dependent kinase inhibitors (CDKi) in combination with an aromatase inhibitor or fulvestrant have demonstrated superior efficacies in increasing progression-free survival, with a safe toxicity profile, in HR+/HER2- metastatic BC patients. CDKi blocks kinases 4/6, preventing G1/S cell cycle transition. However, not all patients respond to CDKi, and those who do respond ultimately develop resistance to the combined therapy. Studies in tumour tissues and cell lines have tried to elucidate the mechanisms underlying this progression, but there are still no conclusive data. Over the last few years, liquid biopsy has contributed relevant information. Circulating tumour materials are potential prognostic markers for determining patient prognosis in metastatic luminal BC, for monitoring disease and for treatment selection. This review outlines the different studies performed using liquid biopsy in patients with HR+ metastatic BC treated with CDKi plus endocrine therapy. We focus mainly on those studies that describe possible resistance mechanisms in circulating tumour-derived material.

**Keywords:** breast cancer; CDK inhibitors; liquid biopsy; resistance mechanisms; therapy

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## 1. Introduction

Breast cancer (BC) has the highest incidence rate and is the second greatest cause of deaths due to cancer among women worldwide, mainly due to metastasis. The five-year prevalence is near 8 million worldwide [1]. The luminal subtype represents approximately 70% of the cases and is characterised by the expression of oestrogen and/or progesterone hormone receptors (HR) [2]. Most of the patients diagnosed with primary luminal BC are treated with adjuvant endocrine therapy (ET), such as tamoxifen, anastrozole, letrozole or exemestane, until surgery and/or radiotherapy to block the hormone receptor or to inhibit oestrogen production [3]. However, patients develop endocrine resistance within 2–3 years, on average. In some studies, it was estimated that 30% of patients would develop metastasis, while 6% were in metastatic stages at the time of diagnosis [4, 5].

Different mechanisms of endocrine resistance have been identified, such as the up-regulation of cyclins, cyclin-dependent kinases (CDKs) and mitogen-signalling pathways (PI3K and RAS); a reduction in CDK-inhibitory proteins (p16, p21 and p27); mutations or the loss of *ESR1*; and epigenetic alterations [6]. CDKs act downstream of oestrogen signalling, controlling cell cycle progression. As these proteins are normally altered in breast

cancer, they have been considered a key target for therapeutic intervention in metastatic settings [5].

The Food and Drug Administration (FDA) and The European Medicines Agency (EMA) approved the combination of cyclin-dependent kinase inhibitors (CDKi) with ET to treat advanced luminal BC. There are currently three cyclin inhibitors on the market (palbociclib, ribociclib and abemaciclib) that target the ATP-binding domain of CDK4 and 6 [7, 8]. Different clinical trials [8–13] have demonstrated the benefits of combined CDK4/6 inhibitors plus ET, such as increased progression-free survival (PFS) and survival rates. Nevertheless, not all patients respond to CDKi, and even those who initially respond ultimately progress. There are factors responsible for endocrine resistance that have not yet been identified [15], which complicates the study of resistance to both therapies. The efforts made mainly using primary tumour tissue samples or cell lines have not produced conclusive results regarding the resistance mechanisms, partly due to tumour heterogeneity [7]. Therefore, a paradigm shift is needed for this emergent drug-resistant patient group. Precision oncology through the analysis of liquid biopsies has emerged as an attractive opportunity for this. Contrary to that in classical oncology, the therapeutic strategy in precision medicine is based on the distinctive molecular characteristics of patients. Thus, the objective is to tailor patient therapy by studying biomarker profiles while reducing the harmful effects on healthy cells. A clear example is BC treatment, where the treatment selection depends on the subtype [16].

In recent years, liquid biopsy has become a tool for elucidating tumour evolution in real time and guiding systemic treatment selection for precision medicine. Moreover, it provides information on the tumour's genomic profile and burden, without invasive procedures. In addition, they can be performed longitudinally whenever needed. Although its analytical and clinical validity is evident, clinical trials that incorporate the analysis of tumour-derived materials such as circulating tumour DNA (ctDNA) or circulating tumour cells (CTCs) are necessary for clinical decision making. For instance, the detection of *PIK3CA* mutations in ctDNA to guide treatment selection is an example of a clinically useful ctDNA assay.

