Improving the CellSearch® system

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REVIEW

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Improving the CellSearch® system

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ABSTRACT

Introduction: The CellSearch® CTC test enumerates tumor cells present in 7.5 ml blood of cancer patients. improvements, extensions and different utilities of the cellsearch system are discussed in this paper.
Areas covered: This paper describes work performed with the CellSearch system, which go beyond the normal scope of the test. All results from searches with the search term 'CellSearch' from Web of Science and PubMed were categorized and discussed.
Expert commentary: The CellSearch Circulating Tumor Cell test captures and identifies tumor cells in blood that are associated with poor clinical outcome. How to best use CTC in clinical practice is being explored in many clinical trials. The ability to extract information from the CTC to guide therapy will expand the potential clinical utility of CTC.

1. Introduction

The CellSearch (Janssen Diagnostics, LLC, Raritan, NJ, USA) test for the isolation of circulating tumor cells (CTCs) from peripheral blood has been on the market for 12 years. The kit has been cleared by the US FDA for clinical use in metastatic prostate, breast, and colon cancer patients and also showed clinical relevance in many other types of cancer. Due to the emergence of personalized medicine in cancer treatment, the need for biomarkers giving accurate and actual insight in the state of the disease is rising quickly [1]. In recent years, many new targeted drugs have come to the market for treating cancer patients, and a lot more are expected in the upcoming years [2,3]. Circulating biomarkers such as CTCs, circulating proteins, and nucleic acids are expected to contain the biomarkers needed to select a suitable therapy. Sampling is minimally-invasive, and can easily be done on a frequent basis for close monitoring. The survival of CTCs in blood is hypothesized to be short, resulting in real-time information of the state of the disease [4–6]. Because the cells can be individually captured and analyzed, heterogeneity can be determined as well as the combinations of aberrations present in each cell. Also when therapy resistance occurs and a change in therapy is needed, the CTCs will most probably reflect the resistant population of cells. An obvious disadvantage of these cells is the low number. Only in advanced patients, a tube of blood contains CTCs in single or in some cases double digits. The CTCs isolated by the CellSearch system have been extensively explored. Immunoassays, single-cell isolation, array-comparative genomic hybridization (CGH), next-generation sequencing, quantitative polymerase chain reaction (qPCR), and fluorescence in situ hybridization (FISH) have been developed on these cells. The CellSearch system has also been used for the isolation of other cell types and subgroups. This review will describe the CellSearch test and will discuss all improvements, extensions, and modifications that were developed using the CellSearch equipment or reagents.

A wide variety of isolation and detection methods have been developed to enrich and enumerate CTCs. Most of these methods end with an enriched CTC population, which is subsequently analyzed and enumerated. The enrichment techniques can be separated in two major categories: enrichment based on affinity, using selective antibodies, and enrichment based on biophysical properties, for example, size, density, deformability, or dielectric properties. An extensive overview of these methods is published by Esmaeilsabzali et al. [7], and a number of these methods have been commercialized. Only one of these commercialized tests has been cleared by the FDA for in vitro diagnostic (IVD) use: the CellSearch test. Even though many studies show that more populations of tumor cells, or tumor-like cells, exist in the bloodstream of cancer patients, only the population of CTCs isolated with the CellSearch test has been proven to be clinically relevant in a variety of different cancer types [8–12].

The CellSearch test has been developed for enumeration of CTCs of epithelial origin. This test is cleared by the FDA for monitoring patients with metastatic breast, prostate, and colorectal cancer. Next to the FDA-cleared CellSearch test, a number of extended, related, and accompanying tests have been developed which will be described in this review. Important to note is that these extended tests are not in any case cleared for clinical prognostic, diagnostic, or predictive use. These tests are either detecting different cell types or cancer types or allowing a further analysis on the isolated fraction of cells or on the contents of these cells. Future analysis of CTCs will probably account for genomic,

transcriptomic, epigenetic, and proteomic aberrations to obtain complete insight in the disease at each specific moment during the course of the disease.

1.1. The CellSearch system

The CellSearch test starts with a blood draw of the patient in a CellSave tube. About 8–10 ml of blood is collected in a CellSave tube, which contains a slow fixing preservative. Leukocytes and CTCs are stabilized, and the sample can be processed up to 96 h after blood draw. The standard CellSearch procedure is illustrated in Figure 1 using the numbers 1 through 9 in the black circles. The green squares A to I show the alternative and extended tests that are discussed in this review. Step 1: 7.5 ml of the fixed blood is pipetted into a specific CellSearch conical tube and 5.5 ml of CellSearch dilution buffer is added to the blood after which it is centrifuged at $800 \times g$ for 10 min without brake. Step 2: The tube is carefully loaded into the AutoPrep system. Step 3: The diluted plasma will be removed until 1 cm above the red blood cell layer. Step 4: Anti-epithelial cell adhesion molecule (EpCAM) ferrofluid and dilution buffer are added to the tubes and mixed by pipetting. In addition, magnets are moved back and forward towards the tube to enhance the collisions between cells and ferrofluids. Step 5: After an incubation period, the magnets remain against the tube, anti-EpCAM-ferrofluids, and the cells that have bound ferrofluid will be pulled to the magnets, and the rest of the cells are removed in a single pipetting step. Step 6: The cells are permeabilized and stained. Step 7: Cells are washed with the use of magnetic stations to retain the cells. Step 8: The cells are resuspended in a small volume. Step 9: In this last step, the cells are transferred to a cartridge, which is placed in a magnetic field to pull the cells in one focal plane against a glass surface. This cartridge is scanned on a dedicated fluorescent microscope scanner (CellTracks Analyzer II; Janssen Diagnostics, LLC, Raritan, NJ, USA) for each individual fluorochrome. The software selects and presents all events that contain phycoerythrin (PE) and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) close together, and a trained operator will select the events according to the CellSearch CTC definition. This definition is described in detail by Coumans and Terstappen [13].

