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Review

Challenges in circulating tumor cell detection by the CellSearch system☆

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ABSTRACT

Enumeration and characterization of circulating tumor cells (CTC) hold the promise of a real time liquid biopsy. They are however present in a large background of hematopoietic cells making their isolation technically challenging. In 2004, the CellSearch system was introduced as the first and only FDA cleared method designed for the enumeration of circulating tumor cells in 7.5 mL of blood. Presence of CTC detected by CellSearch is associated with poor prognosis in metastatic carcinomas. CTC remaining in patients after the first cycles of therapy indicates a futile therapy. Here we review challenges faced during the development of the CellSearch system and the difficulties in assigning objects as CTC. The large heterogeneity of CTC and the different approaches introduced in recent years to isolate, enumerate and characterize CTC results in a large variation of the number of CTC reported urging the need for uniform definitions and at least a clear definition of what the criteria are for assigning an object as a CTC.

1. Introduction

Circulating tumor cells (CTC) are cancer cells that detach from their primary site during the process of cancer metastasis. They enter the circulatory system, migrate through the body and can form secondary tumors at distant sites. If CTC are present, can be isolated and characterized they represent a minimally invasive source of spreading tumor cells and may serve as a liquid biopsy for management of cancer patients. CTC are however rare events compared to the number of hematopoietic cells, therefore, their detection and enumeration is technically challenging.

At present the CellSearch system is the only validated method for CTC detection that has been cleared by the U.S. Food and Drug Administration. The CellSearch system, designed for the enumeration of CTC in 7.5 mL of blood, was first introduced in 2004 where the analytical accuracy, reproducibility, and linearity of the system was shown (Allard and Terstappen, 2015; Allard et al., 2004). There are various challenges when isolating and enumerating CTC, in this review

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these challenges will be discussed using the CellSearch system as an example.

2. Early evidence for circulating tumor cells

Circulating tumor cells were first reported by Ashworth (1869). He described the presence of tumor cells with similarities to the cells from the primary tumor, in the blood of a man with metastatic cancer. Engell (1955) described the occurrence of cancer cells in peripheral blood and in the venous blood that drained the tumor during operation and observed a larger frequency of tumor cells in the draining vein as compared to the peripheral blood. Evidence for CTC in the blood from patients with metastatic and primary carcinoma was found by immunohistochemistry staining several decades ago. Moss and Sanders (1990) found evidence for CTC in 7 out of 10 neuroblastoma patients with known disseminated disease by immunostaining. In 1993, CTC were identified with conventional cytology and cytokeratin staining in patients with colorectal cancer by Leather et al. (1993). They isolated tumor cells from 42 patients undergoing resection for colorectal cancer, using a density gradient followed by cytospin and showed immune histological evidence for CTC in 4 of these patients. In the 1990s, peripheral blood progenitor cells were increasingly used for autografting after high-dose chemotherapy. Brugger et al. (1994) made the observation that tumor cells were detected in blood of a portion of breast cancer, small cell and non-small cell lung cancer patients before mobilization of peripheral blood hematopoietic progenitors and discovered an increase after the mobilization. Braun et al. (2000) reported that the presence of tumor cells in bone marrow was associated with poor prognosis. These studies provided important information that tumor cells could be detected by traditional immunochemistry techniques but also lacked the sensitivity to be used in larger multi-center studies.