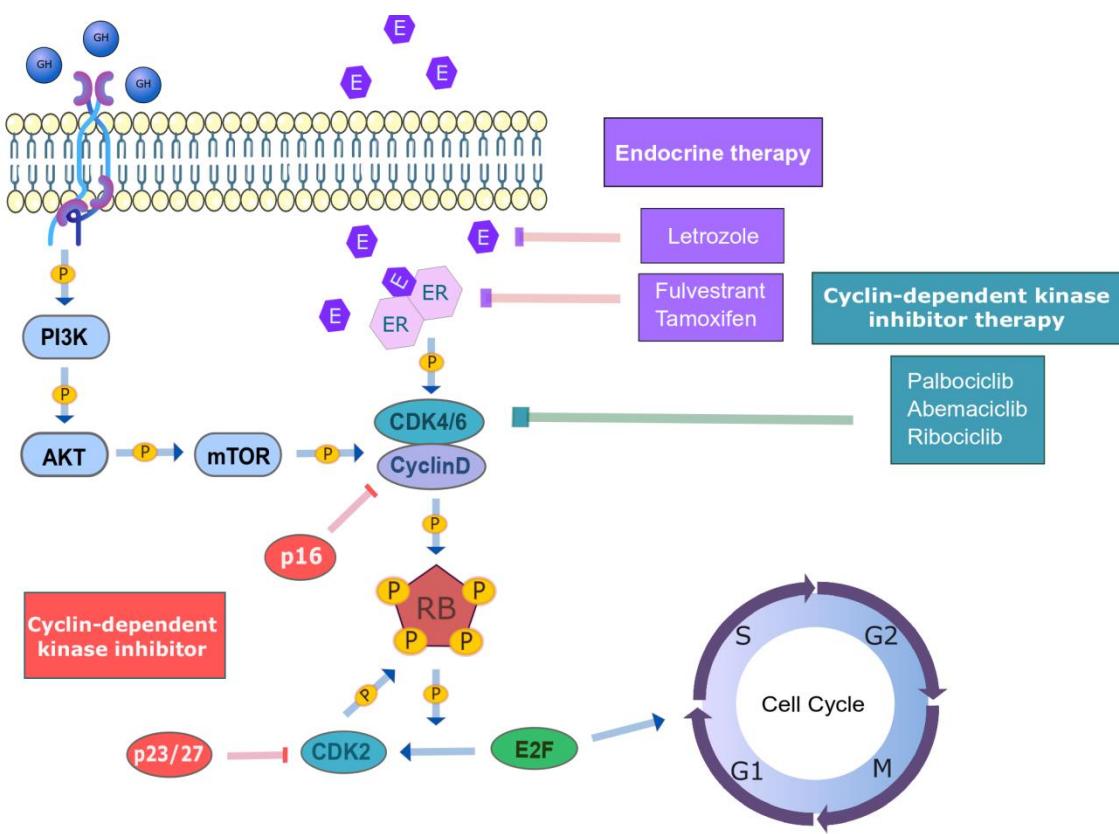
The literature was reviewed to evaluate the use of liquid biopsy, for the analysis of tumour-derived material, to identify predictive biomarkers in HR+/HER2- metastatic BC (mBC) patients treated with CDK4/6 inhibitors plus endocrine therapy.

## 2. Inhibition of Cyclin-Dependent Kinases 4/6 (CDK4/6) in combination with endocrine therapy for HR+/HER2- metastatic Breast Cancer

Cell cycle progression is regulated by cyclin-dependent kinases and cyclins. It has been described that the CCND1-CDK4/6 complex controls the G1/S transition [8, 17], which is normally upregulated in HR+/HER2- BC. Thus, *CCND1* (29% in luminal A and B) and *CDK4* (14% in luminal A and 25% in luminal B) are commonly amplified. The CCND1-CDK4/6 complex phosphorylates the retinoblastoma protein (pRB), a negative regulator of cell cycle progression. The inactivation of RB releases E2F transcription factors, which activate the transcription of genes implicated in DNA replication and cell cycle progression [3, 8, 18, 19] (Figure 1).

