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LABORATORY CLINICAL INTERFACE

Circulating tumor cells (CTCs): Detection methods and their clinical relevance in breast cancer

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SUMMARY

The enumeration of circulating tumor cells has long been regarded as an attractive diagnostic tool, as circulating tumor cells are thought to reflect aggressiveness of the tumor and may assist in therapeutic decisions in patients with solid malignancies.

However, implementation of this assay into clinical routine has been cumbersome, as a validated test was not available until recently. Circulating tumor cells are rare events which can be detected specifically only by using a combination of surface and intracellular markers, and only recently a number of technical advances have made their reliable detection possible. Most of these new techniques rely on a combination of an enrichment and a detection step.

This review addresses the assays that have been described so far in the literature, including the enrichment and detection steps and the markers used in these assays. We have focused on breast cancer as most clinical studies on CTC detection so far have been done in these patients.

Introduction

The outcome of breast cancer largely depends on the development of metastases in the course of the disease. Given this vital importance of metastases, means to detect and monitor their existence are continuously sought for. The detection of circulating tumor cells (CTCs) is one field of research focusing on a new method to detect metastatic disease earlier, less invasive and more reliably than currently available conventional methods, such as clinical presentation, radiographic evaluation and serum tumor markers do. CTCs are defined as tumor cells circulating in the peripheral blood of patients, shed from either the primary tumor or its metastases. Numerous efforts have been made to reliably detect and quantify CTCs in peripheral blood, but development of a suitable assay has proven to be difficult. Unfortunately, there is not one specific feature that universally distinguishes CTCs from blood cells. Ideally, a specific marker would be identified, which is expressed in every cell of every breast cancer type. In reality, different histological and molecular types of tumors express different arrays of markers, and marked heterogeneity of expression exists even within one histological distinct tumor type. Another challenge regarding sensitivity of assays is the fact that CTCs are rare events, with numbers as low as one CTC in 10^6–10^7 leukocytes.[1] In spite of these challenging characteristics, the importance of detecting and enumerating CTCs in breast cancer has been established in several clinical studies, showing a correlation with decreased progression-free survival (PFS) and overall survival (OS).[2,3] In addition to detection and enumeration, molecular characterization of CTCs provides a second much-anticipated application in oncology. Currently, we are dependent on the primary tumor for molecular characteristics in order to determine the type of therapy the patient will benefit from most. However, tumor genotype and/or phenotype may change in the course of treatment as indicated by therapy resistance. CTCs might function as a real-time biopsy of tumor load, and enable oncologists to make better-informed choices regarding therapy.

In addition to CTCs, disseminated tumor cells (DTCs), i.e., isolated tumor cells in bone marrow, are thought to reflect the metastatic potential of tumors. DTCs have also been correlated with prognosis[4,5], but for their detection an invasive diagnostic proce-

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dure, bone marrow aspiration, is necessary. This requirement makes their implementation in the clinic more troublesome. By contrast, CTCs have the advantage of being readily available in peripheral blood and given this, together with mounting evidence supporting their clinical feasibility as reviewed here, the detection of CTCs is anticipated to gain clinical relevance shortly. For a comprehensive review on DTCs and their complementary role to CTCs, we refer to the recent review by Riethdorf et al.[6]

Here we will discuss the principals and technical aspects of the different techniques available for detecting CTCs, in addition to the most frequently used markers in these techniques. Furthermore, we will discuss clinical studies showing the utility of CTC detection in breast cancer patients with these techniques. Reviews focusing on the detection techniques of both DTCs and CTCs, as well as biological relevance, have been published recently.[7,8]

Detection of circulating tumor cells

In general, methods for CTC detection can be divided into cytometric (i.e., whole-cell based) and nucleic-acid based techniques. Both techniques usually include an enrichment step and a detection step.

As CTCs are rare events occurring at rates as low as one cell per 10^6–10^7 leukocytes, enrichment is generally needed to increase sensitivity to an acceptable level. One type of enrichment relies upon the selection of target cells with tumor-specific markers (immunoseparation). Other methods for enrichment are based solely on morphologic criteria, such as cell size or density. Sensitivity and specificity is an issue with both techniques, due to heterogeneity of tumors in size, density and marker expression. Consequently, while enrichment is thought to be required, some tumor cell loss is likely to occur irrespective of the enrichment technique used. The extent of cell loss should be determined with recovery experiments for each technique to validate the results.[9,10]

After enrichment, nucleic-acid based techniques like reverse transcriptase (RT)-PCR or cytometric methods are applied to detect CTCs through putative tumor-specific markers. Here we will discuss the most commonly used enrichment and detection techniques. Some techniques have combined their enrichment and detection steps and these will be presented separately. While a number of studies have compared the performance of different assays,[11–13] not all techniques have been compared directly to each other. In an attempt to clarify the hierarchy in the various techniques, we have depicted their major advantages and disadvantages in Tables 1 and 2.

Increasing assay sensitivity: enrichment techniques

As mentioned before, enrichment can be based on morphologic cell characteristics, such as size or density, or on immunoseparation, using magnetic beads, ferrofluids or rosettes (see Figs. 1 and 2).

Morphology-based enrichment

ISET (Isolation by Size of Epithelial Tumor cells) isolates tumor cells individually by filtration based on their larger size (>8 μm) compared to leukocytes.[14] The Nucleopore assay (Whatman International Ltd., England) is based on the same assumption.[9] However, no validation studies have been executed confirming that CTCs are indeed never smaller than 8 μm, leaving questions about the sensitivity of this method.

Density-gradient based techniques are techniques separating mononuclear cells based on their lower density compared to other blood compartments. Mononuclear cells and tumor cells are separated from blood cells and granulocytes using a density gradient of 1077 g/ml. Similarly, *Oncoquick* (Greiner Bio One, Frickenhausen, Germany) is based on density gradient separation, but adds a porous barrier, which prevents the gradient-separated cells to be contaminated with the whole blood.

