Methodology for the Isolation and Analysis of CTCs

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Abstract

The majority of deaths related to breast cancer are caused by metastasis. Understanding the process of metastasis is key to achieve a reduction on breast cancer mortality. Currently, liquid biopsies are gaining attention in this regard. Circulating tumor cells (CTCs), an important component of liquid biopsies, are cells shed from primary tumor that disseminate to blood circulation being responsible of distal metastasis. Hence, the study CTCs is a promising alternative to monitor the progress of metastasis disease and can be used for early diagnosis of cancers as well as for earlier assessment of cancer recurrence and therapy efficacy. Despite their clinical interest, CTC analysis is not recommended by oncology guidelines so far. The main reason is that there is no gold standard technology for CTCs isolation and most of the current technologies are not yet validated for clinical use. In this chapter we will focus on the most relevant technologies for CTC isolation based on their properties and depending on whether it is a positive or negative selection. We also describe each technology based on its potential use and its relevance in breast cancer. The chapter also contains a future perspective including the challenges and requirements of CTC detection.

Keywords

Breast cancer · Circulating tumor cells (CTCs) · CTC capture · CTC enrichment · Detection · Technology

4.1 Introduction

The vast majority of deaths related to breast cancer are caused by distant organ metastasis. The metastasis of this tumor type is mainly established by the hematogenous dissemination of tumor cells from the primary tumor. Thus, understanding the process of metastasis is key to achieve a reduction on breast cancer mortality.

The current methods used in clinical practice to monitor the disease mainly involve tissue biopsy, imaging techniques and evaluation of

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serum markers such as CA 15-3 and carcinoembryonic antigen (CEA). However tissue biopsy is invasive for patients since entails surgery, the material obtained usually is limited, and does not represent the intratumoral heterogeneity while imaging techniques show limited sensibility. These approaches have been shown to have a limited success, however, in recent years the technic known as "Liquid biopsy" and within this, the study of the circulating tumor cells (CTCs), has emerged as an useful alternative for disease monitoring. CTCs can be isolated from the blood of patients in a longitudinal and non-invasive manner, providing real time information about the status of the disease. In this regard, the prognostic value of the enumeration of CTCs in patients with metastatic breast cancer (MBC) and even in early breast cancer, has been extensively proved [1–3]. Despite of these evidences, CTC enumeration is not currently recommended by oncology guidelines, meaning that further clinical validation is still needed for this approach. However, efforts are being made to advance on the development of technologies used for CTC isolation, in the quest for higher sensitivity and specificity.

The possibility of isolating CTCs for enumeration and analysis is conditioned by the currently available technologies. Although in recent years there have been great advances in this regard, there are still important challenges to be considered. CTCs have an estimated blood frequency ranging from 1 to 10 CTC in 10^6-10^8 blood cells depending on the tumor stage. CTCs are "rare" events in blood, so there is a need for a high sensitive and specific technology for their detection. Another important limitation to take into account is the sample volume to be processed in order to guarantee a successful isolation of CTCs. Additionally, CTCs can be shed either by the primary tumor or metastatic sites, thus showing high heterogeneity regarding expression of markers (epithelial or mesenchymal), as well as different cell sizes, morphologies and plasticity. All this together makes difficult to isolate pure CTCs in one-step approach. To achieve the implementation of CTCs in routine clinical practice, since CTCs enumeration alone is not enough, it is needed to get relevant clinical information out of them, hence improving patient outcome. Ideally, the selected technology for CTCs isolation should also be suitable for downstream analysis such as molecular characterization or functional assays. In fact, the main advantage of CTCs analysis over other circulating biomarkers such as ctDNA is the possibility to perform RNA expression studies, known as transcriptomics, as well as other "omics" analyses.

In this chapter we will review the most relevant technologies used for the isolation of CTCs from the blood of cancer patients, with particular emphasis on those applied for the study of breast cancer dissemination.

4.2 Isolation of CTCs: Enrichment

An ideal CTC detection platform must be capable of isolating and detecting the different subpopulations of CTCs, discarding the background contamination of blood cells. The first step is usually the enrichment of CTCs, which allows the separation of CTCs from blood cells, followed by a step of distinction or identification (and possibly characterization) of the CTCs that allows to determine the tumoral nature of the cells. Currently, there is no system capable of isolating a pure population of CTCs, and indeed, most available technologies concomitantly isolate in a non-specific manner some cells from the hematopoietic fraction together with CTCs. Thus, depending on the purity of the sample obtained, the approach is considered of high or low specificity. To select the most accurate isolation technique it must be taken into account the specificity and sensitivity, as well as the desired downstream analysis to be performed (enumeration, molecular characterization or the performance of functional assays). In addition, the origin of the sample to analyse must also be considered, whether it comes from blood, urine or another biological fluid, and its collection procedure and storage (e.g. if includes a fixation step or not).