Pharmaceutical companies have designed treatments to inhibit CDK4/6 to arrest the cell cycle at G1. The first generation of CDKi was nonspecific, of limited efficacy and affinity, and considerably toxic [5, 8, 20]. Computer-aided drug design is being used to develop CDKi with better potency, selectivity and pharmacological properties, and the spatial structure and inhibition activity of CDKs are also being studied [21, 22]. Palbociclib and ribociclib have more than 100-fold-higher affinities for CDK4/6 than other CDKs, while abemaciclib only has an approximately 6-fold higher affinity. A more profound understanding of molecular differences is necessary for the precise use of this

drugs in clinical settings, although the comparable efficacy of these inhibitors was confirmed by an increase in the PFS independent of the patients' features [23].



**Figure 1. Regulation of the cell cycle in HR+/HER2- mBC patients.** The regulation of the cell cycle is mediated by the CCND1-CDK4/6-RB axis. The CCND1-CDK4/6 complex phosphorylates the RB protein, which releases E2F transcription factors. The latter lead to the G1/S transition of the cell cycle. The cyclin-CDK complexes are, in turn, regulated by other cyclins or intrinsic CDK inhibitors (INK4 and CIP/KIP family members) (in red). The current treatments in HR + mBC are endocrine therapy (in purple) and CDK inhibitors (in green).

It was described that, in mBC patients previously treated with two or more hormonal treatments, CDKi resulted in a higher rate of clinical benefit and PFS than in those patients treated with one hormonal therapy or none. It was also observed that the therapeutic response was independent of the nuclear expression of *RB1*, Ki-67 index, p16 loss or *CCND1* amplification in the tumour tissue. Due to this synergistic effect, several clinical trials were carried out to determine the efficacy of the combined therapy as a first-line treatment in mBC patients [24, 25].

The PALOMA clinical trials (1, 2 and 3) assessed the safety and tolerability of palbociclib plus letrozole or fulvestral as a first-line therapy in HR+/HER2- mBC patients with or without prior treatments. As in previous preclinical studies, a higher clinical benefit rate and PFS were demonstrated in patients treated with the combined therapy than with ET alone or plus placebo [8, 10, 13, 26, 27]. It was also demonstrated that a *CCND1* amplification and/or loss of p16 in the primary tumour did not improve the efficacy of therapy [26].

The MONALEESA trials (2 and 3) assessed the safety and toxicity of ribociclib plus letrozole or fulvestrant in HR+ and HER2- mBC patients. The results reported an im-

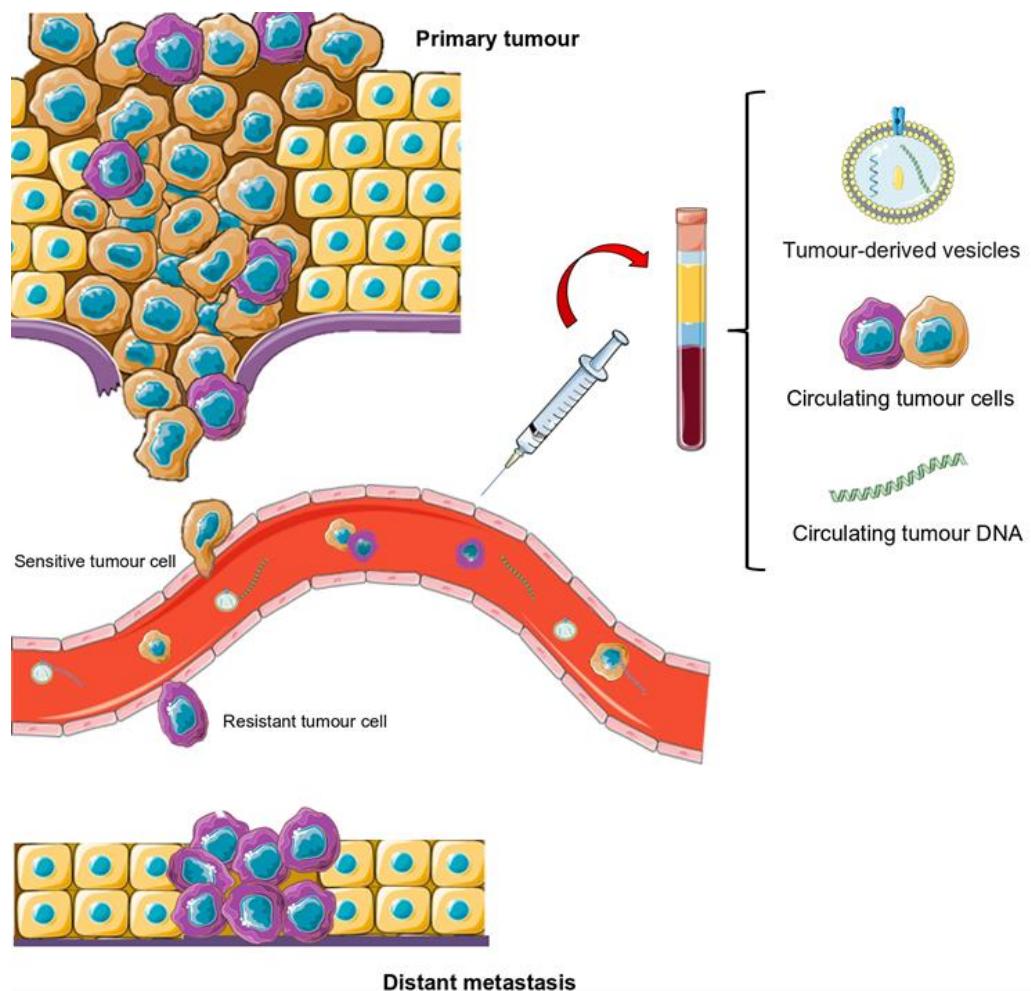
proved PFS and manageable toxicities with the combined therapy than with ET plus placebo [8],[28, 29]. In MONALEESA-7, triplet therapy with ribociclib, goserelin and tamoxifen or aromatase inhibitors (AI) were approved due to resulting in a higher PFS and overall survival than those patients treated with placebo, goserelin and tamoxifen [28, 30].