Immunomagnetic separation

The simplest separation can be done with immunomagnetically labeled monoclonal antibodies and a basic handhold magnet. Negative selection of a blood sample can be done with *magnetic beads* loaded with an anti-CD45 antibody, a pan-leukocyte marker, or against CD61 thereby removing megakaryocytes and platelets.[15]

MACS® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), or Magnetic Activated Cell Sorting system, is a dedicated instrument that captures cells by immunomagnetic labeling with microbeads. It does so by membrane or intracellular staining, the latter requiring a permeabilization and fixation step. Magnetic beads are available linked to anti-epithelial antibodies for positive selection through EpCAM, for example. EpCAM, or tumor-associated calcium signal transducer 1 (CD326), is a cell surface molecule involved in cell-to-cell adhesion and is highly expressed in most epithelial carcinomas.[16] Magnetic beads targeting the tumor-specific cell antigen epidermal growth factor receptor 2 (HER2) are also available.

Table 1
Merits and demerits of CTC enrichment techniques.

Enrichment techniques		Merits	Demerits
Morphology-based	ISET	Applicable for all tumor types	Tumor cells are heterogeneous in size Final result dependant upon detection technique
	Density gradient	Easy and cheap technique Applicable for all tumor types	Relatively low purity of sample Final result dependant on detection technique
	Oncoquick	Porous barrier separates density gradient from whole blood Applicable for all tumor types Easy and cheap technique	Relatively low purity of sample Final result dependant on detection technique
Immuno(magnetic)	MACS	Multiple antibodies available Specific enrichment	Not automated Final result dependant on detection technique
	CellSearch	Semi-automated Specific enrichment	EpCAM negative CTCs may be missed
	RARE	Thorough leukocyte depletion Applicable for all tumor types	Not automated Final result dependant on detection technique
	AdnaTest	Combined MUC1 and EpCAM enrichment Specific enrichment	Not automated
	CTC chip	Controlled flow conditions should prevent trapping Specific enrichment	EpCAM negative CTCs may be missed

Table 2
Merits and demerits of CTC detection techniques.

Detection techniques		Merits	Demerits
Cytometric	CellSearch	Semi-automated Visual confirmation of CTCs Further analysis possible	Subjective CTC analysis
	EPISPOT	Detection of viable CTCs	No morphological analysis possible Proteins have to be actively secreted or shed from CTCs
	CTC-chip	Visual confirmation of CTCs	Subjective CTC analysis
	FAST	No enrichment needed	Subjective CTC analysis
	LSC	No enrichment needed Visual confirmation of CTCs	Subjective CTC analysis Low specificity due to marker limitations
Nucleic-acid based	RT-PCR	High sensitivity Objective assessment of CTC signal	Low specificity Instability of RNA No morphological analysis possible
	qRT-PCR	Higher specificity than regular RT-PCR Objective assessment of CTC signal	Instability of RNA No morphological analysis possible

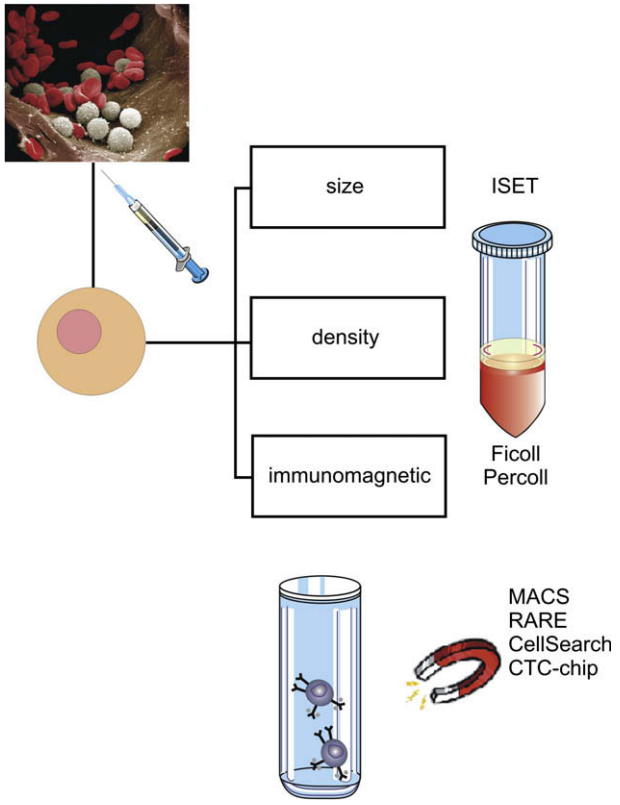

Fig. 1. This figure clarifies the various options for enriching CTCs. After blood is drawn from the patient, CTCs can be separated from hematopoietic cells based on size, density or immunological characteristics. ISET (Isolation by Size of Epithelial Tumor cells) filtrates CTCs based on their larger size compared to hematopoietic cells. Ficoll and Percoll are the most commonly used *density-gradient based techniques*. Immunomagnetic separation techniques separate CTCs from other hematopoietic cells based on marker expression; MACS (Magnetic Activated Cell Sorting) uses microbeads and RARE (RosetteSep-Applied imaging Rare Event) combines magnetic separation with CD45+-cell depletion. CellSearch and the CTC-Chip both enrich based on EpCAM; CellSearch separates cells bound to EpCAM-ferrofluid in a magnetic field, while the CTC-chip does so by binding EpCAM-positive cells to microposts.

The AdnaTest (AdnaGen AG, Laggenhagen, Germany) combines two epithelial and tumor associated antigens. Two antibodies against MUC1 and one antibody against EpCAM are conjugated to magnetic beads. As neither MUC1 nor EpCAM are present on all circulating tumor cells[17], cells expressing MUC1 and/or EpCAM should be isolated with this double-antibody method.

RARE™ (StemCell Technologies, Vancouver), i.e., RosetteSep-Applied imaging Rare Event, is a technique that combines a density gradient separation with an antibody-mediated enrichment step. Enrichment is done through negative selection, as $CD45^+$ cells are cross-linked to multiple red blood cells by bispecific tetrameric antibody complexes, forming rosettes. As the density of these unwanted cells then increases, the CD45-positive cells accumulate in the lower compartment after density gradient centrifugation. CD45-negative mononuclear cells are isolated between the separation medium and plasma.[18] A more extensive negative selection can be done with the same technique, by using a kit containing antibodies directed against CD2, CD16, CD19, CD36, CD38, CD45, CD66b and glycophorin A.