1.2. Commercially available CellSearch kits

The commercially available CellSearch kits are listed in Table 1. The CellSearch CTC kit identifies a clinically relevant population of CTCs. The clearance of this test by the FDA as an IVD test implies that the whole procedure, from reagent production and instrument maintenance to operator training, is carefully described and secured. Therefore, any change to the system, reagents, or analysis would risk a chance of detecting a population of cells that has a different relevance. The circulating epithelial cell kit is the research-use-only version of the IVD kit. Both kits isolate CTCs based on their EpCAM expression, stain the DNA of the cells with DAPI, epithelial cells with PE-labeled antibodies recognizing cytokeratins identified by the clone C11 and A.53B/A2, and leukocytes with allophycocyanin (APC)-labeled antibodies recognizing CD45. The procedure is described in more detail elsewhere [14]. The intended use of the FDA-cleared CellSearch test is: 'the presence of CTC in the peripheral blood, as detected by the CELLSEARCH CTC kit, is associated with decreased progression-free survival and decreased
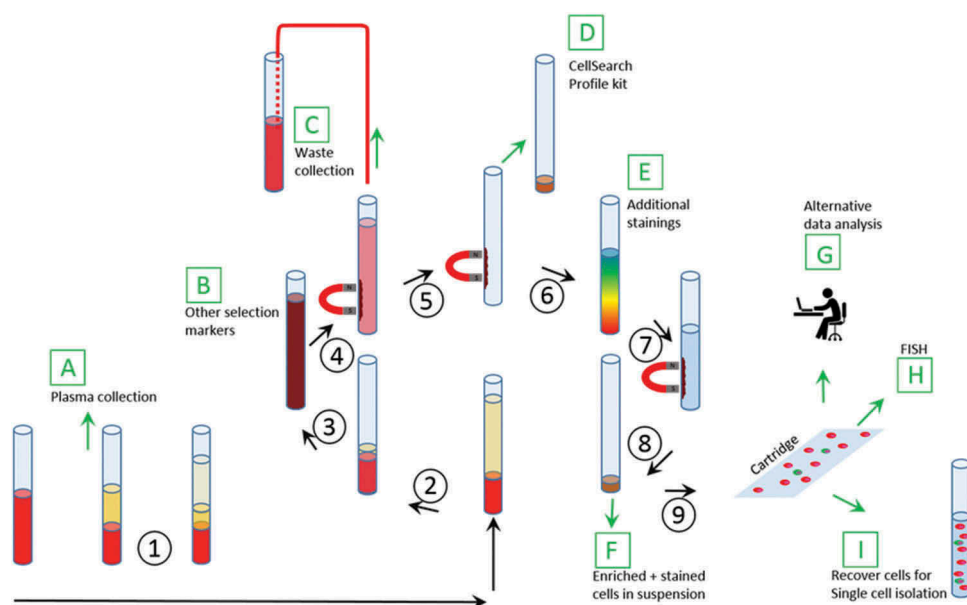
Figure 1. Schematic presentation of the CellSearch procedure. Numbers 1 to 9 in the black circles describe the basic CellSearch procedure. Green squares A to I indicate alternative CellSearch methods, additions to the test and extra analysis of the isolated CTCs discussed in this review. CellSearch processing procedure: 1: Preparation of 7,5 ml of blood by the addition of CellSearch dilution buffer and centrifugation. 2: Removal of the excess of diluted plasma by the CellTracks AutoPrep. 3: Addition of anti-EpCAM-ferrofluid. 4: Magnetic incubation and removal of the unlabeled cells. 5: Washing of the cells. 6: Addition and incubation with staining reagents. 7: Magnetic incubation and washing of the cells. 8: Resuspension of the cells in a small volume. 9: Loading of the cell suspension into a cartridge for scanning. Full color available online.

Table 1. Commercially available CellSearch kits.

Test name CellSearch	Capture antigen	Positive staining antigen	Dye	Negative staining antigen	Dye
Circulating tumor cell kit (IVD)	EpCAM	CK (clone C11 & A.53B/A2)	PE	CD45	APC
Circulating epithelial cell kit	EpCAM	CK (clone C11 & A.53B/A2	PE	CD45	APC
CXC kit	EpCAM	CK (clone C11 & A.53B/A2	FITC	CD45	APC
Circulating epithelial cell profile kit	EpCAM	None	-	None	-
Qualified marker		Her2	FITC	-	-
		EGFR	FITC		
		IGF-1R	PE		
Circulating melanoma cell kit	CD146	RAB38/NY-MEL-1	PE	CD45 & CD34	APC
Circulating endothelial cell kit	CD146	CD105	PE	CD45	APC
Circulating endothelial cell profile kit	CD146	None		None	
Circulating tumor cell control kit	Fixed SKBR-3 cells at low and high concentration				
Epithelial cell control kit	Fixed SKBR-3 cells at low and high concentration				
CXC cell control kit	Fixed SKBR-3 cells at low and high concentration				
CEC/CMC control kit	Fixed SK-MEL-28 cells at low and high concentration				

IVD: *in vitro* diagnostic; CEC: circulating endothelial cell; CMC: circulating melanoma cell; PE: phycoerythrin; APC: allophycocyanin; EpCAM: epithelial cell adhesion molecule; CK:cytokeratin; EGFR: Epidermal growth factor receptor; IGF-1R: Insulin like growth factor 1 receptor; FITC: Fluorescein isothiocyanate.

overall survival in patients treated for metastatic breast, colorectal, or prostate cancer. The test is to be used as an aid in the monitoring of patients with metastatic breast, colorectal, or prostate cancer. Serial testing for CTC should be used in conjunction with other clinical methods for monitoring metastatic breast, colorectal, and prostate cancer. Evaluation of CTC at any time during the course of disease allows assessment of patient prognosis and is predictive of progression-free survival and overall survival.' Twelve years after the FDA clearance, the CellSearch test is however not commonly used in routine clinical practice as its best use and benefits are not sufficiently clear. At present, 868 clinical trials are registered (www.clinicaltrials.gov) in which a role for CTC in routine clinical practice, a surrogate for survival in clinical trials or a guide for therapy, is being explored. The outcome of these studies hopefully will provide clarity of the best use of CTC in clinical practice.

The CellSearch test used for the FDA clearance has also been tested in many other cancers [15], and the presence of CTC is also associated with poor clinical outcome in gastric cancer [16,17], small-cell lung cancer [18–20], melanoma [21,22], endometrial cancer [23], esophageal squamous-cell carcinoma [24,25], cholangiocarcinoma [26], colon cancer, liver metastasis [27], hepatocellular carcinoma [28], pancreatic cancer [29], rectal cancer [30], bladder cancer [31], head and neck cancer [32], and ovarian cancer [33]. Publications referred to above are just examples. In most cases, CellSearch has been used in multiple studies. The CellSearch system has also been used in patients with no known metastasis, and the presence of CTC in these setting also predicted an increased chance of relapse [34,35].

In the CXC kit, the antibodies recognizing cytokeratins are conjugated to Fluorescein isothiocyanate (FITC) instead of PE. This is done to detect antigens on CTC expressed at a low antigen density, which is enabled by the high-quantum yield of the PE dye as in comparison to FITC. The first example for the need of the higher sensitivity was the detection of the insulin-like growth factor receptor-1 on CTC [36], and many have followed since. An overview of tumor marker identification using the CellSearch kit is given in Table 2.

In the circulating epithelial cell profile kit, the protocol is the same up to step 5 (see Figure 1) after which the immuno-magnetically enriched cells are resuspended in a tube for

Table 2. Publications on extra stainings using the CellSearch system.

PMID	Year	Target	Cancer	Author
17079488	2006	UPAR Her2a	Breast cancer	Meng [84]
17906897	2007	Her2	Breast cancer	Stojadinovic [38]
17575225	2007	IGF-1R	Prostate cancer	De Bono [36]
19102715	2009	EGFR	Breast cancer	Payne [92]
20838621	2010	EGFR plus FISH and qPCR	General	Punnoose [93]
20859679	2010	Her2	Breast cancer	Fehm [85]
20406831	2010	Her2	Breast cancer	Riethdorf [82]
20978147	2010	M30	General	Rossi [94]
21264346	2011	Her2	Breast cancer	Ignatiadis [81]
22899576	2012	CD44 M30	Assay development	Lowes [95]
22476856	2012	Her2	Breast cancer	Pestrin [86]
22277196	2012	Her2	Breast cancer	Rink [87]
23275633	2013	Her2	Breast cancer	Ligthart [96]
23538216	2013	M30 BCL2	Breast cancer	Smerage [11]
24023327	2013	EGFR	Colorectal cancer	Kuboki [97]
24201755	2013	Her2	Gastrointestinal cancer	Iwatsuki [98]
24637923	2014	optimization	General	Lowes [70]
25528628	2015	CK20	Colorectal cancer	Welinder [99]
25719830	2015	AR	Prostate cancer	Crespo [100]
25972110	2015	Her2	Breast cancer	Wallwiener [83]
25957999	2015	MCT1 MCT4	General	Kershaw [101]
26093818	2015	PDL1	Breast cancer	Mazel [102]
25896421	2015	Post CellSearch ER, Her2	Breast cancer	Frithiof [89]
25381338	2015	ER BCL2 Her2 KI67	Breast cancer	Paoletti [91]
25450039	2015	Ecadherin CD133	Prostate cancer	Pal [73]
26923772	2016	Vimentin Ki67	Prostate cancer	Lindsay [103]
26695546	2016	Post CellSearch CK7/20, TTF-1, ER, PSA	Cancer of unknown origin	Matthew [104]
27145459	2016	PSA	Prostate cancer	Gorges [105]
26967453	2016	MUC-1	Pancreatic cancer	Dotan [106]
27178224	2016	ER BCL 2Her2 KI67	Breast cancer	Paoletti [90]

[a] Immunicon anti-EpCAM ferrofluids were used for manual circulating tumor cell enrichment and staining.

further alternative analysis. This kit was originally developed to maintain the viability of the cells and enable the analysis of the RNA content of the cells [37]. To maintain viability, the blood has to be drawn in ethylenediaminetetraacetic

acid (EDTA) collection tubes rather than CellSave blood collection tubes. Since the introduction of the profile kit, a variety of alternative technologies have been introduced that use EpCAM-expressing cells from blood. A more detailed description of the applications for which the profile-enriched cells are used is given in Section 2.4.