The MONARCH trials (1, 2 and 3) determined the activity of abemaciclib as a single agent or in combination with fulvestrant or a non-steroidal AI in HR+/HER2- mBC patients as first- or second-line therapies. The combination significantly improved the PFS and overall survival [31], [32, 33], [34].

In summary, CDKi plus ET increases the life span of HR+/HER2- mBC patients, but these patients eventually develop resistance. Preclinical evidence suggests that different cell cycle regulators and oncogenic drivers may be involved in CDKi resistance. First, preclinical cell line studies have revealed some candidate resistance mechanisms such as the upregulation of the Pi3K/AKT/mTOR pathway; the loss of *RB1*; acquired mutations in *RB1* inhibitors; the amplification or mutation of *FGFR*; the upregulation of *PDK1*, *MYC* or *SKYPE*; and the overexpression of *CDK4/6*. Likewise, the formation of *CCNE*–*CDK2* and *CCND1*–*CDK2* complexes can control the cell cycle progression after *CCND1*–*CDK4/6* inhibition [19]. Secondly, Wander *et al.* identified eight possible resistance mechanisms in patients resistant to CDKi, and they confirmed the results in cell lines resistant to this therapy: *RB1* allelic disruption; amplifications and/or mutations in *AKT*, *RAS*, *AURKA*, *CCNE2*, *FGFR2* and *ERBB2*; and the loss of *ESR1* [35]. It is necessary to determine whether these mechanisms are clinically relevant in treated HR+/HER2- mBC patients.

### 3. Liquid biopsy as an innovative tool for deciphering resistance mechanisms

Tumours are heterogeneous and dynamic units that evolve throughout the disease, sometimes due to the selective pressure exerted by the different treatments received [36]. Despite the fact that primary tumour biopsies have been extensively utilised, this technique has multiple downsides: invasiveness, no representation of the tumour's genetic landscape, and an inability to facilitate serial testing. Therefore, primary biopsy data may not provide real information on the current molecular characteristics of a given tumour [37], [36]. However, metastatic tissue biopsies are not always feasible, due to inaccessible tumour sites or the impossibility of sampling multiple metastatic sites, and they do not represent tumour heterogeneity. In the last decade, liquid biopsy overcomes tissue biopsy limitations through the study of tumour-derived material from biological fluids (blood, urine, saliva, etc.). Thus, the main studied tumour entities are circulating tumour cells (CTCs), circulating tumour DNA (ctDNA) and tumour-derived extracellular vesicles (EVs), due to their diagnostic and/or prognostic potential (Figure 2)[38]. Recently, circulating tumour-derived proteins, circulating tumour RNA and tumour-bearing platelets have also been described as potential relevant markers [38]. These tumour entities allow the assessment of tumour heterogeneity, allow the tracking of a tumour's genomic evolution during treatment, and provide information about the biology behind the metastatic development [39, 40]. Therefore, the longitudinal sampling of circulating tumour material may help oncologists to predict disease progression and treatment failure, and tailor patient therapy [41].