Detection techniques

Cytometric methods

The presence of tumor cells in the bone marrow was first identified using conventional imaging techniques.[19] Building on this, detecting tumor cells in the circulation was attempted using simple hematoxylin and eosin staining.[20] This exhaustive method consisted of visually identifying large numbers of gradient-separated cells and comparing them with primary tumor cells morphologically. Nowadays, as previously mentioned, detection of CTCs occurs on a cytometric or a nucleic-acid basis.

Cytometric methods isolate and enumerate individual cells based on their antigen expression, using for example monoclonal antibodies directed against epithelium-specific antigens. The advantage of cytometric methods over nucleic-acid based methods is the possibility to further characterize the cells, as the target cells are not lysed in the procedure. This allows subsequent morphological identification and molecular characterization of CTCs. The major draw-back is the current lack of a tumor specific antibody. The commonly used cytokeratin (CK) antibodies bind specifically and non-specifically to macrophages, plasma cells and nucleated hematopoietic cell precursors.[21,22] The same holds true for Mucin-1(which binds nonspecifically to erythroid progenitors).[23] This problem can be reduced significantly by counterstaining with CD45, a pan-leukocyte marker. Breast cancer specific markers have been used (i.e., HER2, anti-mammaglobin), but as these are not present on all breast cancer tumors or on every cell of a particular tumor, false negatives are likely to occur. Advances in terms of sensitivity and specificity have been made using multimarker assays, which can overcome detection problems due to tumor heterogeneity.[24]

To overcome the problem of high numbers of immunofluorescently labeled mononuclear cells having to be analyzed to identify rare CTCs, FAST was developed. This Fiber-optic Array Scanning Technology locates immunofluorescently labeled cells on glass

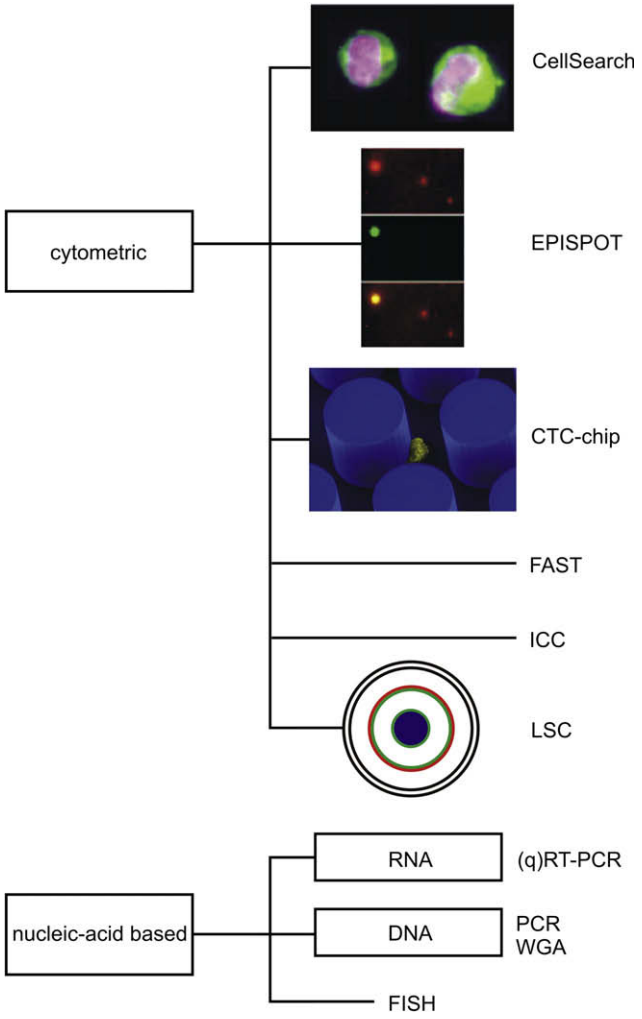

Fig. 2. The techniques for detection of CTCs can be cytometric, nucleic-acid based, or a combination of both. The CellSearch system enumerates CTCs based on morphology, Cytokeratin and DAPI positivity and counterstaining with CD45. EPISPOT is an immunological assay based on the enzyme-linked immunosorbent assay (ELISPOT), which detects a number of proteins released by breast cancer CTCs, such as CK19 or Mucin-1. The CTC-Chip uses controlled blood flow conditions across a chip containing EpCAM-coated microposts. Detection of CTCs then follows by counting CD45-/DAPI+/CK+ cells with a camera. The Laser Scan Cytometer (LSC) analyses fluorescence after the cells are contoured using forward scatter as a threshold parameter and corrects for background fluorescence variation dynamically. FAST (Fiber-optic Array Scanning Technology) is a form of automated digital microscopy using a very large field of view, enabling continuous scanning. Nucleic-acid based detection methods can be based on DNA or RNA. Immunocytochemistry (ICC) allows the identification of CTCs based on marker expression and morphologic features. Part of the major drawback of ICC, the sheer volume of cells needing evaluation, can be relieved by automated microscopes such as ACIS and ARIOL. Nucleic-acid based techniques can be performed on whole cells or on extracted RNA or DNA. (RT)-PCR (Reverse Transcriptase)-Polymerase Chain Reaction) amplifies a specific RNA or DNA sequence. WGA (Whole Genome Amplification) aims to overcome the scarcity of DNA in a sample by nonspecifically amplifying the sample. FISH (Fluorescent In Situ Hybridization) detects the presence of absence of specific DNA sequences.

substrates at rates 500 times higher than conventional automated digital microscopy. The key innovation is a light collection system that has a very large field of view (50 mm), which is large enough to enable continuous scanning without the need to analyze the sample in multiple steps. Because larger volumes of peripheral blood can be analyzed than using conventional microscopy in the same time, purification or enrichment steps are avoided, which reduces the risk of cell loss. In cell line spiking experiments, an average sensitivity of 98% was reached in colorectal and breast cancer after whole blood lysis.[25,26]