Assessment of the presence or absence of targets for therapy is one of the important applications of CTC. The first demonstration of this potential was done by showing the expression of Her2 on CTC in breast cancer patients [38]. The antibodies recognizing Her2-FITC, epidermal growth factor receptor (EGFR)-FITC, MUC-FITC (withdrawn from the market), and IGF-1R have been made available. Assays for other treatment targets such as the androgen receptor, estrogen receptor, bcl-2, and vascular endothelial growth factor receptor (VEGFR) have been developed but are not commercially available. A summary of markers assessed on CTC using the CellSearch system and the expansion of the number of markers that can be assessed simultaneously is described in Section 2.5.

In the CellSearch system, the antibodies on the ferrofluids determine the class of cells that will be enriched. For the enrichment of non-hematopoietic cells, it is of utmost importance that the antibody does not recognize hematopoietic cells specifically or nonspecifically [39,40]. A variety of antibody ferrofluids have been used on the CellSearch system, and those that are not commercially available are described in Section 2.2.

In the melanoma cell kit, ferrofluids conjugated to CD146 are used. The antigen recognized by CD146 is also known as the melanoma cell adhesion molecule (MCAM) and cell surface glycoprotein MUC18. CD146 is expressed on melanoma cells, endothelial cells, smooth muscle cells, and a subset of activated T-lymphocytes. The melanoma cells are identified as nucleated cells that express the high-molecular-weight melanoma-associated antigen and lack CD45 and CD34 [22]. Circulating melanoma cells identified in this manner also associate with poor clinical outcome [21,22,41–43].

The circulating endothelial cell kit also uses CD146 ferrofluids but uses a different staining cocktail, resulting in nucleated endothelial cells defined as CD146+, CD105+, and CD45- [44]. Increased levels of circulating endothelial cells have been observed in patients with cancer and cardiovascular disease [45–48].

Similar to the epithelial cell profile kit, the endothelial/melanoma cell profile kit is available. Only the CD146-enriched fraction is provided that can be used to characterize the enriched cells by other means such as RNA profiling [49].

To ensure that the CellSearch system is performing, control kits are provided that contain a low number (~50) and high number (~1000) fluorescently labeled and preserved cells derived from either the breast cancer cell line SKBR-3 or the melanoma cell line SK-MEL-28. The cells at the low and high number are labeled with different fluorescent membrane dyes. After processing on the CellSearch AutoPrep, they are analyzed on the CellTracks Analyzer II. The high number of cells is automatically counted, and the cells with the low number have to be manually identified.

2. Applications beyond the standard CellSearch assays

2.1. Plasma collection

Circulating biomarkers such as proteins, DNA, or microparticles can also be of great value for monitoring cancer patients. The use of proteins such as carcinoma antigen-125, carcinoembryonic antigen, mucin-1 (MUC1), and prostate-specific antigen is already common in clinical practice. Circulating DNA can be used for the detection of tumor-specific single-base substitutions, insertions, deletions, and translocations. The use of ctDNA as a cancer biomarker has already been shown in a number of larger studies [50–52]. Tumor microparticles (TMPs), tumor-derived exosomes, or tumor-derived extracellular microvesicles (tEVs) are vesicular structures derived from tumor cells. tEVs can be derived from the plasma membrane by apoptosis or cell death or can be actively produced by endosomal pathways. Most of the larger vesicles have high densities and will end up in the cell fraction of centrifuged blood and not in the plasma. For isolation of the small-sized tEVs from plasma, several methods are available, which are done mainly by size exclusion chromatography or ultracentrifugation [53]. The potential of tEVs as cancer biomarkers is currently being investigated [54,55].

In the standard CellSearch assay, the plasma is discarded. It is, however, straightforward to aspirate and collect the plasma before placing the tubes on the CellTracks AutoPrep. To do this, 7.5 ml blood is centrifuged inside a CellSearch conical tube for 10 min at 800×g. Plasma can be taken up to 1 cm above the red blood cell layer. After this the tube can be filled with the CellSearch dilution buffer. Blood draws for CellSearch enrichment of the CTCs using a CellSave blood collection tube are particularly suitable for the collection of a plasma sample to isolate ctDNA. The cell-stabilizing fixative in the tubes will prevent normal genomic DNA to be released in the plasma by cell lysis or apoptosis of leukocytes. The high variation in background DNA is one of the main difficulties in the development of tests identifying tumor-derived plasma DNA. Although the number of patients is still limited, Kang et al. showed that the plasma samples from CellSave tubes contain detectable concentrations of cancer-specific mutations using digital droplet PCR for up to 48 h after blood draw and perform equal or better compared to the Streck cell-free DNA tubes and better than EDTA tubes [56]. Biomarkers assessed in plasma and CTC may provide complementary information, and when it can be obtained from the same blood drawtube, it provides clear advantages.

2.2. Changing the antibodies on the ferrofluids and/or CellSearch assay conditions

The choice of EpCAM as the target for enrichment in the CellSearch CTC kit was based on preliminary work that was performed in which flow cytometry was used as the platform to analyze immunomagnetically enriched cells [57–59]. In these studies, EpCAM antibodies derived from the GA73.3 clone were used that were later replaced by antibodies derived from the VU1D9 clone, recognizing the same EpCAM

epitope. Whether or not a higher recovery of CTC can be obtained when using antibodies with a higher affinity and/or a combination of antibodies recognizing different epitopes has never been thoroughly investigated [60]. The use of controlled aggregation of EpCAM ferrofluids in the CellSearch kits has, however, already made quite an improvement in the recovery of CTC with relatively low EpCAM antigen density [61]. An alternative is the use of antibodies recognizing different antigens expressed on cells of epithelial origin. Jo Hilgers also known as the great 'MUCinier' believed that antibodies against Muc-1 were needed to efficiently capture CTC as they would otherwise be stuck in the MUC. Comparisons of the CTC capture efficiency of ferrofluids labeled with EpCAM or EPCAM & Muc-1 in patients with metastatic breast cancer however showed no improvement in CTC capture (Figure 2 panel a) and was associated with an increase in white blood cell carry-over (Figure 2 panel b). The study was stopped after 11 patients were included as the larger number of white blood cells decreased the specificity of the test, and many more patients would need to be analyzed to determine whether any patients expressed MUC-1, but not EpCAM.

In studies by Mostert et al. and Onstenk et al., the addition of CD146 (MCAM) to EpCAM ferrofluid as a selection marker for CTCs in breast cancer patients was tested [62,63]. The rationale was that breast cancer cell lines with epithelial to mesenchymal transition (EMT) characteristics might express CD146. In the assay, CD34 is used to exclude circulating endothelial cells, which are co-isolated using CD146. Although the number of patients is low, the number of patients positive for CTC increased from ~16% to ~30% when combining the results for EpCAM (ferrofluid)+ CK +/CD45-/DAPI+ and CD146 (ferrofluid)+ CK+/CD34-/CD45-/DAPI+. Both CTC types, however, did not correlate with clinical parameters in this study, and further investigation is needed to determine the clinical relevance of these cells.