The phenotypic characterization of the cells is usually performed using immunofluorescence techniques that permit the simultaneous visual-

ization of different markers. Thereby, cells are immunostained for epithelial, mesenchymal or tumor specific, and blood cell markers, as well as nuclear staining which permits to check the integrity of the cell. The immune-labelling of cells provides additional information about morphology, size and fitness, for example whether they have entered apoptosis (vesicular cells), or about the phenotype (epithelial or mesenchymal traits) [4]. Additional downstream options for molecular characterization of the cells are Copy Number Variation (CNV) analyses or Next-Generation Sequencing (NGS) to verify the tumor nature of the cells. For a more in depth characterization, single cell level analysis is the most suitable methodology to avoid biased results due to low specificity associated to general approaches isolating pools of CTCs.

Current methods for CTC isolation can be divided in two main blocks according to the strategy used for the capture of CTCs, based on (1) biological and (2) biophysical properties of the cells (Table 4.1).

4.2.1 Strategies Based on Biological Properties

The most common technique is the immunoisolation which is based on the use of antibodies against cell surface markers. It can be directly applied to the whole blood or to a previously isolated fraction of peripheral blood mononuclear cells (PBMCs), usually obtained by a density gradient centrifugation protocol. CTCs can be isolated through positive selection, targeting tumor-associated antigens expressed by CTCs; or negative selection, removing background cells by targeting antigens expressed by them but not by CTCs.

4.2.1.1 Positive Selection

Positive selection, the target molecule is an epithelial surface antigen, such as EpCAM (epithelial cell adhesion molecule), HER2 (human epidermal growth factor receptor 2), EGFR (endothelial growth factor receptor) or CEA. Likewise, other mesenchymal markers such as cell surface vimentin [5, 6] and N-cadherin [7], and stem cell marker CD113 have been used to isolate non-epithelial cell populations given the importance of the epithelial-to-mesenchymal transition (EMT) and stem cell markers in different subpopulations of CTCs in relation to metastasis. For the isolation of CTCs from breast cancer patients, the most commonly used antigen for a positive selection is EpCAM, which is a surface protein expressed in epithelial cells. It is assumed that under physiological conditions epithelial cells do not circulate in the bloodstream, thus the presence of EpCAM-positive cells would have to come from tumor cells released into the circulation.

CellSearch® system (Menarini Silicon Biosystems), is one of the most commonly used systems based on immunoisolation, and it is the only system approved by the Food and Drug Administration (FDA) for the isolation and detection of CTCs in metastatic breast, prostate and colorectal cancer. This system uses whole blood and bases the isolation of CTCs on the expression of EpCAM, using particles with a magnetic core functionalized with anti-EpCAM antibodies. EpCAM-positive cells are then sorted in a magnetic field and immunostained for cytokeratins (CKs) and CD45. The current CellSearch® system defines a CTC as an event that has a nucleus (DAPI positive); expresses cytokeratins (CK8, CK18 and CK19); does not express CD45 and it is more than $4 \times 4\ \mu m^2$ in size. This system is mainly used for CTC enumeration, and since blood samples are usually collected in tubes containing fixatives for cell preservation, cells isolated by CellSearch® would no longer be suitable for downstream gene expression or functional analysis. However enriched samples could be used for additional phenotypic characterization or subsequently single cell isolation for DNA sequencing, in combination with other specific technologies.

Although CellSearch® system is widely used for CTCs enumeration, it presents some drawbacks since it only detects CTCs in about 70% of MBC patients [8], and in the non-metastatic setting the sensitivity is much lower. This limitation could be partially explained by the loss or low EpCAM expression in some tumor cell populations (mesenchymal or stem subpopulation).

Table 4.1 Technologies for the isolation of CTCs

BIOLOGICAL PROPERTIES			
POSITIVE SELECTION	**Methodology**	**Advantages**	**Disadvantages**
	CellSearch®	FDA approved Analysis can be made from whole blood Allows fluorescence analysis Clinical relevance, semiautomatic	Based on the expression of cell surface proteins(EpCAM) Expensive equipment Viable cell recovery is not possible
	AdnaTest	Allows gene expression analysis High sensibility	Based on the expression of cell surface proteins (EpCAM, CA 15-3 (MUC1), HER2, ER and PR (optional))
	Dynabeads	Allows the isolation by custom antibodies Isolate viable cells	Based on the expression of cell surface proteins (EpCAM and others)
	CellCollector®	CE approval In vivo sample processing allows the screening of high blood concentrations Isolate viable cells	Based on the expression of cell surface proteins (EpCAM) More invasive for the patients, nowadays is not implemented due the limitations of the time needed for the analysis
NEGATIVE SELECTION	RosetteSep™	Isolate viable CTCs Independent of epitope expression	Antibody-labelling alters cell density
PHYSICAL PROPERTIES			
DENSITY	**Methodology**	**Advantages**	**Disadvantages**
	RareCyte® LeukApheresis, Ficoll-Paque™	Independent of equipment, faster Independent of surface proteins Isolate viable cells LeukApheresis allows the screening of high volumes of blood	Low specificity and efficiency (CTCs loss in plasma fraction) Higher "contamination" with leukocytes
FILTRATION	ISET®, MetaCell®, ScreenCell®, Celsee Genesis system	Preserves integrity Shorter times of sample processing Independent of surface proteins	Processing of the sample need to be done within 4 hours of collection Possible blood clogging on the filter which stops filtration.
MICROFLUIDICS Label-free & label-based	Parsortix™, ClearCell® FX1, CTC-Chip, LiquidBiopsy®, Target Selector™, IsoFlux, ^{HB}CTC-Chip, CytoTrapNano™	Isolate viable cells Controllable and tunable flow patterns Offer ability to multiplex platforms Easy operation	Clogging problems can limit the flow
DIELECTROPHORESIS	ApoStream®, DEPArray™	Viable cell isolated Independent of surface proteins	High-intensity electric field - step-by-step operation needed

