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Review Article

Circulating Tumour Cells for the Monitoring of Breast Cancer, Current Knowledge and Future Prospective; with a Particular Focus on the Her2-Type

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Abstract

Breast cancer (BC) is one the most common malignancies, second only to lung cancer in terms of mortality in women. With a timely knowledge of disease occurrence, recurrence and/or metastases, clinicians could better decide if a patient requires immediate attention and which kind of intervention would be most likely to improve survival. Up-to-now, one of the most promising frontiers achieving this purpose is liquid biopsy. A sensitive methodology related to it is the detection of circulating tumor cells (CTCs), which is based on two major steps: 1) enrichment and 2) detection. These two steps are based on physical and biological properties of the CTCs. Currently FDA has approved only the Cell-Search® system as a method for the detection of CTCs starting from 7.5 mL of blood sample. Higher CTC numbers correlated with worse patients' survival. These promising results show how CTCs could be used as valuable prognostic tool. Therefore, the purpose of this review is to offer an overview of the current methodologies for the enrichment and the detection of CTCs in BC patients for prognostic purposes. A particular attention will be given to HER2 in CTCs in HER2-positive patients as a tool to predict prognosis, monitor the disease progression and/or drug resistance.

Keywords: *Breast Cancer, Liquid Biopsy, Circulating Tumour Cells, Prognostic Value, Monitoring Resistance*

Abbreviations

BC: Breast Cancer; MBC: Metastatic Breast Cancer; CTCs: Circulating Tumour Cells; PLS3: Protein Plastin 3; EMT: Epithelial Mesenchymal Transition; KRT: Keratins 5, 7, 8, 18 and 19; FN1: Fibronectin 1; CDH2: Cadherin 2; SERPINE1/PAI1: Serpin Peptidase Inhibitor, Clade E; OS: Overall Survival; DFS: Disease-Free Survival; PFS: Progression-Free Survival; LKB1: Liver Kinase B1; MRD: Minimal Residual Disease

Introduction

With the recent advances in breast cancer, the 5-years survival rate reaches 90%. The disease usually can be treated with a good prognosis when diagnosed on time. However, when the tumour relapses or becomes metastatic, it leads to a poor prognosis and just in the United States it has been estimated to cause about 41,400 deaths in 2018 [1]. The spread of the tumour in the blood circulation was first recognised in 1869 [2]. These circulating tumour cells (CTCs) have been considered cells detached from the primary or metastatic tumour sites reflecting the heterogeneous characteristics of the tumour source. CTCs derive from tumours are considered to be an early-event during blood-borne metastasis. For this particular reason, their half-life is relatively short, of 1 to 2.4 hours [3]. The concentration of these

cells is considered to be lower than 10 cells per millilitre [4]. Therefore, an enrichment step is usually required in order to raise the CTCs concentration to a detectable amount prior to their characterization. Breast cancer is a heterogeneous disease made of several distinct pathological and molecular subtypes [5] and HER2 is one of the predominant targets for its treatment. Around 70% of BC are considered HR+ (ER+ and/or PR+ and HER2-) [6] and around 20 - 30% are HER2-positive [7]. Even though the HER2 expression is a poor prognostic factor and associated with decreased overall survival (OS) in BC [8], highly efficient HER2-targeted therapies (e.g. Herceptin) became available over the past two decades and the outcome of patients with HER2-positive BC improved noticeably [9].

Unfortunately, up to 70% of HER2-positive patients who receive adjuvant HER2-targeted therapy after chemotherapy develop disease progression or de novo resistance [10]. Furthermore, some cases of tumours with high levels of HER2 overexpression (showed by 3+ IHC assays or by gene amplification using FISH) might not respond to HER2 targeted therapies [11,12] and the HER2 status of patients might change during disease progression [13]. Therefore, nowadays the assessment of CTCs by liquid biopsy could provide useful therapeutic biomarkers for the monitoring of this type of cancer occurrence, progression or recurrences [14]. Several nucleic acid- and protein-based assays have been developed to evaluate HER2 status in CTCs [15].

The main scope of this review is to give an overview of the current state-of-the-art of CTCs-based strategies used for the prediction of clinical outcomes through their detection in the context of the blood circulation in breast cancer with a particular focus over the HER2-type.