To identify disseminated tumor cells (DTCs) in bone marrow, the CellSearch CTC kit was adapted. Assay optimization was performed using bone marrow aspirates from normal donors. The optimal volume of bone marrow aspirates to process on a CellTracks AutoPrep was found to be 3 ml (CellSave added as anticoagulant and preservative); the EpCAM ferrofluid concentration was reduced by 40%, and the antibody recognizing cytokeratin 19 was removed as it was found to be expressed on megakaryocytes as confirmed by expression of CD61 (Figure 3). The staining reagents used in the final assay were cytokeratins antibody C11 FITC and CD45-APC. The larger leukocyte concentration resulted in a two- to threefold higher leukocyte carryover when compared with the CellSearch whole-blood assay, but recovery, accuracy, and linearity of spiked cell lines were similar.

A study was conducted to compare the assay with the assay used in the Pantel Laboratories (University Medical
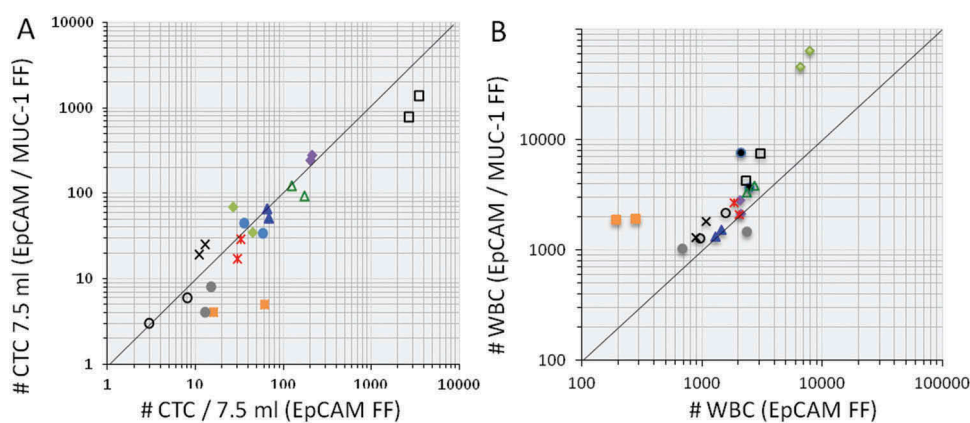

Figure 2. Thirty ml of blood from 11 metastatic breast cancer patients were immunomagnetically enriched. Two 7.5 ml aliquots using EpCAM ferrofluids and two with a combo of EpCAM & Muc-1 ferrofluids. Enriched samples were stained with a nucleic acid dye, antibodies directed against Cytokeratin and CD45. CTC and white blood cells (WBC) were enumerated by flowcytometry. Each symbol represents a patient and duplicates are represented with the same symbol in both panels.
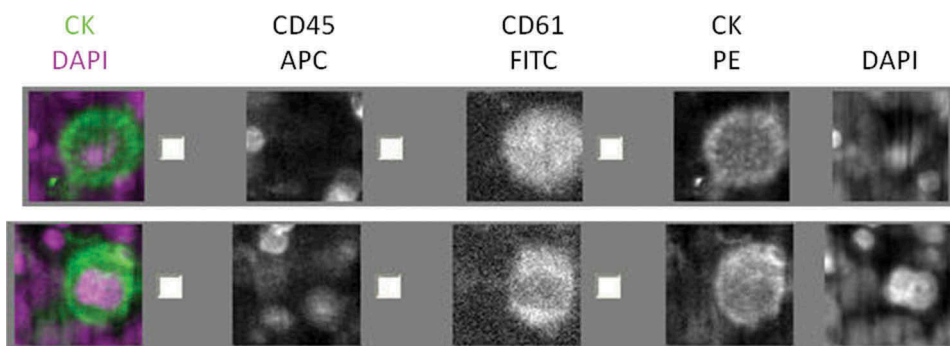

Figure 3. Megakaryocytes in bone marrow enriched by EpCAM ferrofluid and staining with Cytokeratin 19 and CD61 and lacking CD45.

Centre Hamburg-Eppendorf, Hamburg, Germany) on bone marrow samples from 54 healthy donors and 93 patients with primary breast or prostate cancer. Bone marrow aliquots were split into two, one for the CellSearch marrow assay and one for staining and analysis in the Pantel Laboratories. Results are illustrated in Figure 4 and show little concordance, which would imply that a large study would need to be performed to demonstrate a relation with an increased risk for recurrence as was already demonstrated for the slide-based CTC assay [64,65]. At present, DTCs are not used in clinical practice, and besides the availability of a validated assay, studies will be needed to determine the potential benefits above gene signatures obtained from the primary tumor

that recently have been introduced to identify patients that will or will not benefit from adjuvant therapy [66,67].

CellSearch was also tested, optimized, and confirmed to work on small-volume mouse (xenograft) samples [68,69]. Puncture methods were investigated to identify the best way to be able to repeatedly sample the mice. Lateral tail vein, retro-orbital venous plexus, jugular vein, and the left ventricle of the heart were compared. In retro-orbital and jugular puncture samples, epithelial cells were found in the negative control mice. Tail vein samples were too small and therefore obtained no CTC in the positive controls, but the cardiac puncture was found to contain no cells in the negative controls and epithelial cells in the positive controls. Tests on
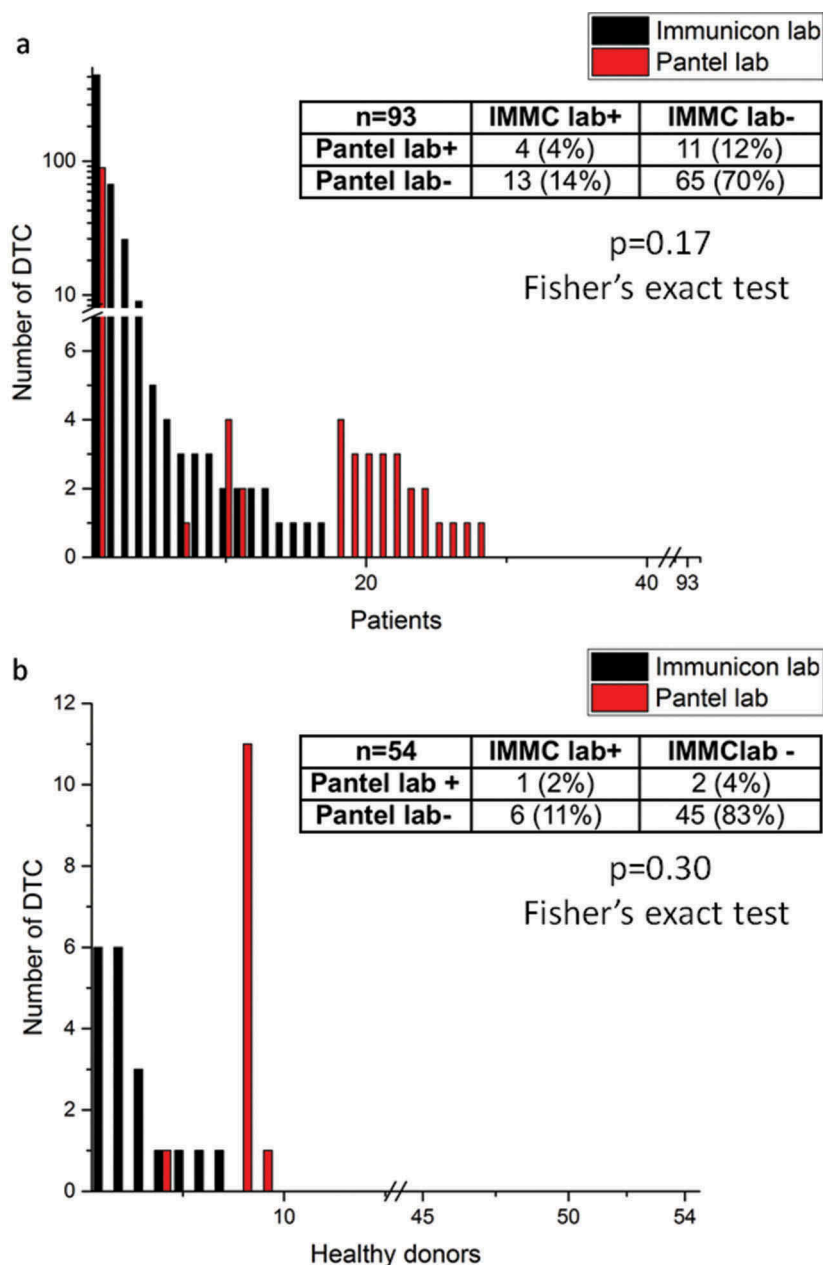