(continued)

Table 4.1 (continued)

SINGLE CELL ANALYSIS	DEPArray™, VyCAP, Celsee Genesis system	Single CTCs and cluster detection Allows single cell molecular heterogeneity analysis	Requires high sample processing time (except VyCAP)

FDA Food and Drug Administration, *CE* abbreviation of French phrase "Conformité Européene" which literally means "European Conformity", *EpCAM* epithelial cell adhesion molecule, *HER2* human epidermal growth factor receptor 2, *ER* estrogen receptor, *PR* progesterone receptor

Regarding its performance in the different molecular breast cancer subtypes, it has been described that CTCs are mainly detected in Luminal subtype and rarely in Triple Negative (TN) patients. Despite of these limitations, the presence of ≥ 5 CTCs/7.5 ml blood was shown to be significantly associated with a shorter overall and progression-free survival (OS, PFS) in MBC patients [9]. Some studies had supplied evidence that the positivity of CTCs 5 years after the diagnosis of Luminal BC provided independent prognostic information for late clinical recurrence [10]. In addition, the prognostic relevance of the CTCs before and after adjuvant chemotherapy has been demonstrated (Success Study Group) [11] and even 2 years after chemotherapy or neoadjuvant therapy [12]. Thus, the prognostic value of CTCs detected by CellSearch® technology is clear in BC patients, which have encouraged other technologies to improve the efficiency of detection of CTCs in MBC patients and to offer other alternatives for downstream analyses.

AdnaTest (Adnagen, Qiagen), is an immune-based method for CTCs isolation and gene expression analysis. It consists of a kit containing magnetic beads conjugated with a cocktail of antibodies (EpCAM, CA 15-3 (MUC1) and HER2; and optional detection of ER and PR), specifically optimized for BC. This system is used for gene expression analysis by real-time multiplexed PCR (RT-PCR) of a panel of relevant tumor markers and characteristic of this tumor type. This technology has been used to identify gene expression signatures in CTCs from MBC patients in association to therapy response [13, 14], and interestingly it has shown that ERBB2 overexpression in CTCs from patients with HER2-negative primary tumors significantly associates with disease progression [14].

CellCollector® (Gilupi) and **Dynabeads** (CELLection™ Epithelial Enrich Dynabeads™; ThermoFisher) are two other methodologies for immune isolation of CTCs based on EpCAM recognition. CellCollector® is the first in vivo CTC-isolation product that has CE approval. It is a medical wire coated with anti-EpCAM antibodies directly placed in the bloodstream of a patient through a permanent catheter (size 20 G). It remains inserted in the vein of the arm for 30 min, getting in contact with a larger volume of blood and allowing the capture of CTCs in vivo. In a study with BC patients, this system successfully enriched EpCAM-positive CTCs in 83.3% of patients, with a median of 5.5 (0–50) CTCs. CTCs were not detected in healthy volunteers but could be isolated from patients at early stages in whom distant metastases have not yet been diagnosed [15]. However, its implementation in the clinic is not simple as it requires manual screening for the detection of CTCs. Dynabeads are EpCAM-coated magnetic beads added to the blood sample and allowed to interact for a short time, then the cells bound to the beads (CTCs) are separated with a magnet. Dynabeads have allowed the detection of EpCAM-negative/low CTCs from MBC patients by customizing the beads coating with different antibodies specific for surface proteins and extracellular matrix proteins [16]. Moreover, this technology has been used to detect CTCs with epithelial-mesenchymal transition and stemness features from BC patients [17].

Both systems, CellCollector® and Dynabeads, allow the recovery of viable cells which are suit-

able for subsequent characterization through assays including gene expression analysis and cell culture.

4.2.1.2 Negative Selection

Negative selection, it is based on the depletion of hematopoietic cells by targeting specific antigens of this cell lineage, allowing the enrichment of circulating epithelial cells. The most commonly used antigen is CD45 that is expressed in hematopoietic cells. Under these strategies, the non-targeted fraction of cells is the one of interest.

