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Review Article

Circulating tumour cells in clinical practice: Methods of detection and possible characterization

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ABSTRACT

Circulating Tumour Cells (CTCs) can be released from the primary tumour into the bloodstream and may colonize distant organs giving rise to metastasis. The presence of CTCs in the blood has been documented more than a century ago, and in the meanwhile various methods have been described for their detection. Most of them require an initial enrichment step, since CTCs are a very rare event. The different technologies and also the differences among the screened populations make the clinical significance of CTCs detection difficult to interprete. Here we will review the different assays up to now available for CTC detection and analysis. Moreover, we will focus on the relevance of the clinical data, generated so far and based on the CTCs analysis. Since the vast majority of data have been produced in breast cancer patients, the review will focus especially on this malignancy.

1. Introduction

The detection of Circulating Tumour Cells (CTCs) in peripheral blood has been described more than a century ago by T.R. Ashworth, an Austrian pathologist who first reported this type of cells [1]. The presence of CTCs in the bloodstream fits very well with the "seed and soil" theory of metastasis formation: tumour cells enter the blood circulation after detaching from the primary tumour and can migrate to reach distant organs, where they can implant themselves and give rise to metastasis. Though metastatic spread represents the ultimate cause of death, the release of tumour cells may happen also at early stages of the disease: about 30–40% of patients, thought to have a localised disease, may in fact present occult metastasis, probably derived from CTCs, which will be responsible for the disease progression [2,3]. According to these considerations, it seems to be likely that CTCs detection and analysis may play an important role in the diagnosis and treatment of cancer patients.

2. CTC analysis

Much of the research of the past decades has been focused on the development of reliable methods for CTC enrichment and identification, mainly trying to overcome severe technical limitations. When present in the patient's blood, CTCs are very rare events with an expected concentration as low as one cell per 10^5–10^7 with respect to mononuclear cells. It is clear that enrichment steps are necessary to increase the isolation success rate. Most of the enrichment methods developed so far make use of specific markers in order to identify the cells and to distinguish them from leukocytes. Among others, the most common epithelial markers are cytokeratins (CKs), cytoskeletal proteins always expressed in epithelial cells, and EpCAM, a cell adhesion molecule present in epithelial cells as well. CTCs identification must be specific and sensitive enough to distinguish the malignant cells from all the other circulating non-tumour hematopoietic cells. Immunomediated, cytometric- and PCR-based strategies have been proposed to increase the chances to distinguish a CTC from the cellular background, with different performance characteristics. Nevertheless, no well-designed comparative investigations have been performed so far, therefore the comparison of results is quite difficult.

3. Enrichment process

Different approaches have been considered to overcome the restrictions linked to the low CTC concentration in peripheral blood. Here the most common methods such as filtration, density gradient and immunomagnetic enrichment are shortly described. Table 1 schematically summarises the different methods, listing their advantages and disadvantages.

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Table 1
Summary of different CTC enrichment approaches.

Enrichment method		Advantages	Disadvantages	References
Size based	ISET	Easy and cheap Feasible with EpCam positive and negative tumour cells	Low specificity Loss of small CTC which can pass through the pores Enrichment of large leukocytes	[4]
	Density gradient	Easy and cheap Feasible for EpCAM positive and negative tumour cells Feasible for negative selection	Low specificity Cross contamination with blood mononuclear cells possible	
	OncoQuick	Density gradient based Feasible for EpCAM positive and negative tumour cells Cross contamination reduced because of additional barrier	Low specificity	[5,6]
	Rosette Sept	Good clean up of unwanted hemapoietic cells	Cross contamination possible	[7,8]
Immunomagnetic based	MACS/Dynal Magnetic Beads/ Easy Sep	Flexible Cell integrity preserved	False positive due to the expression of the same antigens on non-tumour cells False negative due to loss of antigens on CTCs	[10–12]
	AdnaTest	Recognition of fixed markers (EpCAM, MUC1) Downstream analysis (RT-qPCR of MUC1, HER2 and GA73.3–2) Possibility to characterize for stem cell and Epithelial Mesenchimal Transition	No flexibility False positive due to the expression of the same antigens on non-tumour cells False negative due to loss of antigens on CTCs	[13]
	CellSearch	Semi-automated Combination of positive (anti-EpCAM) and negative (anti-CD45) selection FDA approved	Only EpCAM positive CTCs detected False positive due to the expression of the same antigens on non-tumour cells False negative due to loss of antigens on CTCs	[14]
	CTC-Chip	Good enrichment grade 98% cell viability Further analysis possible	Only EpCAM positive CTCs detected Clinical validation not yet available Not yet on the market	[64]

3.1. Filtration

ISET (Isolation by Size of Epithelial Tumour cells) is a size based-direct method for enrichment of epithelial cells. ISET makes use of 8 μm pores filters which enable to separate small leukocytes from the larger epithelial cells [4]. After the filtration step, the putative CTCs can be fixed and stained for different markers such as CKs. Unfortunately, large leukocytes can be trapped by the filter as well, therefore contaminating the CTC fraction or small CTC can pass through the pores therefore depleting the CTC population. This is why this technique is generally considered not highly sensitive and poorly specific.