Figure 4. Comparison of the number of DTCs detected with CellSearch Marrow assay and the standard DTC assay used in the Pantel laboratories in 93 patients with primary breast and prostate cancer (Panel a) and 54 bone marrow aspirated of healthy donors (Panel b). Although a Fisher's exact test showed no significant difference between both DTC assays, the concordance was low which can be expected with the low numbers of DTCs detected [72]. The background of DTCs detected in bone marrow of healthy donors is higher as compared with the CTCs in 7.5 ml of blood of healthy donors [12].

patient-derived xenograft models showed that in a number of patients CTCs can be found in the blood of the mice and that the mice could be monitored in time using cardiac punctures. The CellSearch test adapted for mouse samples was commercially available as mouse/rat cell capture kit but has been discontinued. Lowes et al. have described the use of the CellSearch reagents for a manual isolation based on this kit [70].

To identify circulating multiple myeloma cells (CMMCs), a CellSearch test was developed in which antibodies recognizing CD138 were conjugated to ferrofluids and used to enrich for plasma cells. These cells will be isolated from peripheral blood using CD38 and/or CD138 as was presented at the American association for cancer research (AACR) annual meeting 2016 [71]. These cells are found in elevated levels in the peripheral blood of patients with plasma cell disorders. FISH and transcriptional profiling of these cells show aberrations consistent with those found in the tumors. Multiple myeloma patient groups at risk can be separated using the CellSearch CMMC enumeration data.

2.3. Collection of the immunomagnetically depleted blood

CellSearch captures a clinically relevant group of CTCs based on EpCAM expression. Several studies indicate that there are tumor cells expressing lower EpCAM that are missed by CellSearch [73,74]. To investigate if these cells can indeed be found in the depleted fraction of blood, de Wit et al. introduced a device that collects the blood from the CellTracks AutoPrep after immunomagnetic cell capture [75]. In this study, the standard CellSearch assay was performed; the EpCAM-depleted blood was collected and subsequently passed through a filter with 5 μm pores. The cells collected on the filter were stained for cytokeratins and leukocyte markers and counted. On the filters, about the same number of CTCs could be found as with CellSearch. However, other than the CellSearch cells alone, the cells on the filter or the sum of CTCs did not correlate with clinical outcome. Studies are ongoing to reveal the differences in the EpCAM+ and EpCAM- tumor cells as well as studies in other cancers. A protocol to manually collect the discarded blood is available on the medical cell biophysics (MCBP) website (https://www.utwente.nl/tnw/mcbp/protocolsandtools/).

2.4. Immunomagnetic separation only

The first step in the CellSearch AutoPrep system is the enrichment of the CTCs. After this isolation, the system will perform permeabilization, staining, and washing steps. The CellSearch profile kit will only do the enrichment step and present the enriched cell suspension in the same conical tube. This procedure is comparable to Illumina's MagSweeper system. A user can now do a manual staining procedure or proceed immediately to a lysis step for total RNA analysis. Samples isolated with the regular CTC kit are permeabilized for optimal cytokeratin and nuclear staining and are therefore not expected to retain the mRNAs. Important for RNA work is that the cells are not fixed. Blood draw should therefore not be done in

CellSave tubes but in EDTA, acid citrate dextrose (ACD), or heparin tubes. Doing a total RNA analysis using the profile kit has the advantage that the procedure is fast and standardized. Disadvantage is that the number of background cells is unknown. An estimation of the CTC number could be made by also performing a standard CellSearch CTC test. The first demonstration of the feasibility of this approach was reported by Smirnov et al. [37]. Onstenk et al. recently showed the detection of the splice variant of androgen receptor (AR) (AR V7), which is associated with resistance to abiraterone and enzalutamide, by using reverse transcriptase-qPCR (RT-qPCR) after mRNA isolation of the profile kit products [76]. The same procedure was also used by others [37,49,77,78]. Cho et al. are using a linear T7 preamplification step for the RNA to compare the profile kit CTC mRNA to mRNA from microdissected single bone lesions [79]. They found a high concordance between presence and absence of detectable gene expression in both fractions.

Next to mRNA analysis, the CellSearch profile kit has also been used for the isolation of viable CTC to attempt expansion of the tumor cells. An example is a study by Rossi et al. who used the CTC for xenografts [80]. Blood drawn in EDTA tubes was enriched using the CellSearch profile kit and was injected subcutaneously into nonobese diabetic/severe combined immunodeficient mice. Cells were found back in the peripheral blood, bone marrow, and spleen, which indicates the migratory capabilities of this EpCAM-positive cell fraction.

2.5. Additional reagents for cell labeling

Additional markers can be added to the CellSearch test staining mix. To do this, a fluorescent-labeled antibody can be added to the system in a separate vial. For the normal CTC kit, this antibody needs to be labeled with a dye that fits into the free FITC channel. Janssen has three commercially available CellSearch tumor phenotyping reagents: Her-2/neu, IGF-1R, and EGFR. Protocols for androgen receptor, estrogen receptor Ki-67, and VEGFR-2 are also available. If a target with low expression is investigated, it is possible to use the CXC kit. This kit uses, as described above, the FITC channel for the cytokeratin detection leaving the stronger PE dye free to use for the extra marker. Many studies have been done looking at the Her-2 expression on CTCs using CellSearch [38,81–89]. Most of these studies show that a number of patients develop Her-2-positive CTCs, while the primary tumor was negative indicating a new possible treatment target for this group of patients. A recent article from Paoletti et al. shows the use of the CXC kit for studying the development of resistance of breast cancer patients to the estrogen receptor downregulating drug fulvestrant [90,91]. This small study suggests a heterogeneous cause of resistance based on the expression of estrogen receptor (ER) and B-cell lymphoma 2 (BCL-2) on CTCs. Table 2 gives a complete overview of the studies, research, and development work done on extra target analysis of CellSearch CTCs.