**Figure 2. Scheme of metastatic progression and potential use of liquid biopsy.** In the blood-stream, there are different CTC subpopulations. Those that have survived the therapy and become resistant are responsible for the recurrence and progression of the disease to distal locations. Liquid biopsy permits the sampling of these and other tumour entities such as ctDNA or extracellular vesicles. As such, serial liquid biopsy throughout therapy is useful for studying the appearance of treatment resistance.

#### 4. Circulating tumour DNA (ctDNA) analysis as a tumour biomarker in HR+/HER2-mBC patients

The mechanisms of ctDNA release are not well described, but two release processes are accepted: (a) an active mechanism due to the necrosis or apoptosis of tumour cells; (b) a mechanism of active release by the tumour itself, which could constitute a system of communication with the environment. Thus, the amount of ctDNA depends not only on the number of dead cells but also on the metabolism of the tumour, tumour location, vascularisation, rate of proliferation, etc. [42–44]. ctDNA analysis is a minimally invasive approach for diagnosis, as well as for detecting residual tumours and metastases, but mainly for identifying resistance mutations at clinical progression, permitting therapy selection [43, 44]. For ctDNA analysis, the main techniques used are Next-generation sequencing (NGS) and droplet-based digital PCR (ddPCR) (Table 1). NGS is a multiplex technology that can be used to detect a large number of (novel) genetic alterations covering complex genomes [45]. On the contrary, ddPCR and BEAMing assays are rapid, sensitive and precise with little input material, but the alterations must be known in advance [46, 47].

In recent years, ctDNA has been used as a promising approach to identify resistance to CDKi plus ET in HR+/HER2- mBC patients (Table 1). In primary tumours, an association between biomarkers and therapy responses was not observed [24, 26, 48]; however, in ctDNA, some therapy-related alterations were identified, as described below.

**Table 1.** Summary of the literature review based on the analysis of tumour-derived material from HR+/HER2- advanced breast cancer patients using liquid biopsy.

Tumour-derived material	Biomarker	Therapy	Technique	References
ctDNA	RB mutations	PALOMA 3: Palbociclib plus fulvestrant	Exome sequencing NGS ddPCR	O'Leary B, Cutts RJ, Liu Y, et al. [3]
		Palbociclib plus fulvestrant Ribociclib plus letrozole	NGS	Condorelli R, Spring L, O'Shaughnessy J, et al. [49]
	ESR1 mutations	PALOMA 3: Palbociclib plus fulvestrant	Exome sequencing NGS ddPCR	O'Leary B, Cutts RJ, Liu Y, et al. [3]
			ddPCR	Fribbens C, O'Leary B, Kilburn L, et al. [51]
				O'Leary B, Hrebien S, Morden JP, et al. [52]
	FGFR1 mutations	Palbociclib plus AI/fulvestrant	Exome sequencing NGS	Wander SA, Cohen O, Gong X, et al. [35]
		MONALEESA-2: Ribociclib plus letrozole	NGS qPCR	Formisano L, Lu Y, Servetto A, et al. [56]
			NGS	Hortobagyi GN, Stemmer SM, Burris HA, et al. [57]
	PI3K mutations	PALOMA 3: Palbociclib plus fulvestrant		Neven P, Petrakova K, Bianchi GV, et al. [58]
			Exome sequencing NGS ddPCR	O'Leary B, Cutts RJ, Liu Y, et al. [3]
			BEAMing assay	Cristofanilli M, Turner NC, Bondarenko I, et al. [48]
			ddPCR	O'Leary B, Hrebien S, Morden JP, et al. [52]
EVs	CDK9/4 and TK1 mRNA copies	CDKi plus endocrine therapy	ddPCR	Del Re M, Bertolini I, Crucitta S, et al. [67]
	miRNA 432-5-p	Palbociclib plus letrozole/fulvestrant	mRNA/miRNA array NGS	Cornell L, Wander SA, Visal T, Wagle N, Shapiro GI [68]

O'Leary *et al.* detected subclonal *RB1* mutations in 9 out of 195 (5%) patients treated with palbociclib or ribociclib plus ET at the end of treatment. The clinical prevalence of

*RB* mutations in primary BC tumours is low, while their prevalence in patients resistant to *CDK4/6* inhibitors with prior endocrine therapy is unknown [3]. As *RB* alterations were detected in ctDNA after exposure to CDKi, it was assumed that they were the result of selective pressure from the therapy. However, as these mutations were part of a subclonal population, their analysis in tumour-derived material was complex [3, 49]. Furthermore, *RB1* mutations were only selected in *ESR1* wild-type tumours but not in those with fulvestrant resistance, due to *ESR1* mutations, suggesting that several resistance pathways were involved [49].