3.2. Density gradient

An alternative to filtration can be the density gradient centrifugation using Ficoll-Hypaque: because of their difference in density, it is possible to separate CTCs and mononuclear cells (with a density < 1.077 g/ml) from blood cells and granulocytes (with a density > 1.077 g/ml). However, due to the migration of cells to the plasma layer or to the presence of aggregates, CTCs can be easily lost during this process. Moreover, the centrifugation step must be performed immediately to prevent mixing of the different layers. In addition, Ficoll-Hypaque can be toxic for the cells when they are too long in contact with these chemicals, therefore limiting the method. Density gradient, being independent from the presence of specific markers, is considered a method feasible for the enrichment of any kind of CTC, but because of the lack of markers also a method with low specificity. In order to partially overcome some drawbacks presented by the density gradient enrichment, Oncoquick (Greiner Bio-One, Germany) is a separation method based on density gradient having the advantage to prevent cross contamination of the different layers thanks to a special membrane which keeps them separate [5,6].

A step further is proposed by Stem Cell Technology (Vancouver, Canada) which offers RosetteSep™, a density gradient method based on negative selection [7,8]. When mixed with whole blood, an antibody cocktail forms tetrameric complexes (immunorosettes) recognising cell surface markers on unwanted human hematopoietic cells. The immunorosettes can easily be removed by a density gradient procedure while the CTCs can be collected from the interface between the plasma and the buoyant density medium.

3.3. Immunomagnetic cell enrichment

The immunomagnetic cell enrichment is a magnetic bead-based separation technology. CTCs are positively selected by means of antibodies coupled to magnetic beads targeted to epithelial markers (such as the already mentioned CKs or EpCAM), or to tumour-specific antigens, for examples to the CarcinoEmbryonic Antigene (CEA) or to the Human Epidermal growth factor Receptor 2 (HER2). The CTCs, once bound to the magnetic beads, are then separated from the leukocyte background through a magnetic field. In order to get rid of leukocytes, a valid alternative procedure can be a negative selection, performed by using magnetic beads coated with anti-CD45 and anti-CD61 antibodies. Both molecules recognise surface markers expressed only on leukocytes and megakaryocytes, respectively. In this way the cellular population is depleted of unwanted cells, while the epithelial cells, not expressing CD45 or CD61, are left in solution.

The clear advantage of this approach is that detection and counting of the enriched CTCs are relatively easy, without the requirement of any cellular lysis. However, as it will be discussed later, false positive selection may occur due to expression of the epithelial markers in non-epithelial cells [5]. In addition, false negative selection may be due to the heterogeneity of tumour cells which may variably or not at all express a given marker [9].

Based on this technology, the Magnetic Activated Cell Sorting System (MACS®, Miltenyi Biotec GmbH, Germany) [10] or the Dynal Magnetic Beads® (Invitrogen) [11] are technologies both enabling to catch CTCs by immunomagnetic labelling with microbeads. As described before, the beads, coated with antibodies specific for epithelial or tumour markers, bind to epithelial derived

circulating tumour cells. After the separation in the magnetic field, the cells can be isolated for further analysis. In a similar way works EasySep® (Stem Cell Technologies, vancouver Canada) [12]. This is an immunomagnetic cell selection method where cells are targeted for positive or negative selection using monoclonal antibodies directed against specific cell surface markers. The labelled cells are then interacting with the magnetic nanoparticles and separated with the use of a magnet.

The AdnaTest (AdnaGen AG, Germany) is based on the use of specific antibodies against epithelial and tumour markers, such as EpCAM and Mucin 1 (MUC1), associated to magnetic beads [13]. This test allows a step further: once collected and pooled together, the CTCs are directly lysed, the mRNA isolated, and RT-qPCR is performed for the analysis of the selected markers MUC1, HER2 and the surface glycoprotein GA73.3.–2, in this way genetically identifying the CTCs.

Finally Veridex (Johnson and Johnson, Raritan, NJ) offers the CellSearch System™, a semi-automated analyzer enriching the CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibodies. The enriched population is then stained with specific markers able to discriminate between epithelial cells and contaminating leukocytes. The enriched and correctly labelled cells are then counted as circulating tumour cells using the CellSpotter analyzer (Veridex), a four-colour semi-automated fluorescent microscope [14].

One general limitation to keep in mind when considering the enrichment of CTCs, is that it is still not available a "universal marker" which could be used independently from the type of tumour involved. Tumour markers such as HER2, mammoglobin, CEA, can directly distinguish tumour cells from non-malignant epithelial cells or leukocytes but their use is limited by the general heterogeneity tumour cells are known to show. This limitation applies not only to different genetic characteristics among different tumour tissues, but also to different genetic characteristics within the same tumour tissue, making the identification and enrichment of CTCs based on these markers potentially challenging.

The alternative would be to identify CTCs on the base of epithelial markers like EpCAM and CKs. Nevertheless there are some limitations also in this case. CKs can be found sometime expressed even in leukocytes when these are in an activated state [15]. Moreover, the EpCAM marker can be down-regulated in malignant epithelial cells when these go through the so called Epithelial to Mesenchimal Transition (EMT), typical of the secondary metastatic phase. Cells undergoing this phase lose epithelial characteristics like the expression of specific markers [16–18].

In conclusion, tumour and epithelial markers, widely used in different enrichment methods, are not necessary able to detect all the different types of CTCs.

4. Identification process

Once collected, CTCs can be further characterized, in order to establish their origin and their genetic profile. Most of the available protocols, are focused either on the nucleic acid content or on the protein level. A summary of the different methods is presented in Table 2; advantages and disadvantages of the different methods are listed as well.