RosetteSep™ (EasySep™ Direct Human CTC Enrichment Kit, Stemcells), one of the most commonly used methodologies for negative immunoisolation [18]. This is an immunodensity cell isolation kit designed to enrich circulating epithelial tumor cells from fresh whole blood. The kit contains a cocktail of antibodies that recognizes blood cell antigens. There is a specific version of RosetteSep™ for breast cancer samples that includes the anti-CD56 antibody. Upon a density gradient centrifugation, the unwanted cells settle, and purified tumor cells are present as a highly enriched population at the interface between the plasma and the density gradient medium. Cells obtained are suitable for downstream analyses such as molecular phenotyping, gene expression or single cell analysis. In addition, as the recovered cells can remain viable, it is an optimal strategy for functional analysis, as demonstrated by Ramirez et al. who performed a subsequent secretome analysis using EPISPOT system [19], or by Baccelli et al. and Pereira-Veiga et al. who were able to generate CTC-derived xenograft (CDX) mouse models from CTCs isolated from BC patients [20, 21].

4.2.2 Strategies Based on Physical Properties

The most important advantage associated to the technologies based on the physical properties of CTCs relies in the fact that they are independent of the recognition of surface markers ("label-free methods" or "epitope-independent methods"), therefore aimed to capture a more heterogeneous population of cells. Contrary to immunoisolation-based techniques, this approach allows the isolation of cells with epithelial and mesenchymal phenotypes. Therefore these technologies are appropriate to isolate CTCs with EpCAM-low/negative expression levels, as it occurs on triple negative (TN) BC patients. Additional, these technologies are less aggressive since they are not based in chemical interactions, thereby increasing cell viability. Technologies based on physical properties work by trapping the CTCs in a device to obtain an enriched population, while blood cells are discarded. However, there are some disadvantages related to this isolation approach, as it can cause the deformation and damage of CTCs by filter pores, as well as, the loss of those CTCs with smaller size than average. Also, CTCs have higher plasticity than normal cells so that they can squeeze and pass through the devices being undetected. In the other hand, larger size cells that are not cancer cells could be retained together with the isolated population, often contaminated with megakaryocytes, which are cells commonly found in the circulation of cancer patients who underwent chemotherapy [22, 23].

4.2.2.1 Density Centrifugation

Cell density is one of the first cell physical properties applied for the isolation of CTCs. Technologies based on this physical property take advantage of the differences in density between cell populations for the separation when submitted to a gradient centrifugation. Under these conditions, cells are retained in the buoyant per their relative density. However this methodology has several limitations being the most important the lack of specificity, which means the loss of some CTCs during the process. Currently, density gradient centrifugation is employed as a preliminary step prior to the application of another detection and isolation methodologies.

RareCyte® (RareCyte, Inc.) platform, integrates a density-based cell separation device (AccuCyte®) that allows the separation of the CTC-containing blood fraction due its density difference. This technology allows sample deposition onto microscope slides, automated multi-

parameter fluorescence staining, image scanning, analysis, review and mechanical CTC retrieval [24]. The platform utilizes six fluorescence channels, of which four of them are used to identify CTCs and two are available for custom markers. Single-cell retrieval from fixed slides is compatible with whole genome amplification methods for genomic analysis. This technology has been successfully used to collect CTCs from the blood of a breast cancer patient-derived xenograft (PDX) model, allowing for subsequent analyses which suggested a potential key role for Six1 (an EMT-inducing transcription factor) in metastatic dissemination [25].

Diagnostic LeukApheresis (DLA). The use of CTCs in the clinical practice remains a challenge due to their low frequency, particularly in the non-metastatic setting. DLA has been recently introduced as a more sophisticated strategy for the detection of CTCs. Leukapheresis is a standard clinical method based in the screening of liters of blood for the specific collection of cellular components with various applications such as stem cell harvest. DLA is presented as a possible solution to overcome the low frequency of CTC, since it allows the screening of large volumes of blood [26]. The basic principle of DLA is the collection of mononuclear cells (MNC) from peripheral blood by continuous centrifugation. Since epithelial cells have a similar density compared to MNCs [27], CTCs can be isolated together with the MNCs collected during this procedure. In a study comparing the prevalence of CTCs in DLA products (2 mL) with matched peripheral blood samples (7.5 mL) from different cancer types using the CellSearch® system, CTC could be detected in 72% of DLA samples as compared to a 28% in peripheral blood samples, and with a much higher CTC count per mL in DLA samples [28]. Moreover, analysis of DLA products from non-metastatic BC patients showed that CTCs could be detected in 90% of the samples, and CTC frequency correlated to tumor stage. Importantly, CTCs present in DLA products are viable and after an enrichment step can be used to establish CTC cultures [29].