In principle, any fluorescently labeled antibody can be added to the CellSearch test. The choice of the fluorochrome depends on the fluorescent microscope used to analyze the CellTracks cartridges. The CellTracks Analyzer III that is not

commercially available is, for example, equipped with a 10X objective (numerical aperture (NA) 0.45) and a 40X objective (NA 0.6) and has place for eight fluorescent cubes. The filters in these cubes need to match the fluorochromes used with as little spectral overlap as possible. Interpretation of the images can be quite different between operators, and guidelines have been introduced to define what is and what is not considered a CTC in the CellSearch system [15]. This definition has shown to be quite robust in several ring studies that have been conducted [107,108]. A variety of new technologies have been introduced after the introduction of CellSearch, and most compare the results obtained with those from CellSearch [109–111]. A difficulty here is that the definitions used to define a CTC are not the same, thus comparing apples with oranges. To overcome the image analysis component of this problem, an open-source image analysis program baptized automated CTC classification enuemerating and phenotyping (ACCEPT) is being developed as part of the European union (EU)-funded programs CTCTrap (https://www.utwente.nl/tnw/ctctrap/) and CANCER-ID (http://www.cancer-id.eu/) and will be available at the MCBP and CANCER-ID websites. ACCEPT uses a novel approach to identify objects in the stored images [112], and a variety of features can be extracted from the identified objects. An example of the use of ACCEPT is illustrated in Figure 5. A CellSearch CXC test is done on a healthy donor blood sample spiked with ~200 SKBR3 cells and ~200 MCF7 cells (SKBR3: high cytokeratin, moderate EpCAM, and high Her2/neu) (MCF7: high cytokeratin, high EpCAM, and negative Her2/neu). The extra marker possibility was used to add three markers: polyclonal anti-EpCAM-PE (Sigma cat SAB4700425), anti-Her2/neu-DEAC (Antibody clone Her81 conjugated with DEAC-NHS [Sigma cat. 36801]), and antiCD16-PERCP (BioLegend cat.302030) as an extra negative marker. Figure 5 shows three scatter plots of the objects identified in the cartridge. Only the mean intensity is shown in this figure, but other features such as area, roundness, and overlap with other channels are also measured and can be used to create more complex gates or definitions. In panel a, a gate is set on cytokeratin FITC+ and DAPI+ objects to select possible CTC candidates; the position of these objects can be observed as orange dots in the scatter plots. In panel b, several of the orange cells express CD45 and CD16 or only CD16 and are excluded as CTC. Panel c shows the Her2/neu DEAC and EpCAM PE expression of the cytokeratin FITC+, DAPI+, CD16-PerCP-, and CD45-CTC. Two distinct populations of CTC can be observed based on Her2/neu expression (Her2/neu
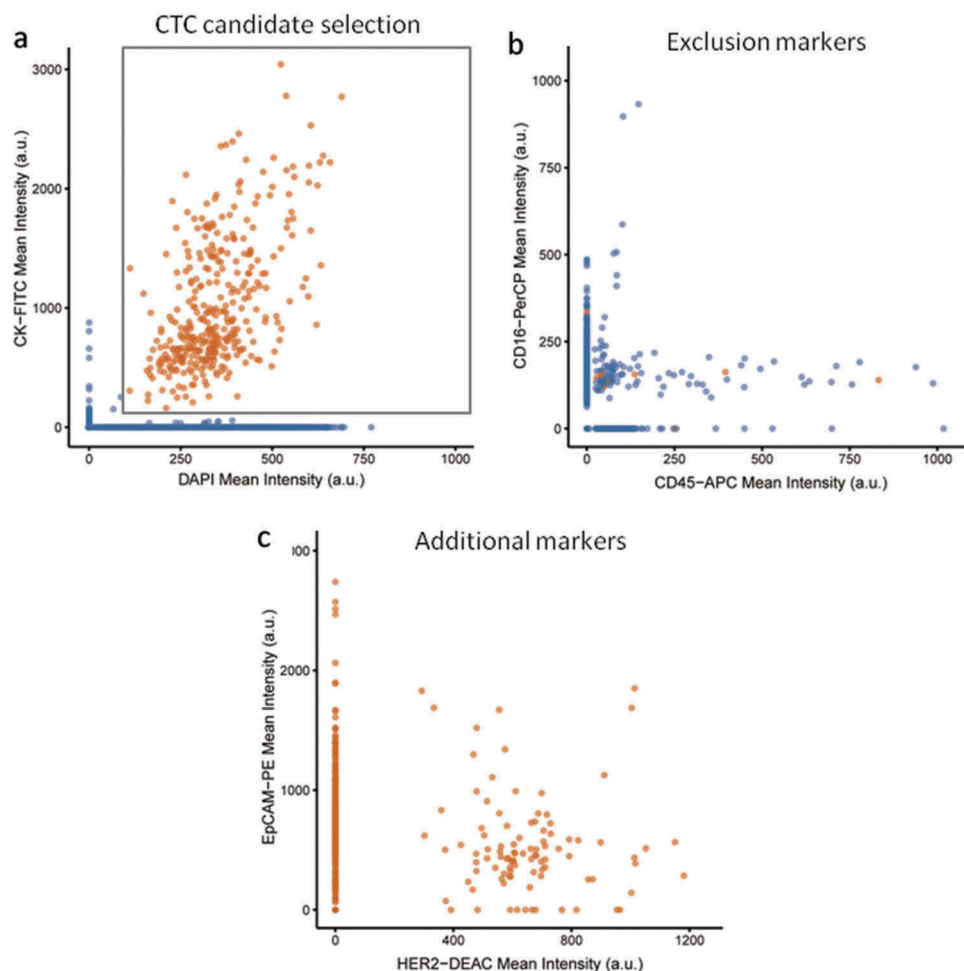

Figure 5. Three scatter plots of objects found in a sample by the ACCEPT algorithm. In panel a, a gate is set around the Cytokeratin FITC and DAPI positive events to identify CTC candidates depicted in orange (all other events are shown in blue). Panel b shows the mean intensity of the exclusion markers CD16-PerCP and CD45-APC, the majority of orange events are present at the origin. Panel c shows the mean intensity of EpCAM-PE, and HER2-DEAC of Cytokeratin-FITC+, DAPI+, CD16-PerCP-, CD45- CTC. Full color available online.
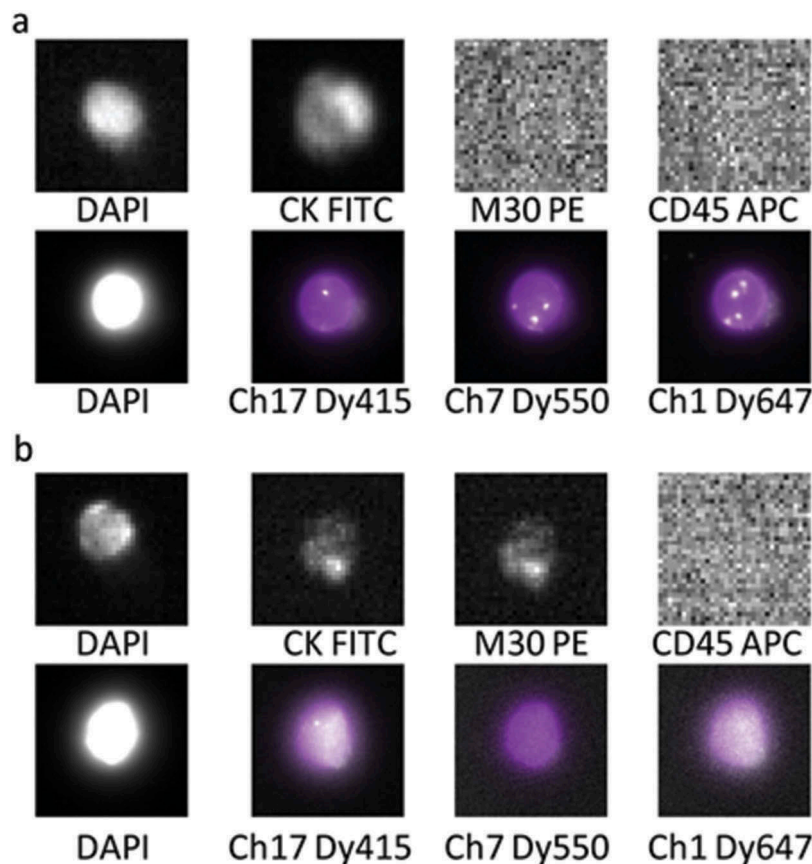
Figure 6. FISH on M30+ and M30- 765 CTCs from 24 prostate cancer patients. Evaluable FISH signals were obtained for 567/590 (96%) of the M30 negative CTC but only 101/175 (58%) of the M30 positive CTC.

positive and Her2/neu negative). A large variation in EpCAM expression can be observed with a relatively higher EpCAM expression of the Her2-negative CTC compared to the Her2-positive CTC. Note that in a portion of CTC, no EpCAM was detected using the polyclonal EpCAM antibody, suggesting a greater sensitivity of the EpCAM ferrofluid to extract CTC with low EpCAM expression compared to the fluorescent detection of EpCAM.