4.1. PCR-based analysis

This is certainly an extremely powerful technique for CTC genetic screening and in several studies PCR has been shown to be more sensitive than immunocytochemistry [19,20]. Specificity is achieved through the design of oligonucleotide primers targeted to the gene(s) of interest. Reverse transcription PCR (RT-PCR) is

Table 2
Summary of different CTC identification approaches.

Identification process		Advantages	Disadvantages	References
PCR- based analysis	RT-PCR	High sensitivity	RNA degradation False positive results due to unspecific amplification, contaminations, pseudogenes No distinction between viable and non-viable cells False negative results due to low expression level	[19–27]
	RT-qPCR	High sensitivity Quantitative	No visualisation of CTCs No further analysis possible	[28–32]
Cytometric analysis	FAST	Scan analysis of large volume of sample Cell loss minimised Quick analysis (up to 300,000 cells/s)	Lack of validation studies in clinical settings	[33,34]
	LSC	Fast High specificity	Technically challenging Low sensitivity	[35,36]
	Flow cytometry	High specificity Multiple parameters	Low sensitivity	[37,38]
	CellSearch	Semi-automated High sensitivity CTC quantification Reproducible Recognition of fixed marker (EpCAM, CKs, CD45) FDA approved	Only $EpCAM^+/CK^+/CD45^-$ CTCs detected Subjective images interpretation No further analysis possible	[14,42–45]
	CTC-chip	98% Cell viability High detection rate Further analysis possible	Only EpCam positive CTCs detected Not commercially available Lack of validation studies in clinical settings	[46]
	EPISPOT	Analysis only on viable cells High sensitivity	CTC isolation not possible, therefore further analysis not possible Need of active protein secretion Technically challenging	[47,48]
	FISH	Genetic analysis	Further analysis not possible	[4,51,52]

generally the method of choice to amplify target mRNAs in CTCs [21–24]. Many "single-institution" reports on the prognostic impact of data obtained with the PCR-based approach are available, but multicenter validation studies have usually not yet been performed. Nevertheless, a variety of biomarkers, both for epithelial cells and for breast cancer cells have been used in many different studies (a comprehensive overview of markers and detection rate has been previously reviewed in [25–27]). In order to increase the sensitivity and the specificity of the assay, multiplex RT-PCR approaches have been often established, giving therefore the chance to screen at the same time for more than one single marker. There are some technical limitations though which may challenge the performance of the PCR-based assays: (1) The need to perform cellular lysis, which prevents from cell counting; (2) The possibility of false positive results due to illegitimate gene transcription in non-tumour cells; (3) The amplification of cell free nucleic acids potentially present in blood; (4) The possibility of false positive results derived from the use of unspecific markers; (5) The presence in the blood of non-malignant epithelial cells, released for example after an invasive procedure; (6) The possibility of false negative results due to low PCR sensitivity connected to limited expression of the tumour marker or to the presence of PCR inhibitors.

Some of the drawbacks can be overcome by combining more than one marker together and by choosing only the cells which are positive (or negative) for all of them in combination. Concerning the possibility to get false positive results because of illegitimate gene transcription in non-tumour cells, a big improvement in the CTC analysis has been achieved with the introduction of the quantitative real time PCR (qPCR) [28–31]. The quantification of markers present simultaneously in tumour and non-tumour cells allows the discrimination between authentic CTCs and contaminant cells. Moreover false positive results can be skipped with an appropriate primer design to avoid the amplification of processed pseudogenes, present in the genome [32].

4.2. Cytometric methods

Cytometric methods isolate and count cancer cells using monoclonal antibodies directed against different antigens, the most common being once more CKs and EpCAM. As lysis is not needed, the cell integrity is always preserved, therefore CTCs can be further scanned and characterized. Also in this case, the major limitation connected to the methodology is linked to the relative low specificity. For example, the most widely used anti-CK antibodies show to bind not specifically also to hematologic cells. This limit can be partly overcome combining the existing antibodies to an anti-CD45 antibody, in this way making possible the leukocytes detection. Concerning EpCAM, as already discussed, not all CTCs express this marker, since it can be down-regulated in malignant cells during the EMT. Not even its association with tumour markers can help to bypass completely this limitation because of their expression variability in tumour cells, with the consequent high risk to generate false negative results.

Nevertheless, once the cells have been immunofluorescently labelled, there are different methods to reliably scan them.

FAST (Fiber-optic Array Scanning Technology) [33,34] is a scanning technology characterized by a large field of view, therefore allowing the analysis of large volumes of sample without any purification step and minimising the risk of cell loss. Moreover, it is a very fast scanning technology with up to 300.000 cells scanned per second. At the moment though, validation studies in clinical settings are still missing.

The Laser Scanning Cytometer (LSC, Compucyte Corporation, Cambridge MA) makes possible to automatically scan and relocate epithelial positive cells immunolabelled for multiple markers such as EpCAM combined with the lymphocyte marker CD45 [35,36].

Also flow cytometry can be a valid method to identify CTCs in a high specific way since multiple parameters can be considered simultaneously, such as size, viability, DNA content, expression of different markers [37,38]. Nevertheless both flow cytometry and LSC, despite their high specificity, show low sensitivity therefore high amount of samples need to be processed every time in order to detect few CTCs.

Finally, additional scanning systems are available on the market such as ACIS (Automated Cellular Imaging System, DAKO, Denmark) and ARIOL (Applied Imaging Corp. San Jose, CA). Both allow an automated and fast cell analysis, based on the morphological evaluation of putative CTCs [39–41].