Enrichment and Identification of CTCs

Based on the fact that CTCs are usually found in very low amounts, their identification requires an enrichment step. There are various enrichment methodologies that are either based on physical or on biological differences inherently present between epithelial-derived CTCs and blood cells. The enrichment methodologies, based on physical differences, include size/deformability-based filtration, electrical property-based di-electrophoresis (DEP) separation and density-gradient centrifugation. Biological enrichment methodologies are based on procedures targeting surface markers that are exclusively expressed on CTCs. In such type of enrichments, either a positive or a negative selection of these targeted markers is used to enrich the tumour cells while depleting blood cells.

Physical enrichment

Initially, the epithelial-derived CTCs were believed to be larger and stiffer compared to leukocytes (diameter of 7 - 12 μm). Therefore various devices for filtration have been developed in order to isolate CTCs in the past decades [16]. With such methodologies, the blood is filtered through pores that are usually 8 μm in diameter, while blocking the passage of larger molecules. In order to have optimal conditions for the filtration, it is important that there is always an adequate blood flow-rate, pore size uniformity and membrane rigidity. Since filtration methods are easy-of-use, high-throughput and provide good recoveries of CTCs, they are continuing to be used and improved over time. Improvements include the use of 3D microfilters to reduce hemodynamic stress on cells and the use of CTC size-amplification strategies to reduce the loss of small-sized CTCs [17]. Density-gradient centrifugation enriches CTCs in the mononucleocyte fraction [18], but inevitably this method loses CTCs even if it is high-throughput. To have a higher purity of enriched CTCs, antibodies that are specific to both erythrocytes and leukocytes have been used in order to form large multicellular rosettes of these populations of blood cells, which are then easily removed by centrifugation from the mononucleocyte fraction [19]. Combined density- and size-based separation methods have been developed in order to reduce the loss of CTCs by inserting porous barriers in centrifuge tubes [18]. DEP method relies on morphological and electrical dissimilarities with high cellular viability [20]. This method combines electric fields with hydrodynamic and sedimentation forces in order to pull apart tumour cells from blood cells. The viable cells with DEP are greater than 97% but the recovery of total cells is still down to 70% [20].

Biological enrichment

Antibody-mediated isolation is the most renowned biological CTC enrichment technology. Surface markers (such as epithelial adhesion molecule, EpCAM) are expressed on carcinoma cells, while lacking from the mesenchymal leukocyte surfaces, and are targeted by antibodies that are frequently used in the positive selection of carcinoma cells from the blood [21]. However through this method EMT-associated CTCs are excluded, which might represent the most aggressive CTC subpopulations [22]. For this particular reason, currently there is a focus on the research for new CTC surface markers that could also detect EMT-associated CTCs. As an example, actin-binding protein plastin 3 (PLS3) has been shown to be a novel marker expressed in CTCs during EMT from patients with colorectal cancer (CRC). In this research study the prognostic values of PLS3 were investigated both in a training sample set (n = 381) and in a validation set (n = 330). The PLS3 was associated also with a greater in-depth invasion of the tissue (p = 0.001). PLS3 expressing CTCs were significantly associated with a shorter OS compared to patients without such cells (Dukes A-D, P < 0.001; Dukes B, P < 0.001, in the training set). PLS3-positive CTCs remained a prognostic factor for both OS and PFS in the entire CRCs population (Dukes B, HR. 4.07; 95% CI. 1.50 - 11.57 and HR. 2.73; 95% CI. 1.16 - 6.24; Dukes C, HR. 2.57; 95% CI. 1.42 - 4.63 and HR. 2.19; 95% CI. 1.10 - 4.34). Furthermore, in the validation set PLS3 was significantly expressed at higher levels in disease recurrence compared to patients without recurrence. By RT-PCR the authors further confirmed that PLS3 was expressed in all solid cancers, including breast cancer [23]. Therefore, PLS3 could be a potentially interesting marker to be tested for the monitoring of BC disease occurrences, recurrences and metastases in larger clinical settings. A second example of an interesting EMT marker is the tumour suppressor liver kinase B1 (LKB1), whose expression in epithelial-negative CTCs isolated from MBC patients, was associated with EMT and stemness features. At the transcriptomic level EpCAM-negative CTCs showed that more than 25% of patients had an enhanced level of LKB1 [24]. A third example of an interesting EMT marker is vimentin. This protein is a mesenchymal marker expressed in CTCs captured from peripheral blood from a cohort of 70 Renal Cell Carcinoma (RCC) patients. This biomarker was also found associated with disease progression. Highest vimentin levels were observed in advanced stages of RCC compared to early stages of RCC (p < 0.001) [25]. Alternatively, the combination of antibodies to successfully capture heterogeneous CTC subpopulations has shown promising results [26]. In fact, Yu M., et al. study collecting blood from 41 BC patients used a cocktail of antibodies against epithelial and mesenchymal markers. In this combinatorial approach, seven epithelial transcripts (keratins (KRT) 5, 7, 8, 18 and 19) were pooled together with three mesenchymal (M) transcripts [FN1 (fibronectin 1), CDH2 (cadherin 2), and SERPINE1/PAI1 (serpin peptidase inhibitor, clade E)]. Notably, by using this analysis the authors were capable to detect CTCs in 41% of patients (17 patients over 41 patients). Moreover, through GSEA database they identified 717 gene signatures (FDR: 0.25) with a dramatic enrichment for the expression of EMT-related genes that significantly overlapped with a core of EMT signature [30]. Of note, more cocktail of antibodies could be made exploiting those that are capable of targeting mammaglobin marker for BC [27], or markers specific against the tumour type (e.g. HER2, EGFRM CD147, CA9, TROP-2, MUC1 and folate-binding protein receptor) to enrich CTCs [25]. Of note, through this latter approach the contamination by blood cells expressing at least one of the markers targeted by the cocktail of antibodies might be a tangible issue increasing the risk of false-positives. This method might also be hindered by the presence of macrophages or platelets that could be physically blocking access to CTCs markers. In such scenario the binding affinity of the antibody and the location of the epitope to which the antibody is bound could greatly influence the efficacy of the methodology for CTCs detection. In the positive selection of CTCs, the antibodies should be chosen on the basis of the biomarkers most likely expressed by the CTCs. On the contrary in the negative selection of CTCs, which is a useful alternative, CTCs are enriched by the depletion of leukocytes from the blood through the use of antibodies that are specifically targeting leukocytes (e.g., anti-CD45) [28].