2.6. Collection of the immunomagnetically enriched and fluorescently labeled cells

The operation of the CellTracks AutoPrep is controlled through a set of commands that can be altered to perform certain tasks. With these so called 'TDefs,' the AutoPrep can, for example, be set such that the stained and enriched CTCs are deposited in a conical cell isolation tube instead of in the CellTracks cartridge and Magnest. The sample can then be analyzed and sorted using for example fluorescence-assisted cell sorting (FACS) or a DEPArray. Advantage of this approach is that the potential loss of cells by retrieval from the CellTracks cartridge can be avoided. Drawback of this approach is that one cannot get the standard CellSearch readout. The CellTracks cartridge and Magnest are designed to immediately magnetically pull and distribute the magnetically labeled cells and free ferrofluid to the surface for analysis on the CellTracks Analyzer and is not designed for the retrieval of cells.

The few CTCs isolated with the CellSearch system could contain valuable information on drug sensitivity or resistance. A variable number of leukocytes will remain in the CTC fraction and will be transferred to the cartridge. The number of these leukocytes can vary from 500 to even around 2×10^5. Tumor cell DNA will be lost in this background. An inevitable step therefore is to pick and isolate the single CTCs in individual tubes or as a pure CTC fraction for genomic analysis. Methods used to isolate single cell for individual analysis in combination with CellSearch-enriched CTCs: Micromanipulation [113], ALS Cellselector [114], the Silicon Biosystems DEPArray [115–118], FACS [28,119,120], and the Punch system using self-seeding Microwell Chips [121]. This step seems a minor intermediate part of the whole workflow but is crucial for the end result. If the step needs to be integrated in the standard laboratory workflow, it will take either very well-trained operators for FACS or micromanipulation or special equipment has to be installed such as the ALS Cellselector, the DEPArray, or the Punch system.

2.7. Alternative image data analyses

The CellSearch software only selects those events out of the scanned cartridge that are both cytokeratin (PE) and DAPI positive, and only those events will be shown for CTC identification and enumeration to the user. The pictures of events that are presented have digitally optimized color brightness for optimal CTC identification, but this makes it difficult to subtract any quantitative

information. Other cytokeratin-positive events can also be found in these cartridges. Coumans et al. manually reanalyzed CellSearch data of 179 patients with metastatic carcinoma's [122]. Here, all cytokeratin-positive particles are counted and sorted into seven classes from large and intact cells to smaller particles with DAPI and smaller and larger particles without DAPI. From this study, it has become clear that all cytokeratin-positive particles are predictive for survival but that the CTC have the lowest background. Ligthart et al. have extracted more morphological features of CTC from the CellSearch images and have identified a clinically relevant definition by combining a number of features which can be extracted from the data by an automated algorithm [88,123]. One of the features was the label intensity data from the cells in the CellSearch cartridge, which can now be used for analysis. Crespo et al. used this type of analysis to quantify staining of the androgen receptor in prostate cancer CTCs [100]. Currently, ACCEPT, a new open-source, platform-independent, automatic CTC identification and scoring software, is being developed. This software should help users of different CTC platforms to come to a more unified CTC definition. An example is illustrated in Figure 5.

2.8. FISH on CTC in CellSearch cartridges

FISH has first been developed on CellSearch cartridges to confirm that the epithelial cells found by CellSearch indeed were tumor cells. To do this, a five-color test for the detection of DAPI and the centromeres of chromosome 1, 7, 8, and 17 was developed together with a protocol to fix and hybridize the cells at the position where they were found inside the cartridge [124]. The cells lose the fluorescence after fixation, but the CellTracks Analyzer software has stored the position of the cells, and specific FISH software is able to relocate these cells after fixation and hybridization. Using the CellTracks Analyzer III, the original position is observed with a 40X objective using filter cubes that allow visualization of DAPI, Dy415 (chromosome 17), Dy550 (chromosome 7), Dy495 (chromosome 8), and Dy647 (chromosome 1), all CTCs can be individually and automatically scanned. Together this enables a five-color FISH on each of the identified CTCs. This study showed a high degree of aneuploidy of the CTCs and also heterogeneity between CTCs from individual patients. A small cohort of the patients used for this study was also stained with M30, an antibody detecting caspase-cleaved cytokeratin 18 which is a marker for apoptosis. Evaluable FISH signals were obtained for 567/590 (96%) of the M30-negative CTC, but only 101/175 (58%) of the M30-positive CTC, which indicates that due to apoptosis, a part of the CTCs cannot be used for DNA analysis (Figure 6).

Attard et al. used the FISH method to detect a combination of phosphatase and tensin homolog (PTEN) deletion, transmembrane protease, serine 2/ erythroblast transformation-specific (ETS)-related gene (TMPRSS/ERG) translocation, and AR amplification in each CTC of castration-resistant prostate cancer patients. Homogeneity in ERG rearrangement, significant heterogeneity of AR copy number, and PTEN loss was found within the CTCs of the patients identifying early and late events in the course of the disease [125].

Recently, Gasch et al. also showed a detection technique of microRNA using locked nucleic acid probes against mir10b on

CTCs fixed in the CellSearch cartridge [126]. To relocate the CTCs, Gasch uses the CellSearch staining reagents again on the fixed and hybridized samples after which the cells are manually examined for FISH signal. They show the ability to relocate and recognize the CTCs after hybridization and re-staining and show heterogeneity of Mir10b expression in breast, prostate, and colon cancer CTCs. So far, all work on RNA in CellSearch has been done on the bulk of cells in the cartridge using the profile kit because of the hypothesis that permeabilized cells would not contain RNA any more. This profile kit uses no staining and most important no cell permeabilization reagent. The presence of RNA in the stained and permeabilized CellSearch CTCs shows a possibility for in-situ detection of more RNAs in single cells.

2.9. Use of CellSearch for other body fluids

Besides in blood, tumor cells can also be found in other fluids using the CellSearch system. A normal CellSearch assay depends on the separation line between the erythrocytes and the plasma. This line is automatically detected, and the plasma is removed until 1 cm above this line. In most other fluids, there are not enough erythrocytes to make a clear layer. A solution to this problem is to run the samples as a control by using 4.5 ml in total or to dilute the sample to this volume. The control cell test skips the layer detection and initial plasma removal step. Patel et al., Tu et al., and Lee et al. show the detection of epithelial cells in cerebrospinal fluid and the possibility of monitoring leptomeningeal metastasis [127–129]. The same method is used to find tumor cells in the fluid of the drain used by a head and neck cancer dissection [130]. Also for leukapheresis samples, which are concentrated mononuclear cells from blood, the same method is used. For these samples, a maximum of 2×10^8 cells is used which is about five times as many as in 7.5 ml of blood [131]. In this study, diagnostic leukapheresis (DLA) was performed preoperatively for 23 patients. Detection rates were 28% for the peripheral blood and 72% for the DLA products and even 90% in the breast cancer patients. This shows great promise for the use of CTCs as a liquid biopsy. Other studies have been done where intraoperative blood samples were taken such as portal venous blood or liver venous blood. These samples can be run as normal CellSearch CTC samples [132,133].