Attempting to improve scanning of fluorescent cells, the *Laser Scanning Cytometer* (LSC) (Compucyte Corporation, Cambridge, MA) was developed. Following whole blood lysis and staining with anti-human epithelial antibody (HEA) in combination with CD45, this cytometer analyses fluorescence after the cells are contoured using forward scatter as a threshold parameter. The cytometer determines background fluorescence dynamically to calculate peak and integral fluorescence on a per-cell basis. This calculation results in improved correction for background fluorescence variation. It is also possible to relocate the cells within the positive population, allowing for visual verification through the microscope.[27,28] In a recent study, three different combinations of techniques were compared; immunomagnetic separation and LSC vs. cell filtration and LSC vs. a multimarker quantitative RT-PCR assay. qRT-PCR was found to be the most sensitive. Samples from patients with metastatic breast cancer were significantly more likely to be positive for one or more of three markers (CK19, mammaglobin, and PIP (prolactin inducible protein) using RT-PCR than to be positive in LSC.[11]

ACIS®[22] (Automated Cellular Imaging System) (DAKO, Glostrup, Denmark) and ARIOL®[29] (Applied Imaging Corp., San Jose, CA) are automated scanning microscopes enabling faster examination of slides. After initial automated scanning and analysis of slides in a manner that can be configured to the assay used, the investigator reviews the presented images and classifies them morphologically. Numerous other automated scanning systems have been used in the *immunocytochemical* detection of rare events.[30–32]

Nucleic-acid based methods

CTCs may be identified through the detection of (epi)genetic alterations that are specific for cancer cells. Alterations in DNA such as mutations in proto-oncogenes or tumor suppressor genes, microsatellite instability and sequences of oncogenic viruses may be detected. Circulating free total DNA in the blood of cancer patients was detected for the first time in 1977 using a radioimmunoassay.[33] In later studies, circulating mitochondrial DNA[34] and amplification of MYC-N (a neuroblastoma-derived MYC oncogene) DNA[35,36] in neuroblastoma patients was detected in greater amounts in patients with cancer than in healthy individuals. Implementing DNA-based CTC detection in clinical practice is difficult however. DNA changes occur in merely dysplastic lesions as well as in full-blown neoplasm. Furthermore, there is uncertainty about the half-life of circulating cells and nucleic acids, which means that the presence of circulating free DNA may reflect merely the presence of nucleic acids, not tumor cells. As a result, the detection of free total DNA has not been implemented into clinical practice.

Detection of mRNA of factors that are overexpressed or mutated in breast cancer using RT-PCR is a more widely used alternative. As RNA disappears quickly from the blood after cell death, detection of RNA is likely due to the presence of a whole tumor cell, not cell fragments or free RNA. In RT-PCR, after cDNA synthesis, the gene of interest is amplified using oligonucleotide primers specific for this gene of interest. The sensitivity of RT-PCR was higher than immunocytochemistry in several studies.[12,13,37] However, RT-PCR is prone to false-positivity, as sample contamination, expression of target genes in normal cells, and pseudo genes (genes without protein-coding abilities) can all occur. The problem of false-positivity was demonstrated very clearly in work on activated peripheral blood mononuclear cells (PBMNCs).[38] A multimarker RT-PCR assay was performed on healthy donors, stimulated PBMNCs and unstimulated PBMNCs from patients with immune thrombocytopenic purpura (ITP). While all markers (SCCA (secondary structure conserved A), EGFR (epidermal growth factor receptor), hMAM (mammaglobin), SBEM (small breast epithelial

mucin) and CA-9 (carbonic anhydrase 9)) were negative in healthy donors, 4 out of 5 (SCCA, EGFR, hMAM, SBEM) were positive in stimulated PBMNCs and 3 of 5 (SCCA, EGFR, SBEM) were positive in patients suffering from ITP. In another study, it was revealed that CK19 and CEA (carcinoembryonic antigen) expression is present in lymphatics following cytokine stimulation, as well as in 50% of bone marrow samples of patients with chronic inflammatory disease.[39] As cancer can induce inflammatory responses,[40] these inducible signals may be the cause of false-positive outcomes in CTC detection. Another possible source of false-positivity is the presence of free RNA or genomic DNA, which can be eliminated by adding a gradient separation step or genomic DNA elimination by DNAse, respectively.[41]

In general, nucleic-acid based methods combine their higher sensitivity with a lower specificity, as background noise due to expression of markers in normal cells is hard to distinguish from a true positive signal. Quantitative RT-PCR provides a way of visualizing low and high expression of a chosen marker, increasing discrimination between mRNA expression of normal cells and tumor cells. Like in cytometric methods, in RT-PCR as well the absence of a true tissue-specific marker has been an issue with regard to specificity. RT-PCR outperformed immunocytochemistry in sensitivity (49.6 vs. 42% positive samples in 133 patients) in a study on CK19 detection. However, no data were provided on results in healthy donors.[12] The importance of the latter was underlined by the findings of another study comparing CK19 detection by immunocytochemistry vs. RT-PCR vs. Nucleic Acid Sequence-Based Amplification (NASBA). While RT-PCR was more sensitive than immunocytochemistry and NASBA, all three methods showed false-positive results in healthy donors,[13] prompting the authors to deem CK19 an unsuitable marker. As these studies show, single-marker assays reach sufficient sensitivity but lack in specificity. Given the heterogeneity of breast cancer, the consistent presence of a specific tumor marker or fusion gene such as in Ewing tumors[42,43] seems unlikely. Instead, the use of multiple marker assays, combining several breast cancer specific markers as well as leukocyte-specific markers, might at least in part resolve the issue of specificity.