In the last years many efforts have been devoted to the development of automated techniques offering at the same time the enrichment, the staining and the scanning of the samples. The CellSearch System™, previously described, enriches the CTCs with ferrofluid nanoparticles coated with anti-EpCAM antibodies. The enriched $EpCAm^+$ cell population is then stained with phycoerythryn conjugated antibodies directed against CK8, 18 and 19, with allophycocyanin conjugated antibodies specific for leukocytes (anti-CD45 antibodies) and with the nuclear dye 4′,6-diamino-2-phenylindole (DAPI) for the nucleic acids staining. The resulting $CK^+/DAPI^{+/}CD45^-$ cells are counted as CTCs using the CellSpotter analyzer (Veridex), a four-colour semi-automated fluorescent microscope [14]. After the scanning, the images taken by the microscope are displayed in a photo gallery which is then further analysed by an algorithm to finally qualify the cells as CTCs (Fig. 1). To date this is the only FDA approved method to monitor patients with advanced breast [42,43], prostate [44] and colon [45] cancer. The permeabilization of the cells, necessary for CKs staining, is preventing any further use of the CTCs. When assays like colony formation are desired, Veridex offers the Cell Profile™ kit which collects viable EpCAM positive cells before the permeabilization step.

Alternative to the CellSearch System™, the new technology named "CTC-chip" consists of an array of 78.000 microposts coated with anti-EpCAM antibodies. Whole blood is pumped through the chip and EpCAM positive cells are captured and detected by cameras recognising their morphology, their viability and the expression of tumour markers [46].The limitation of this system is that the CTC-chip is suitable only for EpCAM positive cells. Moreover, validation studies in clinical trials are also still missing.

Finally, another antibody-based approach is EPISPOT (Epithelial Immunospot), an immunological assay based on the ELISPOT (Enzyme-Linked Immunosorbent assay) technology [47,48]. With EPISPOT it is possible to identify and count only viable and not apoptotic cells. The assay is in fact based on the identification of cells able to secrete proteins like MUC1 and CK19 in short term culture. The information is important since only viable cells would be in principle the one generating secondary tumours. Like with the CTC-chip though, clinical trials are still missing.

5. Genetic characterization

Once the CTCs have been enriched and isolated, the next desirable step is certainly their genetic characterization. Genetic analysis might help to understand, for example, if the isolated CTCs show still malignant characteristics or how genetically similar to the primary tumour the CTCs may be. This information would certainly allow to better monitoring the development of the disease giving the opportunity to analyse relevant gene segments, which might have an influence on the tumour development and possibly on the following therapeutic approaches.

For a PCR-based genetic analysis of single CTCs, AmpliGrid (Beckman Coulter Genomics, Munich, Germany) can be an ideal platform [49,50]. This is a PCR-based chip for direct analysis of sin-