3. Expert commentary

The CellSearch test is being marketed as an FDA-cleared test for the monitoring of metastatic breast, colorectal, and prostate cancer since 2004. The first 6 years after its introduction, studies were published where the test was mainly used in its basic configuration on different cancer types to enumerate CTCs. Figure 7 shows the basic CellSearch publications where the number of CTCs is used as a prognostic biomarker and/or a biomarker to predict the response to therapy after the first cycles of therapy. In the original studies, a CTC cutoff was used to discriminate between patients with a relatively good and bad prognosis. In reality, the peripheral blood tumor load is directly correlated with the clinical outcome. Also survival is
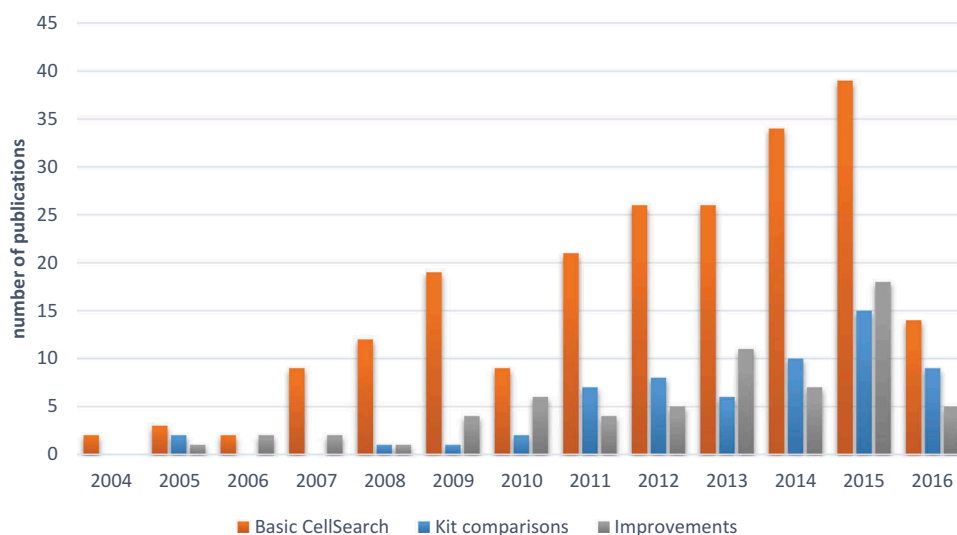
Figure 7. Overview of the number of publications using CellSearch. Basic CellSearch are the papers where CellSearch has been used for a clinical study with enumerating the CTCs without extra staining or analysis. Kit comparisons are the number of publications that were done to compare CellSearch to other CTC isolation methods. Improvements are the CellSearch publications discussed in this review where extra staining or analysis of the CTCs were done. Full color available online.

reduced by 6.6 months for each 10-fold CellSearch-defined CTC increase [72,96,122,134].

This article only discusses CellSearch tests and does not go into detail about other isolation techniques. Clearly there have been many different CTC isolation techniques introduced after the introduction of CellSearch, and many of these techniques compared the CTC numbers obtained to CellSearch, as can be seen in the blue bars of Figure 7. One of the most mentioned reasons for developing a different isolation method is that CellSearch is targeting only the EpCAM positive CTCs and not the CTCs, which have lost their EpCAM expression due to, for example, EMT. Another reason might be that some studies might not need the isolation precision of the fully automated AutoPrep system but allow a variation in quantification precision to gain time and lower the costs. In most cases, a different fraction of cells is detected either by capturing or by detection, which includes possible false positives. It is important to keep in mind that different fractions of cells are enumerated due to different capturing or detection technologies. Finding more cells than CellSearch is often referred to as an improvement over CellSearch. In most cases, there is only the enumeration comparison data with CellSearch, but no clinical data to demonstrate the significance of this subgroup of cells. It is however of high importance that all classes of CTCs will be studied so that more insight can be gained into the metastatic process and the value of these classes of cells as biomarkers for patient treatment.

Biomarkers are needed for the effective use of the growing number of available targeted drugs. Figure 7 The number of publications done on improvements in Figure 7 shows that the CellSearch system is also increasingly used to further analyze the cells captured by this system. Proteins are being analyzed in the form of immunofluorescence, DNA with array-CGH, qPCR, sequencing and FISH, RNA with arrays and qPCR, and microRNAs with ISH. It is shown that the CTCs can be taken out of the cartridge and that pure single cells can be accessed and that the crude CellSearch fractions can be analyzed for the expression of aberrant genes.

CellSearch is considered the golden standard for CTC enumeration, but also has a great potential for the use beyond standard CTC enumeration as is clear from the numerous assays that have been developed but not commercialized or have been introduced by users of the system. One of the large impediments for making use of the full capabilities of the CellSearch system of which some are described in this review is that many of its features are not made available to the users of the system or support has been withdrawn. A valid reason for this is that any change to the system can invalidate the FDA-cleared use of the system. Future will tell whether a solution for this obstacle will be found.

4. Five-year view

At present, several clinical studies are ongoing to provide solid evidence for the use of CTC defined by CellSearch as a surrogate for survival in studies probing new therapies. It is expected that within 5 years, the FDA will accept CellSearch-defined CTC as a surrogate for survival, which will accelerate the development of more effective therapies as well as the use of CTC in the management of cancer patients. The first study conducted to switch to therapy based on perseverance of CTC after the first cycle of therapy in metastatic breast cancer showed no benefit [135]. Although CTC predicted treatment failure, the switch to an alternative chemotherapy did not improve progression-free or overall survival. Lesson learned from this study is that an early switch to an alternative therapy should be guided by the characteristics of the tumor. At present, studies are being designed and conducted in which a switch to a targeted therapy is prescribed. A positive outcome of such studies will boost the CTC field. At present, a large number of clinical studies are ongoing in which CTC are

incorporated (https://clinicaltrials.gov), and we can expect reports of the results from these studies in years to come.

The extraction of information relevant for therapy from the CTC likely will lead to the development of companion diagnostics. In the next 5 years, it is expected that more clinical studies will be done using more protein markers and using DNA analysis techniques. Whole-genome amplification products will be made from single isolated CTCs, captured from the cartridge, from which multiple analysis can be done such as array-CGH, qPCR, and sequencing. There will be more attention to the development of single-cell techniques to analyze other regulation mechanisms such as DNA methylation and microRNAs. Because of its clinical usability, CellSearch will most probably play a leading role in this research. Next to this, there will be continuous research to investigate the clinical relevance of subgroups of CTC.

Key issues

- Plasma collection for ctDNA analysis can be combined with the CellSearch test.
- Various antibodies coupled to ferrofluid have been tested to capture different cells or a different subgroup of CTCs.
- Waste blood from the CellSearch system, depleted from EpCAM+ cells, can be captured manually and automatically, and further investigated for EPCAM- tumor cells.
- The AutoPrep system allows for an immunomagnetic separation without staining and permeabilization.
- Numerous additional immuno-reagents have been tested and used for analysis of isolated CTCs.
- The AutoPrep system allows for isolation and staining of the CTCs without delivery in a CellSearch cartridge but in the original tube.
- Various different analyses have been done to analyze the CellSearch data for either other tumor related particles or automated CTC identification.
- FISH analysis of DNA and RNA is possible inside the CellSearch cartridge with both automated and manual imaging.

Declaration of interest

Leon Terstappen is coinventor of several patents related to CellSearch. The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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