CTC detection

Various methods for detecting CTC are based on similar principles to those adopted for the previously described enrichment steps. In fact, detection also relies on physical (such as size, deformability, electric properties, cell density) or biological (immunomagnetic separation and immunofluorescence/enzyme-linked immunosorbent assays) characteristics of CTCs. Moreover, there are some studies that have been conducted to detect CTCs through surface protein expression (most commonly EpCAM) and RT-PCR assays. In BC settings,

positive and negative biomarkers such as CD45, GA73302, mammaglobin, cytokeratins and MUC-1 have been used for immunohistochemically-based analysis [29]. The CellSearch® system is the only one that up-to-now has received FDA approval for the detection of CTCs. From 7.5 mL of blood using the CellSearch® CTC Test (Veridex, Rarita, NJ), composed of an epithelial kit-detection EpCAM antigen- and a cell spotter analyzer CTCs can be detected. In the identification system by CellSearch®, after immunomagnetically enriching epithelial cell using ferrofluids coated with EpCAM-specific antibodies, fluorescent labelling with phycoerythrin-conjugated antibodies are directed against cytokeratin 8, 18, and 19, an allophycocyanin-conjugated antibody to CD45, and nuclear dye DAPI. Through the multicolour detection with a fluorescent microscope, CTCs are defined as CK+/CD45-/DAPI+ cells. ISET is another technology that for the initial detection of CTCs in BC relies on an anti-cytokeratin 7. In this type of setting CTCs are defined as cells presenting (i) a nuclear size ≥ than two pores, (ii) irregular nuclear contour, (iii) a visible cytoplasm and (iv) high nuclear-to-cytoplasmic ratio (> 0.8) [22]. Additionally, for the detection of only viable CTCs, the functional EPISPOT assay was introduced. This methodology is based on the assessment of secreted proteins from the CTCs during the 24 - 48h short-term cell culture avoiding direct contact with the target cells. This method is based on the measurement of full-length cytokeratin-19 (CK19) from human BC. The CK19-EPISPOT was more sensitive than the CK19-ELISA as proven using 4 cancer cell lines (MCF7, Caco-2, HT-29, HCT-116) [30]. Of note, a high-speed automated digital microscopy based on fibre-optic-array scanning technology has been developed to detect CTCs labelled by fluorescently conjugated antibodies [31]. The most widely used alternatives to immunological detection are assays targeting specific mRNAs. The most common mRNA found in BC clinical diagnosis is the CK19 [32]. However, in the case of events such as EMT the transcription of some genes might be downregulated in CTCs, favouring a multi-markers approach that can be detected by RT-PCR. As an example, Markou., *et al*. described a liquid-bead-array hybridization assay simultaneously detecting the expression of six genes (CK19, ERB2, SCGB2A2, MAGEA3, TWIST-1 and HMBS) from immunomagnetically enriched mRNA in CTCs. This technology enabled the simultaneous detection of the six genes, which is fast and potentially covers many different types of CTCs [27]. Another approach in the detection of CTCs is to investigate tumour-specific DNA aberrations. However, in BC, as for other solid tumours, there is a markedly genetic heterogeneity making it difficult, even in the same tumour, to have consistent genetic aberrations. The challenge would be to set-up a large multi-panel system of different probes against various genomic aberrations. Moreover, a new molecular-based detection technology that has been recently developed is the AdnaTest, which identifies cells expressing putative transcripts of tumour-specific genes by RT-PCR after separation of MUC1-HER2-EpCAM-positive cells using immunomagnets [33]. A major limitation of this latter technique is that MUC1 could also be found expressed on mitogen-activated human T-lymphocytes, thereby influencing the quality of the results [34]. Thus, there are different strategies that can be adopted to enrich and/or detect CTCs. At the moment, negative selection strategies depleting leukocytes surrounding CTCs might be a feasible approach that could be refined with a positive selection of CTCs using multiple biomarkers to obtain the most reliable and relatively high number of CTCs.