3. Challenge of rare events detection

Tumor cells in blood are present in a high background of hematopoietic cells and are found in frequencies in the order of 1–10 CTC per mL of whole blood in patients with metastatic disease (Miller et al., 2010). One of the problems one faces in the development of assays to detect these rare cells is that one does not know whether tumor cells are present, and if so at what frequency. To test whether the developed methods are working, known numbers of cells from cancer lines are spiked in blood and the efficiency of the method is than evaluated by the determination of the number of cells observed after the procedure. A variety of cell lines should be tested in optimization of the methods. For example, cell lines with different densities of the target antigen, such as the epithelial cell adhesion molecule (EpCAM), for methods based on immune selection. Or a range of sizes, stiffness and densities when methods based on physical differences between hematopoietic cells and tumor cells are used. A frequent oversight is the challenge to accurately detect the "rare cell" among all the others. This is visualized in Figure 1, which shows the probability distribution of two cell populations. A lognormal distribution for both staining intensities was assumed. Panel A shows two cell populations present in equal numbers and they can be easily discriminated from each other. In panel B the number of stained cells is reduced to 1 in 1000 and 48.9% of the "rare" cells can no longer be discriminated. In panel C this ratio is changed to 1:10.000 and 70.3% of the cells can no longer be discriminated. In panel D this ratio is changed to 1:1.000.000 and in this case 95.2% of the cells can no longer be discriminated. To improve the separation one could improve the staining intensity. The use of for example Phycoerythrin (PE) instead of Fluorescein isothiocyanate (FITC) conjugated antibodies will improve the separation from autofluorescence due to higher quantum yield of the PE fluorochrome as compared to FITC. The limitation will however still be the number of antigens present on the cell. Amplification of the signal by for example increasing the antibody concentration or adding a secondary antibody to boost the signal will however also give rise to an increase in the background. Consideration of the frequency of the cell, that one needs to identify, is thus of utmost importance for the approach taken with the identification of the cells (Shapiro, 2003; Terstappen, 2000; Tibbe et al., 2007).

The use of multiple markers is therefore a requirement for "rare" cell detection. One of the first techniques used for the detection of CTC in whole blood was flow cytometry. Gross et al. (1995) reported a flow cytometric assay, which allowed for the detection of cancer cells in blood by using multiple markers, each containing a different fluorophore. They showed that detection of cells, down to a frequency of 1 in 10^7, is possible if 4×10^8 peripheral blood mononuclear cells (PBMCs) are analyzed. They used an approach to stain the unwanted subpopulation of the cells with one exclusion color and stain the rare cells of interest with one, two, or three different remaining colors. The drawback of this method is the large sample volume that needs to be analyzed, thereby limiting the number of samples that can be analyzed. In addition, the instrument has to be stable, the parameter settings have to be set in advance and cell settling and clumping must be avoided during the measurement.

The problems due to a large sample volume can be avoided by the enrichment of the tumor cells by either depletion of the leukocytes or selection of epithelial cells targeting for example the EpCAM antigen. The latter approach was reported by Racila et al. (1998). In this study, ferrofluids were labeled with antibodies targeting the EpCAM antigen, incubated with 20 mL of whole blood and immunomagnetically enriched, followed by fluorescent labeling with a nucleic acid dye, PE-conjugated anti-cytokeratin (CAM5.2) and peridinin chlorophyll protein (PerCP)-labeled CD45 and analyzed by multiparameter flow cytometry. It was shown that cells of epithelial origin defined as nucleic acid$^+$, CD45$^-$ and cytokeratin$^+$ could be detected in patients with metastatic and organ confined breast and prostate cancer, whereas only few epithelial cells were detected in healthy controls. Using this assay a first indication was obtained that the presence and changes in these "epithelial cells" related to the clinical status of the patient and response to therapy (Moreno et al., 2001; Racila et al., 1998; Terstappen et al., 2000, 1998).