There is some controversy concerning *ESR1* subclonal mutations. Some studies have detected *ESR1* mutations in patients resistant to combined therapy [35], while other patients were sensitive to CDKi regardless of *ESR1* status [50, 51]. A reduction in *ESR1* ctDNA abundance was also observed after two weeks of therapy, but did not improve the PFS, nor predict the sensitivity [52]. Studies focusing on ctDNA analysis found that substantial *ESR1* loss and gain alterations reflected therapy pressure in different subclones [3]. Likewise, it seems that patients with *ESR1* mutations at baseline exhibited worse PFS than those with wild-type mutations due to therapy pressure [13]. Studies carried out after the completion of treatment suggested that the loss of the *ESR1* mutation was more common in patients treated with palbociclib plus fulvestrant therapy than in those receiving the placebo, but this loss did not improve the PFS or predict the sensitivity [52]. Nevertheless, further analyses are required to understand the role of *ESR1* mutations in resistance to polytherapy. A PADA-1 trial (phase III) assessed *ESR1* mutations in ctDNA to evaluate the efficacy and safety of switching the ET (from AI to fulvestrant) combined with palbociclib. Likewise, *ESR1* mutations (E380, L536, Y537 and D538 hotspots) were monitored by ddPCR [53]. Preliminary results show that *ESR1* mutations were uncommon in patients not treated with AI in the neoadjuvant setting. In addition, a one-month treatment with palbociclib and AI decreased the *ESR1* mutation rate [54, 55].

Other proposed resistance mechanisms imply PI3K alterations. PI3K gene is described as gene with a strong pattern of variant acquisition and a loss of clones during treatment [3, 48]. There was no association between PI3K alterations and PFS, the benefit of the combinatorial therapy or the HR status [48]. However, it was observed that a reduction in the *PIK3CA* ctDNA level after two weeks of treatment predicted the long-term clinical outcome (4 vs. 11 months) [52].

ctDNA *FGFR1* alterations have also been linked to patients' outcomes. Formisano *et al.* observed that, at baseline, 20 out of 247 (5%) patients possessed an alteration in *FGFR1*, which was associated with worse outcomes. Of those patients that progressed to poly-therapeutic treatment, 41% (14/34) possessed an *FGFR1* alteration, suggesting a connection between *FGFR1* mutations and progression [56]. In addition, the analysis of the *FGFR1* mRNA expression in tumour samples showed that patients with high *FGFR1* mRNA expression exhibited a worse PFS when treated with letrozole plus ribociclib. It was also identified that the mRNA overexpression and amplification of *FGFR1* reduced the sensitivity to palbociclib and fulvestrant treatment *in vitro* [56]. Other studies observed that alterations at baseline in *PIK3CA*, *TP53*, *CDH1*, *FGFR1*, cell cycle-related genes or genes involved in receptor tyrosine kinase signalling did not predict the response to ribociclib plus letrozole therapy [57]. However, Neven *et al.* found that, regardless of the ctDNA gene alteration status, the PFS was higher in patients treated with ribociclib [55].