Prognostic value of CTCs

In MBC CellSearch® system sets a cut-off value ≥ 5 of CTCs per 7.5 mL of blood. In early breast cancer, 1 CTC in 7.5 mL of blood is considered to be the cut-off for positivity. Different studies found that an increase in CTCs ≥ 1 in early stage I-III BC correlated with worse clinical outcomes. Such studies mainly used RT-PCR for seeking CK-19 mRNA-positivity or CellSearch® (≥ 1 CTC/7.5 mL). Two recent phase III BC studies have found that having ≥ 2 CTC from the 7.5 mL of blood correlated with worse survival, as measured by OS, and either PFS [38] or DFS [39]. Having 1 or more CTCs before neoadjuvant chemotherapy has been found associated with increased metastases and OS [40,41]. A large meta-analysis has been conducted looking at 49 studies entailing 6825 patients in both early and metastatic stages of BC and found that CTCs only in early stage was associated with a worse DFS (HR 2.86, 95% CI 2.19 - 3.75) and OS (HR, 2.78, 95% CI 2.09 - 2.60) [42]. As to the association between chemotherapeutic treatments and CTCs, the proportion of patients having CK-19 (+) CTCs significantly decreased from 43.5% before chemotherapy to 34.9% after adjuvant chemotherapy ($p < 0.001$). As to clinical relapses after completion of therapy, the incidence was significantly higher among CK-19 (+) CTCs patients compared to CK-19 (-) patients (33.2% and 16.6% relapse rate, respectively; $p < 0.00001$). Additionally, within the CK-19 (+) group the median DFS was significantly higher for patients treated with a taxane-based regimen compared to those treated with a taxane-free regimen (DFS 10 years rate 61.3% and 47.8%, respectively; p =

0.018). On the other hand, within the CK-19 (-) group the difference between DFS was not significant between patients whither receiving taxane-based treatment and those receiving taxane-free therapy (p = 0.481). Moreover, in the subgroup of patients who remained CK-19 (+) after receiving adjuvant chemotherapy, the incidence of clinical relapse was 50.6%, in the taxane-free group, compared to 20.7%, in the taxane-based group (p = 0.000001). In such subgroup, the median DFS was 101 months (7 - 131 months) in patients treated with a taxane-based regimen and 86 months in patients treated with a taxane-free regimen, respectively (HR: 1.8; 95% CI: 1.016-3.341, p=0.044) [35]. Table 1 and table 2 summarise all the currently available studies exploring the prognostic values of CTCs in early breast cancer and MBC, respectively.