4.2.2.2 Filtration

Filtration is a size based methodology, wherein the blood cells can pass through the filtration device because are smaller than the pore size (6.5-8μm), while larger cells like CTCs are trapped. These technologies have the advantage that they work as a kit completely independent of any equipment. Some examples of commercially available technologies that isolate CTCs by filtration are:

ISET® (Isolation by SizE of Tumour cells; Rarecells diagnostics). This technology allows the isolation of rare cells and CTCs by blood vertical filtration of fixed samples, preserving cell integrity for further analysis. ISET® technology enables the isolation of CTCs and circulating tumor microemboli (CTM, potentially important cancer biomarkers; also referred to as CTC clusters) for almost all types of cancer (breast, lung, prostate, liver, etc.). Numerous clinical studies have chosen ISET® technology for isolation of CTCs [30]. A study published by Farace et al. proved clear discrepancies between CellSearch® and ISET® technologies with regard to the enumeration of CTCs in metastatic patients, including BC patients. The study showed that tumor cells undergoing EMT (characterized by the loss of epithelial markers and neoexpression of cytoplasmic mesenchymal markers) are not detectable by CellSearch®, whereas ISET® system is much more efficient at identifying these cells. Hence, the study has validated ISET® effectiveness for CTC isolation and proved how technologies based on the isolation of EpCAM-positive cells populations, show limitations, especially in patients with metastatic lung carcinoma [31].

MetaCell® (MetaCell s.r.o.), this size-based technology allows the filtration of up to 50 mL of blood through a membrane with pores of 8 μm diameter. The technology is fast and the collection of the CTCs on the membrane takes 2 minutes for a 10 ml blood sample. It is a non-aggressive technology, thus, after the separation process, viable intact cells are suitable for subsequent characterization and/or in vitro cultivation over the filtration membrane. A study published by Jakabova et al. demonstrated the efficacy of

MetaCell® for the isolation of heterogeneous CTCs from BC patients, which have lost epithelial antigens as the result of the EMT process. In a cohort of 167 BC patients (stage I to III) they were able to detect CTCs in 76% of patients [32].

ScreenCell® (ScreenCell), is a technology for isolating circulating rare cells (including CTCs) from whole blood or other biological fluids that takes only 3 minutes. The technology is available in three different kits (ScreenCellCyto®, ScreenCell MB®, ScreenCell CC®). This system allows the recovery of live cells for further cytological studies such as enumeration and cell structure characterization, genomic analyses, and cell culturing for functional characterization. This technology has been shown to be useful for enrichment and identification of circulating tumor associated cells as well as for downstream genetic characterization of CTCs isolated from MBC patient [33].

4.2.2.3 Microfluidics

Nowadays microfluidics are one of the most popular technologies because they provide many attractive advantages for CTC studies such as continuous sample processing to reduce target cell loss, and easy integration of various functions as "do everything -on-a-chip". The isolation process is founded on the differences in size and deformability between CTCs and blood cells and the hydrodynamic flow applied in the devices.

Parsortix™ (Angle plc), it is a low cost system and easy to manipulate, based in microfluidic technology, presented as a disposable cassette to capture and then harvest CTCs from whole blood. Cassettes have a critical gap size of 6.5 μm in which CTCs get retained. CTC capture is based on their larger size and less deformable nature when compared to other blood cell components. Additionally, it allows easy harvesting of CTCs providing viable cells for later staining and/or genetic analyses, and the possibility of in vitro cell culture. The system can analyze from 100 μL to 30 mL blood sample. The Parsortix™ reproducibility, high capture efficiency, and ability to produce highly enriched viable cells, has been validated by different groups. Lampignano et al. published a protocol to enrich, detect and isolate EpCAM-negative CTCs from MBC patients, by combining potentials of both the Parsortix™ together with the automated micromanipulator CellCelector™. This workflow allows for further molecular characterization of CTCs such as the evaluation of the heterogeneity of PIK3CA mutational status within patient-matched EpCAM-high and EpCAM-low/negative CTCs in MBC patients [34].

ClearCell® FX1 (Clearbridge Biomedics) system is an automated cell retrieval system that allows the enrichment of CTCs from small amounts of blood in a relatively short time. This microfluidic biochip isolates CTCs based on size, deformability and inertia cell flow, relative to other blood components, by using inherent vortex flows present in their curvilinear channels, termed Dean Flow Fractionation (DFF). Through the process of DFF, blood cells separate and distribute themselves within the channels of the CTChip® FR1S (the chip inserted in the ClearCell® FX1 system), with the larger cells along the inner wall and the smaller cells away from it. As opposed to other microfluidic systems, ClearCell® FX1 requires a chemical red blood cells lysis. The intact CTCs are enriched in suspension, which allows for further molecular analyses and diagnostic assays. The technology is able to isolate viable CTCs allowing for an in vitro expansion of the cells as shown by Khoo et al., who were able to predict patient responses to therapy testing anti-cancer therapies on short-term CTC cultures [35].