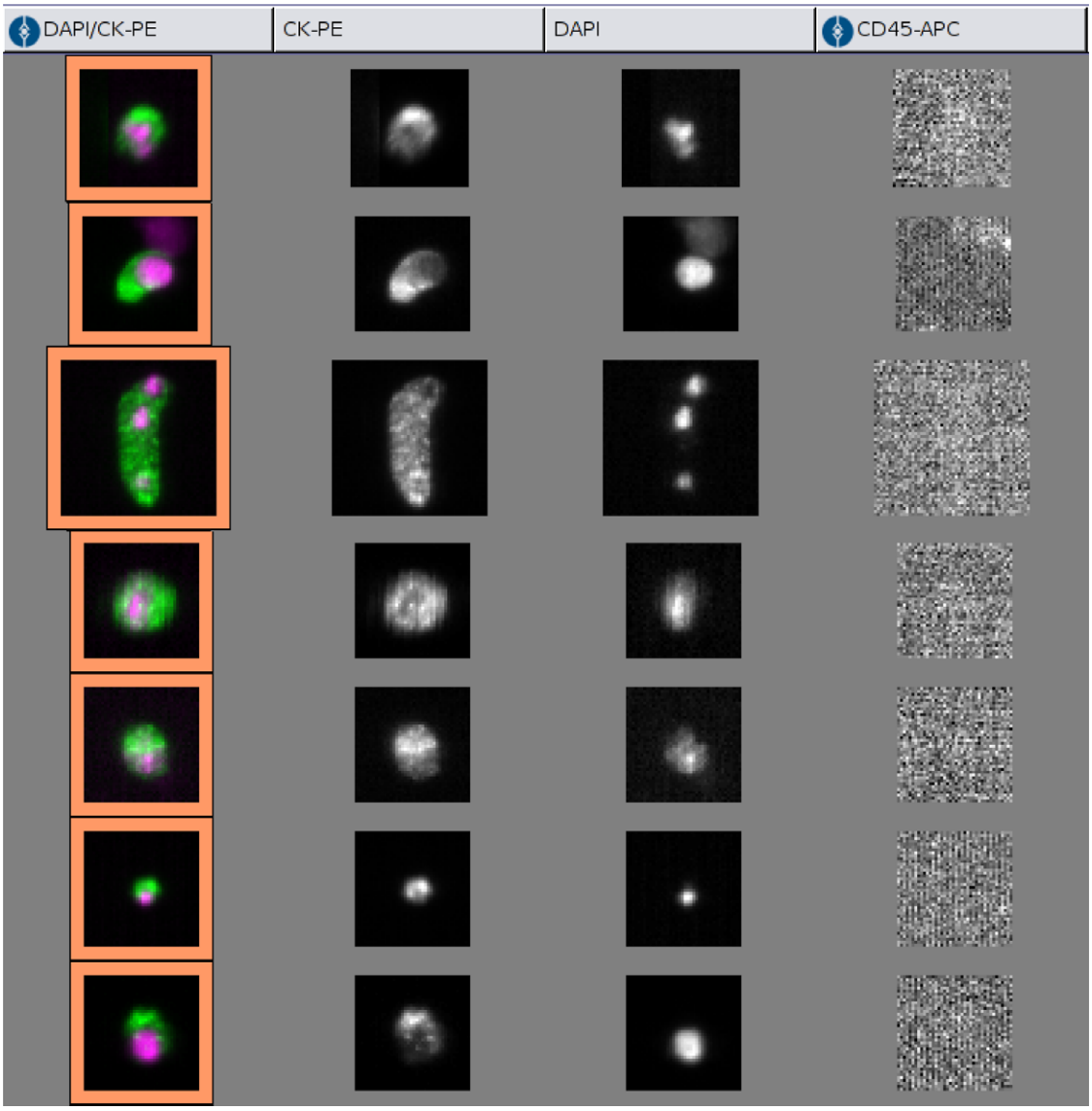

Fig. 1. CTCs identification using the CellSearch system. After the scan performed by the automated fluorescence microscope, the software present on a computer screens a gallery of images to be interpreted: The cytockeratin-PE positive, DAPI positive and CD45 negative images are counted as CTCs.

gle CTCs which allows to genetically determinate possible differences among the isolated CTCs. After deposition on the reaction site, single CTCs can be analysed by multiplex RT-PCR or RT-qPCR, unravelling for example cellular heterogeneity, epigenetic features, genetic fingerprint or microRNA levels (Fig. 2). Preliminary data obtained in breast cancer patients showed that it is possible to genetically characterize isolated CTCs by RT-qPCR run on AmpliGrid, detecting specific tumour markers such as Keratin 5 and 18 (Görner and Bonizzi, data not shown). Nevertheless while the AmpliGrid platform is already commercially available, its use for CTCs genetic analysis is under evaluation and large-scale clinical trials are still missing.

FISH (Fluorescent *In Situ* Hybridization) has been proposed as a valid method for CTC genotyping [4,51,52]. FISH is a fluorescent technology thought to detect and localise the presence or absence of specific DNA sequences on chromosomes or their alteration due to gene translocation or amplification.

An alternative method frequently used in genetic characterization and therefore potentially useful for CTC analysis is CGH (Comparative Genomic Hybridization) [53]. This is a cytogenetic method for the analysis of changes in copy number in a given DNA.

Finally, immunostaining can be a valid approach for detection of tumour markers. A cellular protein can be detected by the corresponding specific antibody making possible to unravel the presence or absence of the protein, its sub-cellular localisation, changes in its expression or finally its degradation.

The different discussed methods for enrichment and analysis of CTCs are summarised in Fig. 3.

6. Clinical data

CTCs have been evaluated in different malignancies, however the larger number of data are available from breast cancer. A summary of the clinical study results is presented in Table 3.

6.1. Prognosis

Despite all the technical limitations, numerous clinical studies have been published in a relatively short period of time. However, it is quite difficult to draw firm conclusions: because of different assays, alternative definition criteria for CTCs, different clinical populations, limited number of patients, different stages of the dis-