Author	Year	Number of Patients	Methods	Tumour markers	Positive rate (%)	Follow-up (months)	Prognostic relevance
Walter., *et al.*	2018	58	CellSearch	CK8/18/19+/CD45-	9%	30	OS, DFS
Hartkopf., *et al.*	2016	202; 383	AdDNA Test; CellSearch	MUC-1, GA 33-42, HER2; CK8/18/19+/ CD45-	22%	35	PFS, OS
Rack., *et al.*	2014	2026	CellSearch	CK8/18/19+/CD45-	21,5%	35	PFS, OS
Karhade., et al.	2014	113	CellSearch	CK8/18/19+/CD45-	25%	40	PFS, OS
Xendis., *et al.*	2013	545	RT-PCR	CK19 mRNA	43.5%	71	DFS
Franken., *et al.*	2012	404	CellSearch	CK8/18/19+/CD45-	19%	48	DDFS, BCSS
Molloy., *et al.*	2011	733	RT-PCR	CK19, p1B, EGP-2, PS2, Mammaglobin, SBEM	7.9%	91	DFS, BCSS
Bidard., *et al.*	2010	115	CellSearch	CK8/18/19+/CD45-	23%	36	DFS, OS
Rack., et al.	2010	1489	CellSearch	CK8/18/19+/CD45-	9%	32	PFS, OS
Daskalaki., *et al.*	2009	165	RT-PCR	CK19 mRNA	55%, 52%	59	OS
Pierga., *et al.*	2008	118	CellSearch	CK8/18/19+/CD45-	23%, 17%	18	DFS
Benoy., *et al.*	2006	116	RT-PCR	CK-19, mammaglobin (MAM) mRNA	12-14%	26	none
Ntoulia., *et al.*	2006	101	RT-PCR	Mammaglobin A mRNA	14%	24	DFS
Nieto., *et al.*	2004	242	ICC	CK+	7%	84	DFS, OS
Stathopoulou., *et al.*	2002	148	RT-PCR	CK19 mRNA	30%	28	DFS, OS
Zach., *et al.*	2002	218	RT-PCR	Mammaglobin mRNA	2%	>12	DFS

Table 1: *CTC detection in primary breast cancer patients' prognostic significance.*