Combined enrichment and detection techniques

CellSearch (Veridex™, Warren, PA) is a semi-automated technology by which whole blood is enriched for CTCs by adding ferrofluids loaded with antibodies directed towards EpCAM. Currently, CellSearch is the only FDA-approved assay for CTC detection. CTCs in the enriched population are stained with CK and DAPI using fluorescent antibodies, while hematopoietic cells are counterstained with CD45. The CK+/DAPI+/CD45- cells are then enumerated with an automated fluorescence microscope. The semi-automated character of this system enables samples to be analyzed rapidly and reproducibly. When CellSearch was compared directly to Oncoquick followed by labeling with CKs, EpCAM and DAPI, both methods reached 100% specificity in 15 healthy donors, but CellSearch detected more samples with >1 CTC in a group of 61 heterogeneous carcinoma patients (14 vs. 33 positive samples). The mean number of CTCs per sample was also higher using the CellSearch technique.[44]

A technologically advanced and novel method to isolate CTCs is the 'CTC-chip'. This chip consists of 78,000 microposts, each coated with EpCAM antibodies. As whole blood is pumped across the chip under controlled flow conditions, EpCAM-positive cells bind to the microposts, which are then detected by a camera based on their morphology, viability and tumor markers. This system uses CKs and DAPI for positive selection together with CD45 for negative selection.[45,46] The micropost system should prevent trapping of EpCAM positive cells among leukocytes. According to the developers, sensitivity was remarkably high with this method, as CTCs were detected in every patient, including those with localized disease.[46]

Another new approach is the epithelial immunospot (EPISPOT) assay, an immunological assay based on the enzyme-linked immunosorbent assay (ELISPOT). The assay is preceded by immunomagnetic depletion of $CD45^+$-cells and enrichment for $CXCR4^+$-cells (a chemokine receptor involved in the homing of metastatic tumor cells[47]). EPISPOT detects specific proteins released by breast cancer CTCs, such as cathepsin-D (a cysteine protease) or Mucin-1, thus counting only viable, protein-excreting cells.[48,49] In theory, viable cells have more clinical relevance than apoptotic cells as they should still be capable of forming metastases. In a first study on breast cancer CTC detection, this assay was performed using Mucin-1 and CK19 as markers, showing high sensitivity and specificity.[48]

In conclusion, no enrichment or detection method for CTCs has yet proven to be the golden standard, and continuing efforts are made to improve the reliability of these methods.

Markers

The effectiveness of tumor cell enrichment and detection depends upon the choice of markers, tools to identify and characterize CTCs. Many different markers have been explored in the field of CTCs. To date, no one marker has proven to be ideal for the detection of breast cancer CTCs. This is not unexpected given the heterogeneity of the disease and the rarity of CTCs. In breast cancer, a wide array of markers has been studied, especially with nucleic-acid based techniques. In Table 3, we present an overview of markers for enrichment and detection of breast cancer CTCs in cytometric techniques. Table 4 depicts the methods of enrichment and the markers for detection of breast cancer CTCs in nucleic-acid based techniques. The heterogeneity of experiments in studies to date does not allow drawing conclusions on superiority of one marker. Study populations, sample handling and preparation and use of markers differ so strongly that any comparison would be misleading. However, all these markers do represent a specific quality of tumor cells, and can therefore offer essential information. As a consequence, the combination of multiple markers seems promising when this results in an increment in specificity and sensitivity.

Clinical applications of CTC detection

The presence of occult metastases cannot be deduced from the finding of CTCs alone, as CTCs must pass through several stages before forming a metastatic colony. Cells must extravasate from the circulation into target organs and subsequently proliferate whilst evading immunological response and overcoming metabolic difficulties. It has been estimated that only one in 10,000 CTCs is able to form a metastasis.[50]

Despite all this, the clinical usefulness of CTC detection has been demonstrated in metastatic breast cancer,[2,51] metastatic colorectal[52,53] and metastatic prostate cancer.[54] In addition, CTCs have been studied in pancreatic,[55] gastric,[56–59] bladder[60–63] and lung cancer,[27,64,65] among others, with variable results. Of all tumor types, breast cancer is the tumor type in which CTCs have most strongly proven their value, and in which the largest variety of techniques has been applied.

Notably, while reaching technically good results with respect to sensitivity and specificity, no studies in large patient series have been conducted using RARE, Histopaque, Percoll, ISET, Nucleopore or MACS as enrichment techniques. Furthermore, with regard to detection techniques, EPISPOT and FAST have not been correlated to clinical outcome. In contrast, CTC detection using other techniques such as CellSearch or PCR-based techniques has been extensively studied in large series of patients. For this review we have

Table 3
Markers for enrichment and detection in cytometric techniques.

	Techniques								
	ARIOL	ICC	IF	Flow cytometry	ELISPOT	CellSearch	FAST	CTC chip	LSC
Enrichment	IMS-CKs[29] IMS-EpCAM[29]	Density gradient[13,37,44,76,91–94] IMS-CKs[91,95,96] IMS-EpCAM[88,97,98] IMS-CD45[94]	IMS-EpCAM[99]	Density gradient[100] IMS-CKs[101] IMS-EpCAM[102]	IMS-CD45[49] IMS-CXCR4[49]	EpCAM[16,29,44,103,104]			Density gradient[11] IMS-EpCAM[11]
Detection	CKs[29]	CKs[13,37,44,76,88,91–98] EpCAM[44] HER2[88,91]	CKs[99] HER2[99] uPAR[99]	CKs[100–102]	Cath-D[49] MUC1[49]	CKs[16,29,44,103] IGF-IR[104]	CKs[25]	EpCAM[46]	EpCAM[27,28,67] CKs[11]

ICC, immunocytochemistry; IF, immunofluorescence; FAST, Fiber-optic Array Scanning Technology; LSC, Laser Scan Cytometry; IMS, immunomagnetic separation; CKs, cytokeratins; EpCAM, epithelial cell adhesion molecule; HER2, c-erbB-2; uPAR, plasminogen activator receptor; Cath-D, cathespin-D; MUC1, Mucin 1; IGF-IR, insulin-like growth factor I receptor.

Table 4
Markers for enrichment techniques and detection markers in nucleic-acid based techniques.