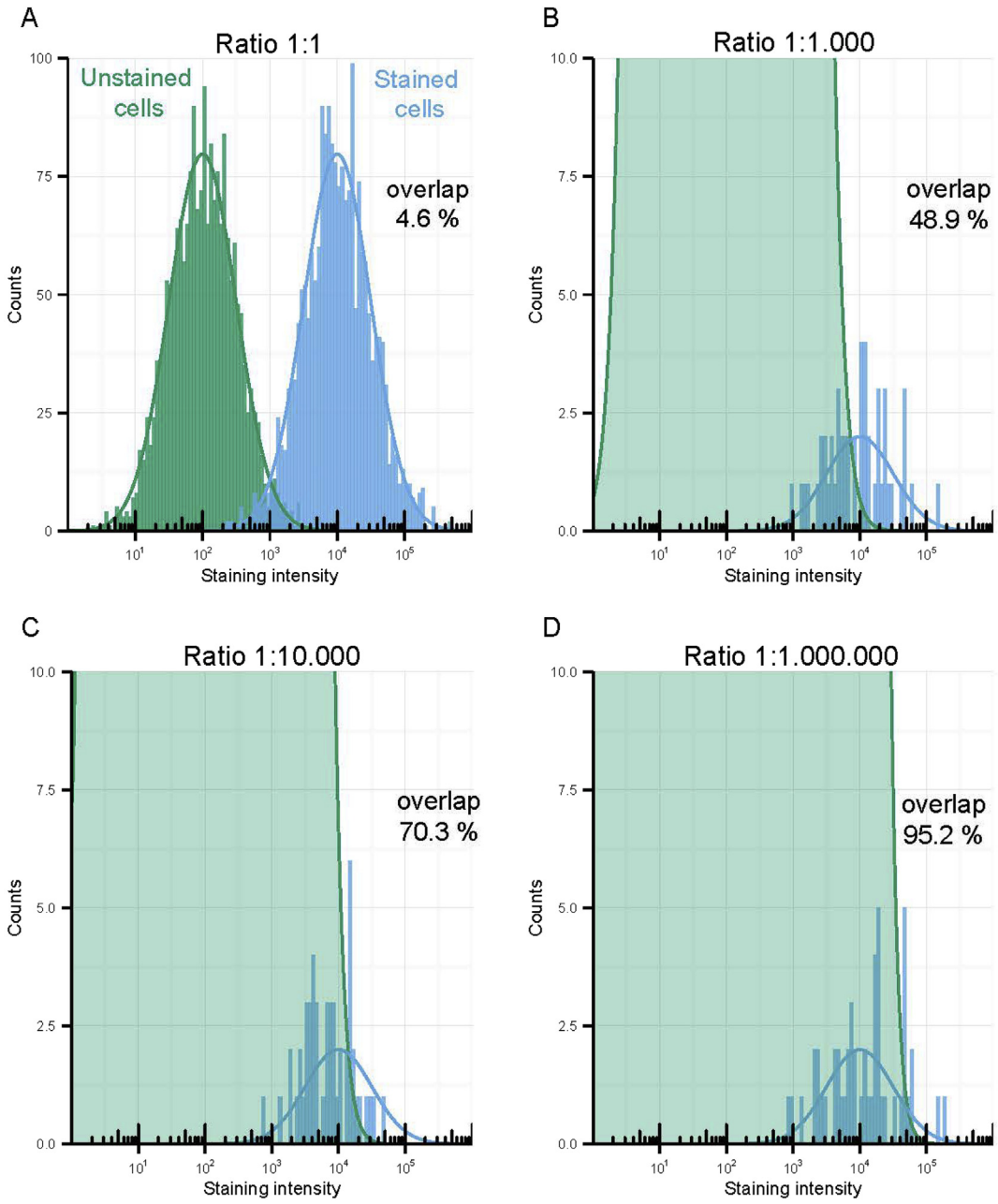

Figure 1 – Rare cell detection. Probability distribution of two cell populations one unstained (green) and one stained (blue) appearing at a ratio of 1:1, panel A; 1:1.000 panel B, 1:10.000 panel C, and 1:1.000.000 panel D. The blue and green lines depict the probability distribution.

First evidence that these epithelial cells indeed were tumor cells was obtained by cytospin preparations after immunomagnetic enrichment targeting the EpCAM antigen. The cytospins were stained by immunocytochemistry to confirm that the circulating epithelial cells found in the cancer patients had the morphologic appearance typical for cancer cells, Figure 2. The figure shows 20 thumbnails each containing a cell staining with cytokeratin (red) containing a relative large nucleus (blue) with some of them having clear nucleoli suggestive of the cells being active. Although these images did not provide sufficient evidence of these cells being cancer cells, it surely excluded that these cytokeratin expressing cells were, for example, derived from the venipuncture in which case cells with a large cytoplasm and small nucleus were to be expected. Further evidence was obtained by demonstration of cytogenetic aberrancies in the nucleic acid^{+}, CD45^{-} and cytokeratin^{+} cells (Fehm et al., 2002). Exploration of further utility of circulating tumor cells was demonstrated by the ability to detect treatment targets on the circulating tumor cells (Hayes et al., 2002; Meng et al., 2006, 2004b).