Author	Year	Number of Patients	Methods	Tumor markers	Positive rate (%)	Follow-up (months)	Prognostic relevance
Huebner., *et al.*	2018	60	CellSearch	CK8/18/19+/CD45-	56.7%	87.8	OS
Iwata., *et al.*	2017	148	CellSearch	CK8/18/19+/CD45-	44%	18	PFS, OS
Mango., *et al.*	2017	65	CellSearch	CK8/18/19+/CD45-	61.5	19.4	OS
Riethdorf., *et al.*	2017	213	CellSearch	CK8/18/19+/CD45-	21.6	67.1	DFS, OS
Rossi., *et al*.	2017	91	CellSearch	CK8/18/19+/CD45-	88%	9	OS, PFS
Paluchowski., *et al.*	2017	252	CellSearch	CK8/18/19+/CD45-, HER2	49.8%	19	OS, PFS
Hall., et al.	2016	509	CellSearch	CK8/18/19+/CD45-	24%	48	RFS, OS
Shiomi-Mouri., et al.	2016	98	CellSearch	CK8/18/19+/CD45-	45.9%	NS	OS
Jansson., *et al.*	2016	52	CellSearch	CK8/18/19+/CD45-	27%	6	OS, PFS
Jueckstock., *et al.*	2016	1221	CellSearch	CK8/18/19+/CD45-	20.6%	64	DFS, DDFS, BCSS, OS
Zhang., *et al.*	2016	101	CellSearch	CK8/18/19+/CD45-, HER2	57.4%	24	PFS
Wallwiener., *et al.*	2015	107	CellSearch	CK8/18/19+/CD45-, HER2	35%	28.5	OS, PFS
Helissey., *et al.*	2015	56	CellSearch	CK8/18/19+/CD45-	45%	20	OS, PFS
Paoletti., *et al.*	2015	52	CellSearch	CK8/18/19+/CD45-	36.5	1	PFS
Salvador., *et al.*	2015	90	CellSearch	CK8/18/19+/CD45-	NS	NS	OS
Wallwiener., *et al.*	2015	107	CellSearch	CK8/18/19+/HER2+CD45-	35%	28.5	PFS
Mu., *et al.*	2015	115	CellSearch	CK8/18/19+/CD45-	17.4%	77.6	PFS
Dalum., *et al.*	2014	403	CellSearch	CK8/18/19+/CD45-	19%	273.6	RFS, OS
Magbanua., *et al.*	2014	102	CellSearch	CK8/18/19+/CD45-	44%	26	OS
Giuliano., *et al.*	2014	492	CellSearch	CK8/18/19+/CD45-	38.4%-61.6%	32.2	OS
Wallwiene., *et al.*	2014	393	CellSearch	CK8/18/19+/CD45-	34%	26	PFS, OS
Smerage., *et al.*	2014	595	CellSearch	CK8/18/19+/CD45-	53.6%	NS	OS
Bidard., *et al.*	2014	1944	CellSearch	CK8/18/19+/CD45-	46.9%	23	PFS, OS
Hartkopf., *et al.*	2014	33	CellSearch	CK8/18/19+/CD45-	52%	NS	PFS, OS
Peeters., *et al.*	2014	154	CellSearch	CK8/18/19+/CD45-	More than 45.5%	NS	PFS, OS
Lustberg., *et al.*	2014	32	CellSearch	CK8/18/19+/CD45-	NS	NS	OS
Wallwiener., *et al.*	2013	486	CellSearch	CK8/18/19+/CD45-	42%	11.13	PFS, OS
Tryfonidis., *et al.*	2013	83	CellSearch	CK8/18/19+/CD45-, HER2	57%	28	PFS, OS
Jiang., *et al.*	2013	294	CellSearch	CK8/18/19+/CD45-	39.1%	NS	PFS, OS
Zhao *et al.*	2013	98	RT-PCR	EpCAM, CK19, hMAM	87.8%	24	PFS, OS
Giordano *et al.*	2012	517	CellSearch	CK8/18/19+/CD45-, HER2	40%	24.6	PFS, OS
Pierga *et al.*	2012	267	CellSearch	CK8/18/19+/CD45-	44%	14.9	PFS, OS
Müller *et al.*	2012	254	CellSearch; AdnaTest	CK8/18/19+/CD45-	CSS:50%; AT:40%	NS	CellSearch: OS; AdnaTest: none
Androulakis *et al.*	2012	298	RT-PCR	Ck19	67%		
Hayashi *et al.*	2012	52	CellSearch	CK8/18/19+/CD45-, HER2	26.9%	22	PFS, OS
Giuliano *et al.*	2011	235	CellSearch	CK8/18/19+/CD45-	40%	18	PFS, OS
Reinholz *et al.*	2011	86	RT-PCR	CK19	56-75%, 23-38%	27.6	OS
Nakamura *et al.*	2010	107	CellSearch	CK8/18/19+/CD45-	37%	ns	PFS
Liu *et al.* [	2009	74	CellSearch	CK8/18/19+/CD45-	NS	13.3	PFS
Tewes *et al.*	2009	42	AdnaTest	EpCAM, MUC1, HER2	52%	9	OS
Bidard *et al.*	2008	37	ICC	Pan-CK (CK8/18/19)	41%	24	OS
Nole *et al.*	2008	80	CellSearch	CK8/18/19+/CD45-	61%	13	PFS
Hayes *et al.*	2006	177	CellSearch	CK8/18/19+/CD45-	54%	7	PFS, OS
Budd *et al.*	2006	138	CellSearch	CK8/18/19+/CD45-	43%	10.1	OS
Benoy *et al.*	2006	32	RT-PCR	CK-19, mammaglobin (MAM)	25-40%	26.3	none
Cristofanilli *et al.*	2004	177	CellSearch	CK8/18/19+/CD45-	49%	9	PFS, OS

Table 2: *CTC detection in metastatic breast cancer patients' prognostic significance.*