4.2.2.4 Dielectrophoresis

Dielectrophoresis (DEP) is an isolation technique based on physical properties that takes advantage of the distinct electrical properties of cells (dielectric properties). DEP relates to the movement of cells induced by electric field gradients since CTCs have a unique surface charge that distinguishes them from other cells. Thus, a dielectrophoretic flow field can be used to fractionate CTCs from blood cells based on their differential electrical properties.

ApoStream® (Apocell, Inc.) technology, for the isolation of CTCs based on the different dielectric properties (polarizability) of cells. The

system can analyze from 50 μL to 10 mL of blood sample but need step-by-step operation by the user. It can be applicable for different cancer types including breast cancer. In a preliminary report, ApoStream® allowed the isolation of a heterogeneous population of both EpCAM-positive and EpCAM-negative CTCs in relation to the expression of EMT and stem cell markers, from the blood of patients with primary BC [36].

DEPArray™ (Menarini Silicon Biosystems) system. DEPArray™ is usually a second purification step, used in combination with other enrichment methods (e.g. CellSearch®). This technology combines microfluidics and dielectrophoresis trapping individual cells in dielectrophoretic cages for latter recovery. Cells of interest are identified by image-based selection, isolated and recovered as single cells or pools of cells enabling downstream analysis. This technology has been successfully used in diverse clinical studies in BC. Notably, Mu et al. performed a genomic analysis which detected the TP53 R248W mutation from single and pools of CTCs by targeted sequencing on CTCs isolated from a patient with MBC, matching the mutation on patients´ primary tumor [33].

4.2.3 Dual Combination Technologies

Despite the several technologies that have appeared in the market in recent years, it does not exist a technology either based on the physical or biological properties that can be applied as a standard for the isolation of CTCs. Therefore, in recent years, technologies combining both properties, immunoisolation and the different physical characteristics of CTC, have been developed to increase the efficiency in the isolation process.

The LiquidBiopsy® (Cynvenio Biosyntems, Inc.) platform relies in the immunomagnetic capture of CTCs on blood flow and it is performed on a chip that includes antibodies against EpCAM, Trop2, HER2, and MUC1/CD227. The system allows standard and customized assays. This platform achieves high target cell recovery and purity, and it enables downstream molecular characterization of rare cells and cell-free DNA (cfDNA) using NGS, FISH and immunohistochemistry. Analyses are performed for whole-blood samples. This system has been recently used for the detection of HER2-positive CTCs in BC, showing that these cells can be detected at all stages, including early BC, although detection rate was higher in metastatic patients [37].

Target Selector™ (Biocept, Inc.) platform, has the capability to improve cell capture because it combines two steps, first the sample is passed through a blood collector tube for CTC and ctDNA isolation (CEE-Sure™) and next through a microchannel. The novelty is that this technology inhibits cell clumping and clogging of the microfluidic devices. The blood collection tube is designed to keep the sample at room temperature after blood collections from 5 to 7 days and to prevent blood coagulation preserving cells from lysis during storage or transportation.

IsoFlux (Fluxion Bioscience, Inc.), is based on microfluidics and immunomagnetic capture. The system combines the power of antibody-based magnetic bead separations with the precision of flow cytometry. The system allows the CTC recovery even during early-stage disease and it is applicable to different cancers such as breast, lung, colorectal, prostate, pancreatic, kidney, liver, bladder, etc. [38–42]. IsoFlux allows for CTC detection and enumeration as well as analysis through qPCR or NGS techniques. The system permits up to 4 analyses at the same time and processes from 7 to 10 mL of blood in less than 2 hours and a half.

The CTC-Chip is a dual technology that combines the use of microfluidics chips coated with antibodies for immunoisolation. The chip increases the sensitivity and the performance of the capture of CTCs from whole blood, using a smooth laminar flow that preserves the viability of 98% of the isolated CTCs. The chip is a silicon chip, about the size of a standard microscope slide containing an array of microposts

with a specific geometric pattern coated with antibodies against EpCAM, allowing high-throughput capture of CTCs directly among posts. Staining can be performed to confirm CTCs origin and for enumeration or molecular characterization. The platform is flexible, since different antibodies could be potentially used to functionalize the microposts, resulting in the ability to detect a wide variety of CTC populations. The CTC-chip was shown to successfully identify CTCs in the peripheral blood of patients with MBC, among other cancers; as well as to capture cells EpCAM-low or EpCAM-positive with the same efficiency [43].