	Techniques			
	RT-PCR		qRT-PCR	
Enrichment	Density gradient[92,105–116] Adnatest[88]		Density gradient[37,117–120] Adnatest[17] IMS-EpCAM[121]	
Detection	β-HCG[109,122–124]	HER2[88,145–147]	ANKRD30A[156,157]	Mammaglobin[11,17,120,139,142,156–160,167]
	c-MET[122,125,126] CD44[127]	MAGE-A3[73,122,148,149] Mammaglobin[110,112,116,124,126,133,142,149–152]	B305D[156,157] Bmi-1[74,128]	Maspin[130,139,149,167] MUC1[51,121,158,159]
	CEA[128–133] CK7[118,126,134,135] CK19[13,92,106–108,111,112,115,123,124,127,129–133,136–142]	Maspin[17,105,128,130,133,137] MUC1[88,114,127,129,131–133] PTHrP[114,153]	CEA[158] CK7[37,51,117,118] CK19[11,37,51,119,134,142,157–165]	MUCL1[160] p1B[51] PIP[11,158,159]
	CK20[113,123,129,133,138] EGFR[17,133,138,142–144] EpCAM[122,129] GalNAc-T[17,73,88,122]	Survivin[154] Telomerase[155]	EGFR[142] EGP2[17] EpCAM[17,119,161] GABRP[156,157] HER2[158,161,166,167]	Secretoglobin[149,158–160] SPDEF[159] TTF1[119,161,168] TTF3[119,161] uPAR[169]

ANKRD30A, ankyrin repeat domain 30A; B305D, antigen B305D; β-HCG, chorionic gonadotrophin; Bmi-1, B lymphoma Mo-MLV insertion region 1 homolog; c-MET, proto-oncogene met; CEA, carcino-embryonic antigen; CK, cytokeratin; EGFR, epidermal growth factor receptor; EGP2, epithelial glycoprotein 2; EpCAM, epithelial cell adhesion molecule; GABRP, GABA A receptor pi; GalNAc-T, UDP-N-acetyl-D-galactosamine; HER2, c-erbB-2; IMS, immunomagnetic separation; MAGE-A3, melanoma antigen family A subtype 3; MUC1, Mucin 1; MUCL2, Mucin-like 2; PIP, prolactin-induced protein; PTHrP, parathyroid hormone receptor protein; SPDEF, SAM pointed domain containing ets transcription factor; TTF1, Trefoil factor 1;TTF3, Trefoil factor 3; uPAR, plasminogen activator receptor.

chosen to discuss only major CellSearch- and RT-PCR-based studies.

CTC detection in localized breast cancer

Neoadjuvant setting

For patients presenting with locally advanced breast cancer, i.e., tumors presenting with extensive regional lymph node involvement, skin involvement or a large size (>5 cm), resection of the primary tumor is frequently either not possible or only at the cost of an amputation of the breast. Systemic therapy given prior to management of the primary tumor, also known as neoadjuvant therapy, aims to reduce tumor size thereby rendering the residual tumor amendable for a breast-conserving resection. In addition, neo-adjuvant systemic therapy aims to eradicate micrometastases, which may otherwise have resulted in incurable, overt metastatic disease later on. Until now, the value of CTC detection in the neoadjuvant setting has not been extensively studied. Recently, CTCs were detected before and/or after neoadjuvant chemotherapy with CellSearch in 118 patients included in a phase II trial.[66] In 23% of the patients, one or more CTC per 7.5 ml blood was detected before the administration of neoadjuvant chemotherapy, while 17% had >1 CTC per 7.5 ml blood after neoadjuvant chemotherapy. The persistence of CTCs during neoadjuvant chemotherapy was not correlated with treatment response, but the presence of CTCs either at base-line or after neo-adjuvant chemotherapy was an independent prognostic factor for distant metastasis-free survival.[66] In another study, CTCs were monitored by *Laser Scanning Cytometer* (LSC) before each of three therapy cycles in 30 patients.[67] CTCs were detected in all patients prior to the start of therapy, but the decrease in number of CTCs for different patients varied up to several 100-fold. A strong correlation was however shown between a reduction in the number of CTCs and a favorable pathological response at surgery.[67] This correlation suggests that CTCs may serve as an early marker to assess response to neo-adjuvant therapy. However, the remarkable high CTC detection rate in these primary breast cancer patients, as well as in another 91 patients treated in the adjuvant setting using the same technique as discussed below, has been questioned.[68] It was suggested that further characteriza-

tion is needed to confirm that the cells assigned as CTC using this technique are tumor cells indeed. The authors explained their findings by stating that the lack of enrichment in their method accounts for less cell loss and, consequently, the high CTC counts.[68]

Adjuvant setting

Adjuvant chemotherapy refers to systemic therapy after primary surgery for early stage breast cancer patients who are considered to have a high risk for metastatic disease developing from micro-metastases that are already present at initial presentation. The intent of adjuvant therapy is to cure patients by eradicating these micrometastases. Currently, it is not possible to adequately identify patients who do not harbor micrometastases and therefore should be spared from adjuvant therapy and the accompanying toxicities. On the other end of the spectrum, 20–30% of patients treated with adjuvant therapy will develop overt metastasis, in spite of adjuvant therapy.[69] Detecting this population not cured by the administered adjuvant therapy could open the door for additional treatments with new drugs. Several studies have recently been conducted to establish whether or not CTC detection and enumeration may guide treatment in this setting.