Prognostic Significance of CTCs in HER2+ Metastatic BC

Punnoose., *et al*. in 2010 founded that CTC counts to be lower in patients with HER2+ metastatic breast cancer than in those with ER+ metastatic BC [43], probably due to the low expression of EpCAM and a higher percentage of epithelial-mesenchymal transition (EMT)-associated phenotype of tumour cells. Giordano., *et al*. in 2012 analyzed the relationship between basal CTCs count and breast tumour subtypes in a retrospective analysis of 517 patients affected by MBC. They founded that the median overall survival for patients with < 5 and ≥ 5 CTCs was respectively 32.4 and 18.3 months but no clear difference was observed in median overall survival in patients with HER2+/ER- breast cancer with ≥ 5 CTCs against those with < 5 CTCs [44]. CTCs seemed to have the least prognostic value in patients with HER2+ breast cancer treated with HER2-targeted therapy and the most value in basal-like and ER+ breast cancers. According to authors, targeted therapy with trastuzumab and lapatinib might have removed most of circulating cells overexpressing HER2 and thereby reducing the prognostic value of CTC enumeration [44]. Other studies have demonstrated that HER2-targeted therapy has a significant effect in reducing CTC counts [45,46]. In the Wallwiener's study of 468 MBC patients, the detection of CTCs can predict OS associated with HER2+ expression only in those who had not received trastuzumab [47]. In the neoadjuvant phase III Neo-ALTTO trial, no evident correlation was observed between CTC detection at any point and clinical complete or FDG-PET/CT scan response after HER2-targeted therapy with lapatinib and trastuzumab [48]. According to the more recent meta-analysis conducted by Lv., *et al*. HER2 expression in primary tumour was a considerable risk factor for a greater CTC number, while no significant associations were founded between CTC number and triple negative and hormonal receptor expression BC [49]. They also confirmed that CTCs presence was correlated with greater risk of cancer progression and death.

Prognostic Significance of CTCs in HER2+ Early BC

Currently, the largest clinical trial to evaluate the prognostic impact of CTCs in early BC was performed by the German SUCCESS study (EUDRA-CT No. 2005-000490-21, NCT02181101). They showed that women with worse DFS and OS were those with detectable CTCs before chemotherapy. In addition, they assessed the relationship between survival and CTC enumeration for the purpose of defining the optimal cut-off. Women with five and more CTCs had the largest risk for disease recurrence after a median follow-up of 36 months [50]. Even other smaller trials with longer follow-up confirmed the link between clinical outcome and CTC presence [51,52]. Janny., *et al*. in 2016 conducted a multicenter pooled analysis and confirmed CTC presence as an independent predictor of poor disease-free, overall, breast cancer-specific, and distant disease-free survival [53]. Moreover, HER2 status was an additional relevant independent prognostic factor in the multivariate analysis whereas menopausal status, histologic type, adjuvant and neoadjuvant chemotherapy were not significantly correlated cancer relapse or survival. Of considerable importance is the fact that clinical outcome in a patient with low-risk with small node-negative tumours was not influenced by the presence of CTCs, recommending that early stage breast cancer can be treated successfully even with a minimal residual disease (MRD) in the blood. In addition, Janni., *et al*. calculated CTC presence in different BC subtypes and found that their presence predicted survival in triple-negative and luminal subgroups but not in patients with HER2-positive, hormone receptor negative tumours [53]. In 2007 Ignatiadis., et al. on the contrary, found a correlation between CTC presence and survival in HER2 and triple negative subgroup but no relationship between CTCs presence and prognosis in patients with luminal subtypes [54]. Banys-Paluchowski M., *et al*. in 2015 reported similar findings [55].

HER2+ CTCs in breast cancer

While the correlation between survival and CTC enumeration has been shown in several clinical studies, clinical tests investigating the impact of specific CTCs phenotypes have shown contradictory results. Undoubtedly, a pivotal role in the personalized oncological treatment has been played by the competence to assessing tumour subtypes. In this regard, several efforts have been made to characterize CTCs phenotype and investigate prospective clinical applications over the ordinary tumour cells count. There is some discordance between HER2 status and CTCs in primary tumours; genotype, phenotype, metastatic lesion and CTCs often differ. According to the SUCCESS trial phase III, of the 257 patients with early HER2 positive breast cancer and detectable CTCs, only 57,2% proved to have CTCs with HER2