^{HB}CTC-Chip. The CTC-chip was further developed in a redesigned version, the herringbone-chip or "HB-Chip". The novelty of HB-Chip is its design that applies the microvortices generation to increase the number of interactions between target CTC and the antibody-coated chip surface. Its capability to isolate CTCs in patients with metastatic disease for different cancers was proved, as well as to isolate CTC clusters [44]. Due to its design, the low shear process allowed to detect clusters of CTCs difficult to detect by applying more aggressive technologies. Using this technology it was reported the presence of mesenchymal traits both in CTCs and CTC clusters from MBC patients, and the association of mesenchymal CTCs with disease progression [45]. In addition, using this technology to capture CTC clusters, Aceto et al. showed how the continuous presence of CTC clusters in the blood of metastatic prostate and breast cancer patients was associated with an adverse clinical outcome. Moreover, it helped to prove that CTC clusters are important oligoclonal precursors of BC metastasis [46].

CytoTrapNano™ (CytoLumina Technologies). This is a technology in premarket validation. The concept of CytoTrapNano™ is cell-affinity substrates with the ability to target a specific type of cancer cell due its morphology. It is a semi-automatic system and coupled with a microfluidic mixer is able to capture and quantify CTCs from a standard blood sample with a high level of sensitivity and specificity.

4.3 Single Cell CTC Isolation

Single cell isolation allows to analyze single cell molecular heterogeneity in a specific manner (without blood cells background). Three of the most popular technologies for single cell isolation base their approaches in the physical properties of the cells.

DEPArray™ system (Menarini Silicon Biosystems), previously described (see Sect. 4.2.2.4).

VyCAP Puncher system (VyCAP B.V). The system allows the isolation of individual CTCs through specific hardware and software. It requires a pre-enrichment step of the CTCs (e.g. Cellsearch®, Parsortix™, RosetteSep™, etc.) that then are sorted applying hydrodynamic forces to drag and distribute single cells in individual microwells of the isolation chip. After sorting, the chip is transferred to the Puncher system. This system allows imaging of the cells for the identification and recovery of individual CTCs in a fully automated manner for their subsequent molecular characterization. VyCAP is a versatile system since it also allows the capture and enumeration of CTCs by filtering cells according to size and stiffness.

Celsee Genesis system (Celsee diagnosis). This is a less known technology also applied for single cell isolation. It is a technology that bases the isolation in gravity forces and size-based exclusion allowing to capture individual cells into individual chambers in a microfluidic slide. The system allows for the capture and retrieval of CTCs for single cell downstream analysis.

4.4 Futures Perspectives in the Technologies Applied to CTCs Isolation and Their Clinical Application

Liquid biopsy is becoming an useful tool for the detection and management of breast cancer. In particular CTCs and ctDNA have gained remarkable attention as biomarkers. This is reflected in the increased number of technologies that have

been patented for CTC isolation in the last decade. Despite so, the only one approved for its application in the clinical practice is the CellSearch®, which was patented more than 14 years ago. This shows the technical challenges that still nowadays need to be solved in order to take some other technologies into the clinic. In spite of the numerous clinical studies related to the analysis of CTCs in cancer patients and the proven usefulness of this biomarker, there are still certain technological limitations related to sensitivity and specificity. Currently, there is a lack of consensus regarding different methodological aspects about the isolation technique to be used, the type of sample, the conditions of collection or storage of the samples or the most suitable candidate biomarker to be used. However, it is anticipated that the clinical importance of CTCs will increase, especially in early stage cancers (when CTCs are present at extremely low frequencies) due to the dynamic development of techniques for the detection and analysis of CTCs, enabling prediction of disease progression. An effort should be made to improve the technologies allowing characterization of CTCs (in addition to enumeration) in order to obtain a clinical benefit in patients with early and advanced BC.

Given the growing interest of the clinical and scientific community on the information provided by the analysis of CTCs, technological advances are being made and large-scale clinical trials are underway. Although CTCs have great potential as biomarker for the diagnosis and prognosis of cancer, CTC enumeration has only informative application in patients with MBC, and enumeration alone does not offer suggestions on treatment selection or predict treatment failure. For this reason, technologies need to be validated in order to overcome the gap between the number of novel technologies developed and the number of them that enter the market and are being used in the clinical practice [47]. Reasons for this are the lack of standardized protocols for technologies validation, difficulty to access patients' samples for technology validation, and the high cost to manufacturing the technologies (takes a long time and money to build a manufacturing facility). Therefore, it is necessary a change on the strategy, and commercialization, as a final goal, should be taken into consideration by researchers from the initial steps of technology development. Moreover several reports have been published making comparison between the different isolation technologies assessing their performances with no clear conclusions, instead the technology of choice should be selected according to the purpose of each individual study, i.e. enumeration, downstream molecular analysis or cell culture [48, 49].