In a study of 91 clinically non-metastatic primary breast cancer patients, CTCs were quantified by LSC before adjuvant therapy, before each new cycle and at the end of the chemotherapy.[70] There were three distinct patterns of response: 28 patients showed a decrease in cell number of 10-fold or more, 30 patients showed changes less than 10-fold in cell number and 33 patients had an increase of more than 10-fold. The pattern of CTC counts during therapy correlated significantly with relapse, and in multivariate analysis, an increasing CTC count of 10-fold or more at the end of therapy was associated with shorter relapse-free survival.[70] If confirmed, this group of patients may be candidate for additional therapy. As in another study of the same group as discussed before,[67] the remarkably high rate of CTC positivity in the current study[70] has been subject of discussion.[68]

The prognostic relevance of the detection of CTCs with RT-PCR has recently been demonstrated in 444 early-stage breast cancer patients.[71] After a median follow-up of 53.5 months, patients with CK19 mRNA-positive CTCs experienced significantly reduced disease-free survival (DFS) and OS compared to those without CTCs. In multivariate analysis as well, the detection of CTCs was associated with decreased DFS and OS.[71] It should be noted, as discussed before, that using only CK19 as a marker does raise concerns about specificity.[13]

As HER2 has become an important target for therapy since the introduction of trastuzumab (Herceptin®), the determination of HER2 expression on CTCs has caught the interest of research groups. Apostolaki et al. showed that the detection of HER2 mRNA-positive cells with RT-PCR after the administration of adjuvant chemotherapy was correlated with shorter disease-free interval (DFI) in 214 stage I and stage II breast cancer patients.[72] However, this prognostic value of HER2 on CTCs could not be reproduced in a more recent multimarker qRT-PCR based study.[73] Simultaneously studying mammaglobin A (MGB1), HER2 and CK19 in 175 patients with stage I–II breast cancer after primary surgery and before adjuvant therapy, marked heterogeneity was seen in the CTC phenotypes inter- and intra-individually. In multivariate analysis, CK19 mRNA+ and MGB1mRNA+ cells were independent adverse prognostic factors, whereas HER2 mRNA+ cells were not.[73] This apparent difference in significance of HER2 might be due to the fact that the presence of HER2 mRNA+ cells after adjuvant therapy, which was only determined in the first study, could reflect resistance to chemotherapy and therefore be stronger associated with prognosis.

In a single-marker assay, using CK7 as a marker for qRT-PCR, CTCs were detected in 37 of 206 primary breast cancer patients.[74] Ninety-eight patients were followed up 24 months after primary surgery. Of those, the CK7-negative group showed significantly longer DFS than the CK7 positives. This difference was even more profound in 61 lymph node-negative patients observed over 24 months after surgery. This suggests that CK7+ CTCs are a prognostic marker for early recurrence after primary surgery.[74]

Employing the CellSearch technique, a large trial is currently being conducted to assess the value of CTCs in the adjuvant setting.[75] This SUCCESS trial has enrolled 1767 primary breast cancer patients. Preliminary results show detection of >1 CTCs per 7.5 ml blood with CellSearch in 10% of 1500 patients before the start of systemic therapy. Of those, 10% remained positive after chemotherapy. Persistence of CTCs after chemotherapy was associated with decreased PFS and OS. As follow-up of this trial is ongoing, the prognostic value of these promising data cannot yet be determined.

Oncoquick was combined with *immunocytochemical* staining with anti-cytokeratin, CD45 and Ki-67, the latter being a proliferation marker, to detect CTCs in 60 primary and 63 metastatic breast cancer patients[76] CTCs were detected in 8.3% of primary and 39.7% of metastatic breast cancer patients, but this did not correlate with prognosis or tumor characteristics. Remarkably, in a subset of 47 randomly chosen patients, none of the 9 CTC-positive patients expressed Ki-67 on their CTCs, suggesting that CTCs are at the very least rarely proliferative.

Studying 341 primary breast cancer patients included within 3 years after primary surgery, CTCs could be detected by *immunocytochemical* staining with anti-cytokeratin following enrichment with Ficoll and immunomagnetic CD45-depletion in 10% of the patients.[77] While the presence of CTCs was correlated with DFS and breast cancer specific survival in the whole group of patients, when 23 patients who had had a breast cancer-related event prior to the collection of peripheral blood were excluded, CTC detection no longer correlated with DFS.

CTC detection in metastatic breast cancer

In metastatic disease, the intention of treatment is essentially palliative, striving to optimize quality rather than duration of life. Assessing prognosis in patients with metastatic breast cancer with CTCs can be helpful in the individualized management of these patients. In a multicenter, prospective study conducted by Cristofanilli et al.,[78] CTC count was assessed using CellSearch in 177 progressive metastatic breast cancer patients who were to start a new line of systemic therapy. CTCs were enumerated before the start of new treatment and at first follow-up visit. Patients with a level of CTCs before the start of treatment of $\geqslant 5$ cells per 7.5 ml blood had a shorter median PFS (2.7 vs. 7.0 months) and shorter OS (10.1 vs. >18 months). Maybe even more interestingly, patients with initially elevated CTC levels at baseline that had declined below five cells per 7.5 ml blood at first follow-up after the first administration of therapy had a PFS and OS similar to the patients with low levels of CTCs at baseline and follow up. These results indicate the potential role of CTCs as a prognostic marker, and as a marker establishing at an early stage whether a patient benefits from anti-tumor therapy.[79] Recently, the patients in this cohort whose CTC levels were determined at the time of newly diagnosed recurrent or de novo metastatic disease, and therefore being treatment-naïve, were analyzed retrospectively.[80] Also in these patients, it could be confirmed that a CTC level of >5 was an independent prognostic factor for death (HR 3.64), and median OS was 28.3 vs. 15 months in patients with CTCs <5 vs. >5.

Even more so than in localized disease, in metastatic breast cancer the clinician should carefully evaluate the effects of chemother-

apy taking into account its side-effects. It is important to assess response or lack thereof as early as possible in order to avoid exposure of the patient to unnecessary toxicity. The current methods to evaluate this response, namely clinical presentation and radiographic imaging, are suboptimal, as they are often only helpful late in the disease process. Ideally, CTCs could predict response to therapy after one or two cycles of therapy, and do so more reliable than traditional parameters. Budd et al.[81] compared the prognostic value of the presence of >5 CTCs per 7.5 ml blood with radiologic response on OS in 138 metastatic breast cancer patients in the same cohort as Cristofanilli et al.[78] Radiologic evaluation was conducted 10 weeks after initiation of therapy, CTC counts were determined with the CellSearch system 4 weeks after initiation of therapy. CTC determination showed lower inter-reader variability than radiologic evaluation (0.7% vs. 15.2%, respectively). In patients who were non-progressive according to radiological evaluation, the median OS was significantly shorter for patients with $\geqslant$5 CTCs than for patients with <5 CTCs per 7.5 ml blood (15.3 vs. 26.9 months). In patients with radiological progressive disease, a similar significant difference in median OS was objectified in patients with $\geqslant$5 CTCs vs. patients with <5 CTCs per 7.5 ml blood (6.4 vs. 19.9 months).[81] This study strongly suggests that CTCs are a good tool to evaluate tumor response to therapy, and in fact better than radiologic evaluation.