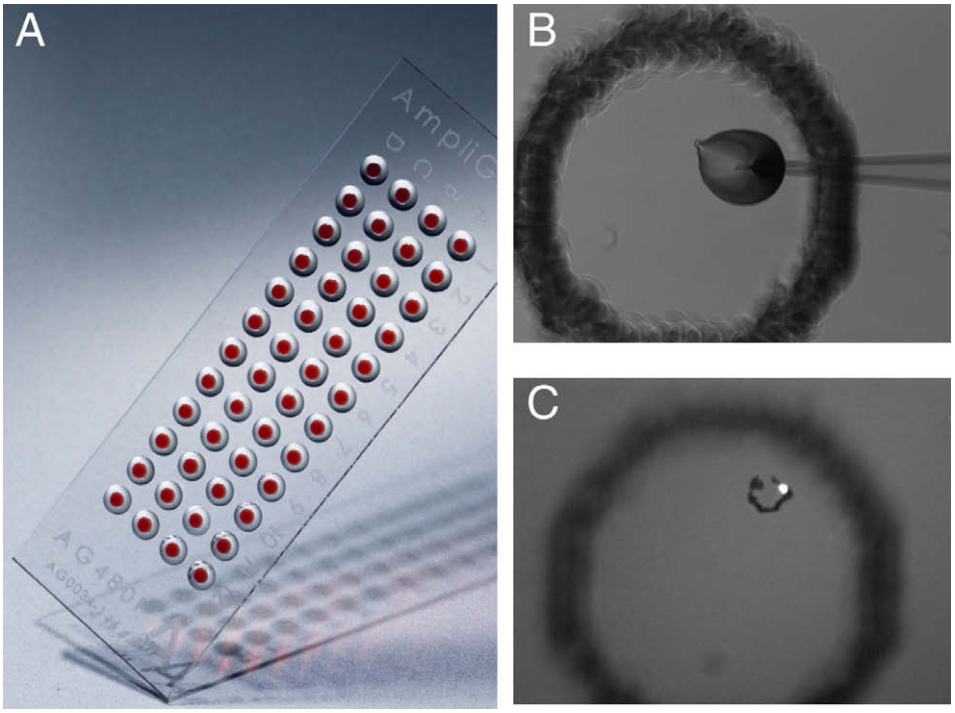

Fig. 2. Deposition of a single CTC on the AmpliGrid chip. (A) AmpliGrid chip with 48 reaction sites for single cell deposition and PCR analysis. (B) A single CTC is deposited with a capillary on the reaction site of the Ampligrid chip. CTCs are normally resuspended in physiological buffer and the volume of deposition is approximately 20 nl. (C) A single CTC can be easily visualised on the reaction site when the DNA is stained with Hoechst dye.

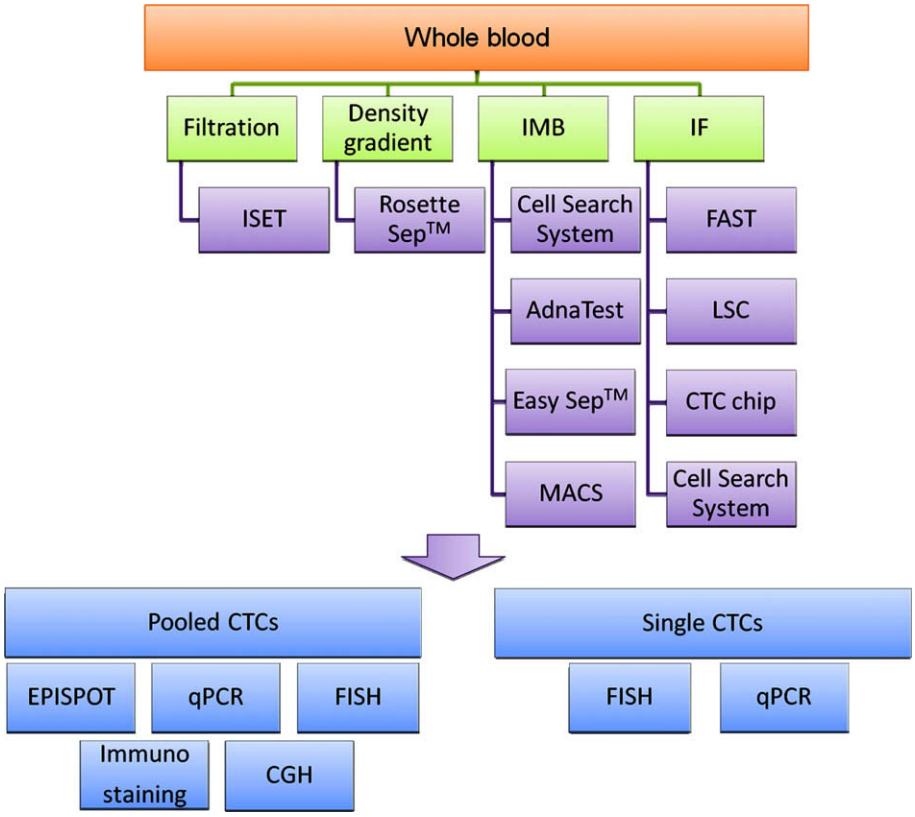

Fig. 3. Overview of the different methods for enrichment and analysis of CTCs. In order to run any kind of analysis, CTCs must be as a first step enriched from the whole blood. Different protocols based on filtration, density gradient or immunomagnetic beads (IMB) can be followed in order to isolate these rare cells. The CTCs can be processed then by RT-PCR or real time PCR for messenger quantification, by FISH and by CHG for analysing chromosome sequence and number aberrations, by immunostaining for protein detection or by EPISPOT for detection of viable cells. CTCs can also be analysed single wise by FISH or by RT-PCR and real time PCR for example when placed on the AmpliGrid slide.

Table 3
Clinical relevance of CTC detection in breast cancer patients.

Study	Tumour stage	Method	Number of patients	CTC detection rate (%)	Clinical results
Cristofanilli et al. [42]	Metastatic breast cancer	CellSearch	177	49	CTCs $\geqslant$ 5/7.5 ml associated with reduced PFS ($P < 0.001$) and OS ($P < 0.001$)
Nolè et al. [54]	Metastatic breast cancer	CellSearch	80	61	CTCs $\geqslant$ 5/7.5 ml associated with reduced PFS ($P = 0.002$)
Budd et al. [60]	Metastatic breast cancer	CellSearch	138	25.4	CTC evaluation after 4 weeks from the start of chemotherapy are better predictor of OS compared to radiologic response evaluation performed at 10 weeks
De Giorgi et al. [61]	Metastatic breast cancer	CellSearch	115	21	Univariate analysis: CTCs levels at 9–12 weeks ($P < 0.0001$) and FDG-PET/CT ($P \leqslant 0.001$) associated with OS Multivariate analysis: only CTCs levels at 9–12 weeks ($P < 0.001$) associated with OS
Pierga et al. [63]	Locally advanced breast cancer	CellSearch	118	27	CTCs $\geqslant$ 1/22.5 ml associated with reduced DFS (0.017)
Stathopoulou et al. [55]	Early breast cancer (stage I–II)	RT-PCR (CK19)	148	29.7	Reduced DFS ($P = 0.0007$) and OS ($P = 0.01$)
Xenidis et al. [23]	Early breast cancer (stage I–II)	RT-PCR (CK19)	167	21.6	Reduced DFS ($P < 0.00001$) and OS ($P = 0.008$)
Ignatiadis et al. [56]	Early breast cancer (stage I–III)	RT-PCR (CK19)	444	40.8	Reduced DFS ($P < 0.001$) and OS ($P = 0.001$)
Apostolaki et al. [57]	Early breast cancer (stage I–II)	RT-PCR (HER2)	214	21	Reduced DFI ($P = 0.006$), no association with OS ($P = 0.2$)
Xenidis et al. [58]	Early breast cancer (stage I-III)	RT-PCR (CK19)	437	32.7	Reduced DFS ($P < 0.001$) and OS ($P = 0.001$)
Rack et al. [59]	Early breast cancer (stage I–III	CellSearch	1500	9	CTCs > 1/22.5 ml associated with reduced DFS ($P = 0.04$) and OS ($P = 0.03$)