As previously seen, some technologies based only on epithelial markers recognition, fail to reflect all the potential CTCs subpopulations, e.g., EpCAM-negative or EpCAM-low cells [50]. Thus, technologies such as the gold standard, CellSearch®, might underestimate those CTCs with the highest metastatic potential and more invasive phenotype, such as tumor cells that underwent epithelial-to-mesenchymal transition (EMT) or with stemnes features. Indeed, changes in EMT status of CTCs during treatments of individual patients with MBC have been reported, and a correlation between the number of EMT CTCs and therapeutic outcome showed [45]. Therefore, it is very important to elucidate which populations of CTCs are responsible of forming metastasis. The number of CTCs detected in MBC may increase if the EpCAM-high and EpCAM-low CTC populations were considered. A proof of this are studies showing improved CTC detection by employing EMT markers in addition to epithelial markers [51, 52]. However, we should not forget that only the presence of EpCAM-high CTCs correlates with poorer overall survival [53]. Antigen-independent approaches could eliminate the risk of underestimation of the different CTC populations; however, they could increase the isolation of a non- specific population. Although it is well defined that bigger cells mainly correspond to CTCs, there are technologies as Nanovelcro chips [54] or Epic CTC platform [55] that have shown how the presence of small nuclear CTCs correlates with the presence of visceral metastasis, mainly in prostate cancer patients. In this regard, isolation technologies based in the physical properties of CTCs are on

the rise in recent years, with microfluidic-based platforms having a prominent impact in the field.

Therefore, all these evidences highlight the importance of the molecular characterization of CTCs, as different subpopulations of CTCs may relate differently to the clinical outcome. The molecular characterization of CTCs could be instrumental to assess tumor heterogeneity, to predict site-specific metastasis, to detect treatment-resistant profiles and to identify new drug targets. In this sense, technologies allowing single CTC analysis are being put forward, as genetic information gathered from single cell analyses can be used for the accurate monitoring of cancer progression and treatment efficacy. Moreover, it may be useful for a precise selection of molecular prognostic and/or prediction markers, thereby improving the clinical outcomes of patients. Hence, studies are now focused on the molecular characterization of CTC with a clear clinical objective. There are several studies which demonstrate that molecular characterization of CTCs will provide useful information transferable to the clinic, but since different methods are applied both for enrichment and identification of CTCs, it is difficult to compare those results. The molecular characterization of individual CTCs with relevance in therapy has advanced a lot in recent years and it seems technically robust enough to be applied now in the clinical setting. However, detection and isolation of CTCs remains a challenge. In fact, not a single method of enrichment is able to collect each of the possible CTCs present in the bloodstream. In addition, no method will work with 100% efficiency, leading to loss of target cells and the isolation of unspecific cells. However, for treatment decisions it will be of utmost importance to improve CTC isolation efficiency to minimize the number of metastatic cancer patients reported with negative CTC-Test. In addition, an increased yield in CTC isolation will be relevant for the reproducibility of CTCs assays and for the analysis of the heterogeneity of CTCs. In this sense DLA allows for a more reliable detection of CTCs since when analyzing only a small part (around 2 ml) of the DLA product, a 2.5-fold increase in the detection frequency is already observed and 30-fold in median CTC values [28]. This might allow to obtain CTCs in those metastatic patients who tested negative for CTCs in a 7.5–10 ml blood sample or have very few CTCs, for prediction and valid molecular diagnosis, respectively.

In addition, the use of diverse technologies and markers in the detection of CTCs has led to some discrepancies about the classification of a cell as a CTC (at phenotypic level). In this regard, new approaches are being developed as for example the ACCEPT software, a tool for automated CTC classification which was developed within the EU Cancer-ID project. It is an open source image analysis set that performs an Automated CTC Classification, Enumeration and PhenoTyping (ACCEPT). Zeune et al. showed how the ACCEPT image software allows a more reproducible quantification of CTCs analysis offering new fully automated and reproducible approaches. The study was done with a cohort of 132 MBC patients from whom blood samples were processed by CellSearch® and stained for HER2 expression. Images were digitally stored and were sent to six independent investigators to score the HER2 expression with and without ACCEPT. Concordance rate of the operators' scoring results for HER2 on CTCs was 30% and using the ACCEPT tool could increase to 51% [56].

Finally, all technologies developed for isolation of CTCs should fulfill some specific requirements on their performance. Thus, expert researchers in the field have suggested a standard set of performance criteria allowing the comparison and evaluation of technological platforms [30]. Assessment of these criteria, including aspects such as capture efficiency, purity, enrichment, throughput, cell viability, and release efficiency, will impact on the development of systems with a higher sensitivity and specificity, which will ultimately represent a benefit on the results of CTC detection studies. However, a major drawback on these studies is represented by the fact that when evaluating a system´s performance for these parameters, blood samples from healthy donors "spiked" with known num-

bers of tumor cells from cancer cell lines are used, possible over-predicting the device performance. The reason is that samples from patients cannot be directly employed for this purpose since the actual number of CTCs in the sample is unknown. Thus, it is important to validate systems using clinical samples, and this is why these technologies are usually compared to the CellSearch®, the only system cleared for the FDA for clinical use. Therefore, it is clear that there are still challenges that need to be solved in order to implement in the clinic the new technologies developed by researchers and companies.

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