The study of multiple rather than single markers to increase sensitivity of CTC detection assays, studying multiple markers seems promising. Combining CK19, p1B, PS2 and EGP2, CTCs were detected by qRT-PCR in 94 metastatic breast cancer patients. After combining the four expression levels into a single discriminant value, a positive value correlated with a significantly worse PFS and OS.[51]

Discussion

Circulating tumor cells are being recognized as a promising diagnostic tool in oncology, and thus many efforts have been made to detect them reliably. In breast cancer, several techniques (both cytometric and nucleic-acid based) have been explored in different settings yielding interesting results. However, in addition to independent confirmation of these results, several issues remain to be resolved.

Studies have shown remarkably varying CTC counts, ranging from <5 to thousands per ml in the same patient category. Whether these differences are caused by cancer biology or varying sensitivity of the techniques used, awaits clarification. Limited studies have been performed comparing the sensitivity and specificity of the different detection techniques. The ultimate goal is to set up an assay that generates inter-individually interpretable results for each individual patient, underlining the necessity for consensus on the exact technique for enrichment and detection that should be applied and on the markers that should be used. Given the heterogeneity between breast cancer subtypes, it is anticipated that assays cannot rely on a single, universally expressed and specific marker. Therefore, efforts should be made to develop a marker set, as multiple markers probably do more justice to the heterogeneity of breast cancer. EpCAM seems to insufficiently detect some molecular subtypes of breast cancer[24] prompting the need for a combination of cellular markers by which all breast cancer subtypes can be detected.

Despite these challenges, enumeration of CTCs has shown to bear prognostic information, and may inform oncologists about response to systemic therapy shortly after its start. Building on this, it would be of great benefit if the increase or decrease in CTC count after the first cycle of systemic treatment would be a better guideline for continuation or switch of therapy than conventional (radiologic) evaluation after 2–3 cycles. Currently, a study is being conducted by the Southwest Oncology Group, measuring CTCs at baseline and after the first cycle of chemotherapy. Patients with >5 CTCs per 7.5 ml blood at baseline who remain at >5 CTCs per 7.5 ml blood after completing one course of chemotherapy are randomized to either continue their current chemotherapy or to switch to a different regimen. The results of this trial (www.clinicaltrials.gov search for NCT003820128[82]) could prove a big step forward in the clinical field.

Another interesting possibility in the CTC field is their molecular characterization. Klein et al. have applied comparative genomic hybridization and discovered remarkable heterogeneity between individual tumor cells in patients treated in the adjuvant setting over time as well as between patients.[83] In these patients, only a few TP53 mutations were revealed. In patients treated in the metastatic setting, genetic heterogeneity over time was less marked, but TP53 mutations were encountered more frequently.[83] Smirnov et al. were the first to generate global gene expression profiles of CTCs from three cancer patients, resulting in 35 cancer- and CTC-specific genes.[84] The expression of these genes was subsequently confirmed by qRT-PCR in 74 metastatic breast cancer patients and 50 healthy donors, ultimately generating 16 genes (such as AGR2 and FABP1), which may be useful to distinguish individuals without cancer from cancer patients, as well as distinguishing breast cancer from colon cancer and prostate cancer patients.[84]

Molecular characterization of primary tumor tissue by gene expression profiling has shown to yield prognostic and predictive models in breast cancer.[85–87] The level of expression of various genes is determined, which results in a number of differentially expressed genes, which can classify patients into a poor-prognosis and good-prognosis group. Gene expression profiles have also shown a correlation with response to therapy. However it is likely that in the metastatic setting, molecular characterization of CTCs better represents tumor genetics than of primary tumors. Studies have shown that expression of clinically relevant markers such as ER, PR and HER2 can differ between the primary tumor and its metastases.[17,88] For HER2, nearly one-third of patients whose primary tumor was HER2 negative, had amplified HER2 on CTCs.[17] Furthermore, tumor characteristics may change over time under pressure of therapy. In a preliminary report, 27% of patients with HER2-negative primary tumors acquired HER2 overexpression during the course of chemotherapy.[89] Among 23 patients obtaining less than a pathologic complete response on neoadjuvant chemotherapy with concomitant trastuzumab, 7 (30.4%) had HER2-negative residual tumors at surgery.[90] It is likely that when the metastatic tumor cells gain or lose such important markers, treatment should change accordingly. Prospective studies should clarify whether it is indeed of benefit, for example, to start trastuzumab therapy when the CTCs of a prior HER2-negative primary tumor do express HER2. A predictive and prognostic gene expression model for CTCs could be of great help to the oncologist in making treatment decisions as the disease progresses. In addition, comparison of molecular profiles of primary tumors to CTCs may provide better insight into those mechanisms involved in dissemination. Tumor cells are thought to lose and/or gain specific gene expression as they evolve to enter the circulation and proliferate in a target organ. Identifying these changes in gene expression for each point in the evolution toward overt metastasis could improve our understanding of the metastatic cascade.

In conclusion, several assays enabling the detection and enumeration of circulating tumor cells in breast cancer have been introduced during the past 10 years. With some of these, already promising results with potential clinical relevance have been obtained. If confirmed, this may pave the way for the introduction of such assays in daily clinical care. However, much is still uncertain in this field and in particular consensus is required on the most optimal assays for CTC detection. In addition to enumeration,

characterization of CTCs forms an interesting possibility to clarify the metastatic cascade and to improve prognostic and predictive models enabling more individualized treatment of breast cancer patients.

Conflicts of interest statement

None declared.

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