ease, the comparison among different studies and publication results quite challenging. However, keeping these limitations in mind, it's worth to note that the vast majority of studies find an association between the presence of CTCs and the clinical outcome parameters such as the Disease Free Survival (DFS, i.e.the time span between the end of a therapy and the relapse), the Progression Free Survival (PFS, i.e.in the metastatic the time span between the end of a therapy and the progression) or the Overall Survival (OS, i.e. the time span between the end of the therapy and the final patient's death).

6.1.1. Metastatic breast cancer

The study of Cristofanilli et al. [42] showed that the presence of CTCs, detected using the Veridex CellSearch System™, in patients with metastatic breast cancer has an adverse prognostic impact. The authors analysed the blood samples of 177 patients beginning a new therapy. Based upon a training set, the threshold for elevated CTCs was defined as $\geqslant$5 CTCs per 7.5 ml of whole blood. Elevated CTCs at baseline (i.e. before starting any kind of therapy) predicted an extremely short median PFS and OS (2.7 and 10 months, respectively), in comparison to low/negative CTCs corresponding to PFS and OS of 7 and >18 months, respectively ($P < 0.001$). Even more interesting, CTC values, obtained after one cycle of therapy, predicted in which patients the therapy was likely to fail. Patients with elevated CTCs after one cycle of therapy had median PFS and OS of approximately 2.1 months and 8.2 months, respectively, when measured from baseline. In contrast, median PFS and OS of 7.0 months and 22 months, respectively, were observed in a patient group with <5 CTC at the first follow-up visit. These differences were statistically significant ($P = 0.001$) for patients receiving chemotherapy. The data were further confirmed by an independent study [54] in a group of 80 metastatic breast cancer patients. In this study the prevalence of metastatic breast cancer patients with CTCs $\geqslant$5 per 7.5 ml was 61%; baseline CTCs value were predictive of response to therapy, and changes of CTCs level during treatment were found to be associated with response to therapy. Moreover, baseline value of CTCs and CTCs variations (both at 1 month and during follow-up) were the most significant predictors of PFS.

6.1.2. Early breast cancer

In patients with early breast cancer undergoing adjuvant chemotherapy, CK19 mRNA-positive cells detected by means of RT-PCR were shown to be an independent prognostic factor for both PFS and OS, both in node-negative and node-positive patients. Stathopoulou et al. [55] analysed the blood of 148 stage I and stage II patients before the administration of adjuvant chemotherapy: CK19 mRNA-positive cells were detected in 29.7% of the patients, and their presence had a prognostic implication, because they were significantly associated with reduced PFS ($P = 0.0007$) and OS ($P = 0.01$). The same findings were later confirmed by an independent study run in a group of 167 node-negative breast cancer patients undergoing chemotherapy [23]: the detection of CK19 mRNA-positive cells was shown to be an independent predictive and prognostic factor for poorer clinical outcome. Furthermore the same group confirmed these results in an extended cohort of women (444 patients) presenting node-negative and node-positive breast cancer [56]: all patients received chemotherapy +/− hormone therapy. The percentage of patients with detectable CTCs was 40.8% (181/444). The adverse prognostic impact of CTCs detection was demonstrated in the triple-negative and HER2-positive but not in Oestrogen Receptor positive/HER2 negative tumours. Moreover, Apostolaki et al. [57] demonstrated that HER2 mRNA-positive CTCs were detected in 21% (45/214) of Stage I–II breast cancer patients after the completion of adjuvant treatment, and this fact was associated with reduced disease DFS in node-negative, but not in node-positive patients. Furthermore, no association was found between presence of HER2 mRNA-positive CTC and OS. Similar results were found by Xenidis et al. [58], who demonstrated that, after the completion of adjuvant chemotherapy, CK19 positive mRNA CTCs were found in 32.7% (143/437) of the patients, and their detection was significantly associated with increased risk of disease recurrence and death owing to disease progression.

As part of the SUCCESS trial (a large translational research project run in Germany) the amount of CTCs, isolated with the CellSearch system was evaluated in patients before, during and at the end of adjuvant chemotherapy. Using a cut-off of >1 CTC/23 ml of blood, CTCs were detected in 9.5% and 8.7% of 1500 node-positive and high-risk node-negative women, respectively. After a 12-month median follow-up, the detection of >1 CTC/22.5 ml after the end of chemotherapy was associated with a shorter DFS and OS [59].