## 5. Circulating Tumour Cell (CTC) analysis as a novel biomarker for managing HR+/HER2- mBC patients

Cancer heterogeneity results in tumour cell subpopulations with different genomics, rates of proliferation, aggressiveness and drug sensitivities. These cancer tumour cells are released into the blood circulation actively, via epithelial–mesenchymal transition, or passively, detached from the primary tumour or metastasis as single cells or clusters, which have greater metastatic potential. Thus, the presence of  $\geq 5$  CTCs per 7.5 mL of blood was associated with poor outcomes in metastatic breast and prostate cancer, while  $\geq 3$  CTCs per 7.5 mL of blood was so in colorectal cancer patients [42]. The CellSearch® system (Menarini Silicon Biosystems, Inc) is the only platform validated by the FDA for CTC enumeration. It is an immunomagnetic method that uses the epithelial antibody EpCAM to positively enrich CTCs. Next, it is performed a staining for cytokeratins 8, 18 and/or 19; CD45; and nuclei for CTC enumeration [42]. However, it ignores CTC subpopulations with mesenchymal or stemness phenotypes [42]. Despite the technological advances, the low number of CTCs in the blood is still a hindrance to their isolation and characterisation [59]. Thus, sampling higher volumes of blood by leukapheresis is an alternative being explored [42, 60, 61]. Likewise, studies at the single-cell level have been of great relevance to unravelling the tumour heterogeneity in BC and other types of tumours, studying resistant clones, and determining resistance mechanisms and therapeutic responses [42, 62]. Thus, a study by De Luca *et al.*, carried out in a patient with breast cancer, observed that most of the CTC mutations detected at the beginning of the study disappeared during treatment, while new mutations emerged [63].

The study of CTCs will allow omics analysis (gene expression, proteins, metabolites, etc.), as well as functional tests *in vitro* and *in vivo*, to elucidate the metastatic process and the underlying resistance mechanisms (Supplementary Table 1). Regarding HR+/HER2-mBC patients treated with combinatorial CDKi and ET therapy, no study based on CTCs enumeration or molecular analysis in a representative cohort has been reported. Interestingly, in 2020, Koch *et al.* established an ER+ breast CTC line derived from a patient with luminal metastatic breast cancer. The cell line was demonstrated to be genetically identical to the original CTCs, a case that had never been described before. In addition, it was observed that palbociclib reduced the cell line growth even at low doses in this novel CTC line [59].

## 6. Analysis of extracellular vesicles (EVs), a possible biomarker of CDKi

EVs play an important role in the communication between cells in both healthy tissues and tumour microenvironments. They can be generated within endosomes, forming smaller EVs of 50–100 nm in diameter called exosomes, or through budding directly from the plasma membrane, resulting in microvesicles that can vary from 50 nm to 10  $\mu\text{m}$  in diameter [64]. The EVs contain a wide variety of biomolecules including RNA, lipids, proteins and DNA [65]. They allow cancer cells to establish crosstalk between the tumour and the stroma, and to take part in processes such as tumorigenesis, angiogenesis, invasion and metastasis. Likewise, it was described that they can transmit drug resistance through functional proteins and microRNAs (miRNAs) [66].

Del Re *et al.* identified miRNAs from exosomes in plasma from mBC patients treated with CDKi plus ET. These patients, with elevated levels of CDK4 expression, had prolonged PFS and better therapeutic responses. In addition, increases in CDK9 and TK1 mRNA copies were related to clinical resistance [67]. These preliminary results established exosomes as a promising biomarker for monitoring the outcomes of CDKi therapy [67]. Another recent study demonstrated a new exosome-mediated mechanism of resistance to CDKi acquired through extracellular signalling, involving exosomal miRNA [68]. Increases in CDK6 protein and mRNA expression were observed in palbociclib-resistant BC cell lines. CDK6 knockdown re-sensitised the cells to palbociclib

treatment, indicating this cyclin as a key mediator of the resistance mechanism. Through co-culture experiments, it was found that palbociclib-sensitive cell lines could acquire resistance to the drug when co-cultured with resistant cells or with their exosomes, suggesting that the resistance could be transmitted through extracellular vesicles. EV analysis in these cell lines identified the miRNA miR-432-5p as a possible mediator of CDKi resistance [68]. The expression of miR-432-5p was found to be 1.8-fold higher in biopsies from luminal BC patients with intrinsic or acquired CDKi resistance than in those from patients with sensitive tumours. A 2.7-fold decrease in *SMAD4* expression was also observed in resistant tumours, indicating a TGF- $\beta$  pathway suppression mediated by miR-432-5p, as it interacts with numerous genes from the TGF- $\beta$  pathway. Using both *in vitro* and *in vivo* models, the authors provided evidence of the loss of acquired resistance following drug removal, suggesting that CDKi could be used again after adequate drug breaks [68].