expression and 21.4% were doubtful [56]as this may have important implications for the use of HER2-targeted therapy. Methods The number and HER2 status of CTCs out of 30ml peripheral blood were assessed in 642 patients using the CellSearch System (Janssen Diagnostics, USA. In regard to the prognostic value of CTCs, in 2013 Liu., *et al*. demonstrated that patients with primary HER2-positive breast cancer receiving HER2-targeted therapy, those with HER2+ CTCs had substantially longer PFSs than those without positive CTCs only [57]. Wallwiener., *et al*. investigated HER2 expression in CTCs over 107 patients with MBC who had been starting a new line of therapy. Patients with HER2-positive CTCs had substantial longer PFS than those with HER2-negative CTCs and the HER2 status did not influence OS [58]antigen profiles of metastatic tissue and primary tumor differ in up to 20 % of patients. Reassessment of predictive markers, including human epidermal growth factor receptor 2 (HER2. Accordingly to Wallwiener., *et al*. Beiije., *et al*. investigated HER2 and ER status of CTC from 157 patients with MBC showing no correlation with their clinical outcome [59]the prevalence and prognostic significance of HER2-positive CTCs were explored in a chemotherapy cohort, as well as the prognostic impact of the estrogen receptor (ER. On the contrary, Hayashi., *et al.* reported worse survival in HER2-positive CTCs patients [15]. Apparently, CTCs are the predominant tumour cell population in metastatic disease and consequently, their expression profile might predict response to therapy in more depth [60]. Georgoulias., *et al*. reported HER2 positive CTCs were found in 89% of HER2 negative and these had greater survival after trastuzumab therapy [61]. Therefore, evaluating CTCs might provide further information for the tumour HER2 status and managing trastuzumab therapy [62]. Agelaki., *et al*. in 2015 showed that lapatinib markedly reduced the number of HER2-positive CTC in MBC, notwithstanding the HER2 status of a primary tumour [63].

Moreover, Pierga., *et al*. in 2015 [64] showed the effectiveness of a combination treatment made of epirubicin, fluorouracil, docetaxel, cyclophosphamide, bevacizumab and trastuzumab, followed by adjuvant trastuzumab, bevacizumab and eventual hormone therapy in primary HER2-positive IBC patients. Their trial demonstrated the importance of CTC enumeration as a prognostic marker for DFS: in patients with ≥ 1 CTC/7.5 mL at baseline, DFS rate after 3 years was significantly lower (43%) than DFS rate estimated in patients with no CTCs observed at baseline (81%).

Discussion

With the progress in the field of breast-cancer care new and more efficient methods for the monitoring of disease progression and prediction of the emergences of recurrences and/or metastasis are becoming of paramount importance for improving the quality of life of patients. Liquid biopsy has shown a great potential with the advent of technologies that are capable of predicting relapses and/or metastases in certain solid tumours. Cell-free tumour DNA integrity and circulating tumour DNA are stunning examples of how a diagnosis can become always a more efficient reality in clinical settings where patients shall always have the best quality of life offered by cutting-edge technologies.

Interestingly, as to the HER2-subtype, the CTC cells have been demonstrated to have a high potential for metastasis [13] and intratumoral heterogeneity in HER2-positive patients might explain unexpected failures of HER2 targeted therapy with trastuzumab [65]. For this subtype, only a few studies have demonstrated the effects of therapies on CTCs [46,61] and unfortunately, there is no data available on the effect of HER2-targeted therapyon the HER2 expression of CTCs [56]. In the case of early BC, the assessment of the targets is up-to-date reserved to the primary tumour even though evidence of heterogeneity of primary tumour and MRD are rising, especially those correlated to hormone receptor and HER2 status. On the contrary, in the metastatic disease, one of the most noteworthy possibilities is the CTC-guided treatment. By evaluating the correlation between CTC with HER2 expression and efficiency of targeted therapy, we can suppose that it is pivotal to assess the grade of heterogeneity for better predicting the efficacy of targeted therapies. Large-scale clinical trials are therefore warranted for predicting the clinical outcome based on the CTCs targets such as HER2 that are currently available. Properties and nature of CTC in every singular patient are unknown; CTC is likely to shift from an active to a sleeping status, becoming resistant to chemotherapy and our immune system. Molecular studies of CTCs may discover unknown molecular targets for novel drugs and poor prognostic predictors on account of emerging resistant clones, while yet giving the chance to choose the proper therapy [66].

Conclusion

In conclusion liquid biopsy through CTCs is a valid approach that could be used for predicting BC occurrence, recurrence, metastasis or resistances to treatments against targets that could be found within the CTCs, such as the HER2 in the HER2-type. Different lines of studies, using prevalently the FDA approved CellSearch® system and the RT-PCR technology, have proven that a relatively higher level of CTCs expression from the blood samples correlated with a worse clinical outcome of BC patients. Moreover, as to the HER2-type, there are few promising studies showing that HER2 lack of expression over time has been associated with a worse response of patients to treatments such as Herceptin, which are working targeting the HER2 receptor on a type of cancer that had been initially diagnosed such as HER2-positive.

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