6.2. Follow-up and therapy monitoring

Different studies showed that presence or absence of CTCs determined during the follow-up of patients receiving chemotherapy for advanced breast cancer seems to be more informative than conventional imaging methods (such as radiologic assessment, including CT or PET) for response evaluation [42,43]. An analysis

performed in a group of 138 out of the 177 patients included in the study published by Cristofanilli et al. [42], demonstrated that CTCs count at 4 weeks after the start of chemotherapy was a more robust and earlier predictor of survival, compared to imaging studies done before and after a median of 10 weeks from the initiation of the chemotherapy treatment [60]. Recently De Giorgi et al. [61] compared the prognostic value of CTC, determined at 9–12 weeks after the start of treatment, and of FDG-PET/CT monitoring during systemic therapy in 102 patients with metastatic breast cancer (MBC). The authors found that in these patients CTCs could accurately predict prognosis, beyond functional response assessed by FDG-PET/CT: the latter deserving a predictive role in patients with less that 5 CTC during follow-up. A very recent study [62] confirmed that CTC counting in patients with metastatic breast cancer was strongly correlated with radiological signs of disease progression, and this holds true also for the results of CTC determined 7–9 weeks before imaging. Taking all these data together, it could be suggested that CTC evaluation during therapy for metastatic breast cancer may emerge as a clinically useful tool in conjunction with standard imaging methods to better assess treatment benefit.

To provide definite evidence on the importance of CTCs determination and in the management of patients with advanced breast cancer, the American South West Oncology Group has recently started the *SWOG 0500* trial. In this study, patients undergoing first-line chemotherapy for a newly diagnosed metastatic disease are tested for CTCs: women showing elevated CTCs ($\geqslant$5/7.5 ml of whole blood) before starting the chemotherapy, are re-tested after 3 weeks. If CTCs are still elevated, the patients are randomized to continue the same therapy or to switch to a different chemotherapy regimen. The clinical endpoints will be the comparison between the DFS and OS of the two groups of patients, those continuing on the same therapy and those switching to another regimen, to see if the early change in therapy based on CTC count will improve patient's outcome.

Another possible application based on CTCs is to monitor minimal residual disease in patients with locally advanced breast cancer treated with neoadjuvant chemotherapy. A recent paper [63] examined 118 patients undergoing primary chemotherapy. The authors showed that at least 1 CTC per 22.5 ml of whole blood was present in 23% of pre-chemotherapy blood samples and in 17% of post-chemotherapy blood samples. Persistence of CTCs at the end of neoadjuvant chemotherapy was not correlated with treatment response; however their pre- or post-chemotherapy detection was an independent prognostic factor for shorter DFS. These results are in line with what reported from the first analysis of the SUCCESS trial, in which the presence of >1 CTC at the end of the adjuvant treatment was associated with earlier relapse. However, we still do not know if other therapeutic options offered to women with persistence of CTC at the end of therapy would have any impact on their outcome, as no study has yet addressed this issue.

6.3. CTC as a "real-time" biopsy

Apart from detection or counting of CTCs, one of the most interesting and possibly challenging application will be the phenotypical and molecular characterization of CTC, which will allow to obtain more detailed information on CTC biology. In this light, CTCs may represent a "real time biopsy": especially in the metastatic setting, CTCs may better represent tumour genetics than primary tumours. It has been shown that expression of clinically and therapeutically relevant markers such as ER, PG and HER2 can differ between a primary tumour and its relative metastasis [52,64–66]. For example, Meng et al. [52] studied CTCs HER2 status in 24 patients with HER2 negative primary tumours who developed recurrent disease. Nine patients showed HER2 gene amplification on CTCs, and 4 of them were treated with trastuzumab-containing therapy: one had complete response and 2 had partial response. Other similar reports strongly suggest that in a small group of patients HER2 status may change at disease progression [67,68]: the major caveat in analysing these studies relies on the fact that low number of patients are included. Only large clinical trials will be able to verify if acquisition of HER2-positive CTC in patients with HER2 negative primary tumours can be considered predictive of response to trastuzumab-based therapy.

Gaining or losing some markers, potential targets for specific therapy, may lead to a change of the following therapy. On this base, Smirnov et al. [69], looking at gene expression profiling by means of the microarray technology, studied CTCs from patients with breast, colon and prostate metastatic cancer: from the generated profiles, they selected genes expressed in the CTCs of all the three cancers, and proposed a list of genes CTC-associated.

Recently the concept of cancer stem cells responsible for tumour origin, maintenance and resistance to treatment has gained prominence in the field of breast cancer research [70]. It is supposed that these cells represent a minor subset of cells in the tumour, and have different characteristics compared to the more differentiated tumour cells. In particular while cancer therapies are now effective in debulking the tumour mass, they may be non-effective in producing long-term remission, maybe because they do not affect the cancer stem cell population. Recently, it has been reported that a subpopulation of CTCs from patients with metastatic breast cancer express putative stem cell progenitor phenotype [71]. The authors found that most patients in an advanced disease phase show a $CK^{+}/CD^{44+}/CD^{24-/low}$ phenotype, while in a separate group of patients, were found CTCs distinguished by an $ALDH1^{high}/CD^{24-/low}$ phenotype. These finding may represent a first clinical step toward a better characterization of CTCs, but further molecular studies are needed to define the possible prognostic impact, and more importantly, to define targets which may render these cells sensitive to specific therapy and therefore susceptible of definitive elimination.

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