## 7. Conclusions

Liquid biopsy is a fundamental tool for studying tumour heterogeneity, the main cause of therapeutic failure in cancer patients. Therefore, changes in the molecular profiles of primary tumours and metastasis can be studied longitudinally via a non-invasive and real-time approach [57]. Various trials have demonstrated the benefits of combined CDK4/6 inhibitors plus endocrine therapy in HR+/HER2- mBC, such as increasing the PFS regardless of menopausal status, prior therapies, endocrine sensitivity and the site of metastasis [23, 54]. However, certain limitations remain to be resolved, such as the lack of predictive biomarkers with which to select patients or detect resistance [24]; these are among the current topics in the context of luminal metastatic breast cancer.

In this regard, studies carried out on ctDNA point out that mutations acquired at the end of treatment were related to a longer PFS in patients who progressed to polytherapeutic treatment. It is likely that tumours that progress early do not acquire mutations, due to the lack of treatment pressure. Therefore, other resistance mechanisms can affect early progression, so it is important to consider the intrinsic resistance when selecting the next line of treatment. O'Leary *et al.* described that resistance to fulvestrant boosts resistance to combinatorial therapy, mainly in tumours that could progress during CDKi treatment with active ER signalling. One possible explanation is that tumours can adapt to CDK4/6 inhibitors if ESR1 signalling is not correctly suppressed, but considering the lack of consensus regarding ESR1 status and CDKi sensitivity, further studies are needed. However, this suggests that ET could be a resistance driver [3]. Other proposed biomarkers, such as *PI3K*, indicate that a reduction in the mutated fraction extends the time to progression, while the data for *FGFR* are contradictory, preventing reliable conclusions from being drawn. Despite the significant potential of CTCs, there are no reported studies on CTC gene expression analysis in a metastatic clinical setting. Deciphering changes in expression after combined therapy, especially in patients with intrinsic resistance, may be a milestone that allows the interpretation of the underlying resistance mechanisms (González-Conde *et al.*, unpublished data). This is of particular interest, as CTCs that survive therapy can colonise distal organs and contribute to disease progression. Likewise, CTC lines are functional models with which to test drug activity and decipher intrinsic mechanisms involved in the metastatic cascade. Finally, EVs are being considered as novel biomarkers for determining the therapeutic response and identifying resistance mechanisms, but it is necessary to continue with these investigations. In summary, for data validation, the study of tumour-derived material via a comprehensive approach could be of great interest due to ctDNA, CTCs and EVs providing complementary information.

Concerning combined therapy, one limitation is the lack of knowledge about the contribution of each treatment or if the resistance is due to the action of both drugs.

Furthermore, in the clinical context, we must assess whether the mutational state prior to treatment determines the therapeutic efficacy. In addition, patients with intrinsic resistance should be studied to detect novel resistance mechanisms, as the evolution of driver gene mutations is infrequent due to the lack of selective pressure.

Owing to the genetic complexity of cancer and possible mechanisms of acquired resistance, simple models of genetically encoded sensitivity do not reflect patients' genetic landscape [5]. Knowing the profile of each patient at a given point in time will allow the selection of the most beneficial therapeutic sequences. Thus, the future clinical outlook should be based on the molecular characterisation of primary tumours and metastasis, as well as tumour-derived material (ctDNA, CTC or EVs) at different time points in the metastatic clinical setting. The holistic liquid biopsy analysis of tumour material will change the current clinical paradigm for luminal BC patients, in such a manner that the best treatments will be selected and resistance will be overcome. Several clinical trials consider ctDNA to be a major informative biomarker, but other circulating tumoral entities that could provide transcriptomic data related to the metastatic cascade or resistance acquisition have not yet been accounted for. The clinical implementation of liquid biopsy is underway, and, despite current technological limitations, it only is a matter of time before their use becomes universal [24, 42, 44].

**Supplementary Table 1.** Summary of advantages and disadvantages of different circulating tumour entities (ctDNA, CTCs, EVs).

	CTC	ctDNA	EVs
<b>High concentration</b>	NO	NO	YES
<b>Study of tumour mutations and methylation patterns</b>	YES	YES	YES
<b>Study of tumour RNA transcription profile</b>	YES	NO	YES
<b>Detection of systemic changes and inflammation</b>	NO	NO	YES
<b>Use of biobanked samples</b>	YES*	YES	YES
<b>Functional assays</b>	YES	NO	YES
<b>Morphological characterisation</b>	YES	NO	NO
<b>Validated predictive value</b>	YES	NO	NO

[69–71] \*Biobanked samples do not allow performing functional analysis.

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