These studies formed the basis for the development of the CellSearch system. The manual sample separation was

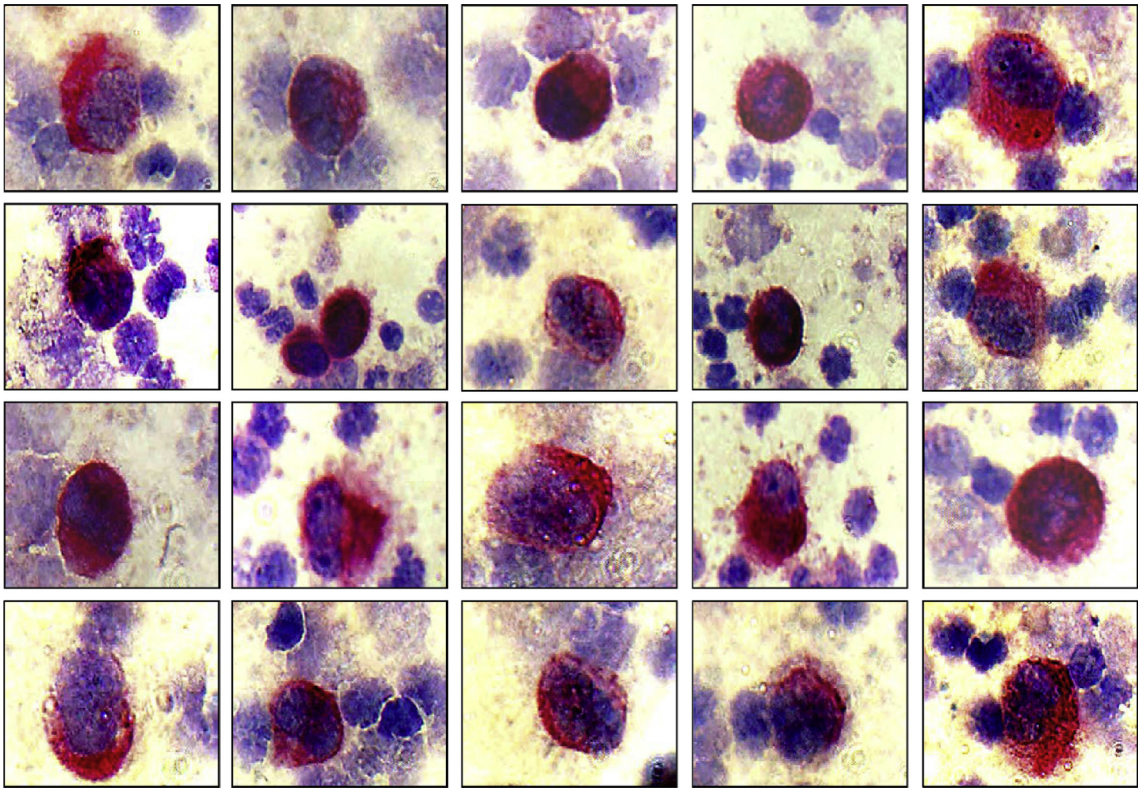

Figure 2 – Immunocytochemistry. Typical images of CTC immuno-magnetically enriched using EpCAM labeled ferrofluids and stained by immunocytochemistry with hematoxylin nuclear stain (blue purple) and cytokeratin (red). The brown yellow color can be contributed to the ferrofluids that are approximately 175 nm in size and is visual due to the accumulation.

replaced by fully automated sample preparation to avoid errors made by manual sample processing (CellTracks Autoprep) (Kagan et al., 2002). Cell loss, accompanied by making cytospins was avoided by introduction of the CellTracks Magnest (Tibbe et al., 2002) and the flowcytometer was replaced by a semi-automated fluorescent microscope to enable morphological confirmation that the nucleic acid^{+}, cytokeratin^{+}, CD45^{-} objects were indeed cells (CellTracks Analyzer II) (Kagan et al., 2002).

4. CellSearch system

The system was designed for the immunomagnetic enrichment, fluorescent labeling and detection of rare cell populations. To enable the processing of blood samples up to 96 h after blood draw, the CellSave blood draw tube was developed. In case viable cells are needed or extraction of RNA from the enriched cells, an EDTA blood draw tube should be used. The immunomagnetic enrichment was optimized such that cells expressing low as well as high antigens were selected (Liberti et al., 2001). For enrichment of carcinoma cells (CTC kit, CXC kit, CTC profile kit) the VU1D9 antibody recognizing EpCAM is used, for melanoma cells (CMC kit) and endothelial cells (CEC and CEC profile kit) an antibody recognizing CD146 is used and for myeloma cells (CMMC kit) an antibody recognizing CD138 (Weiss et al., 2014).

For enumeration of CTC the CTC kit is used in which reagents are provided to stain and fix the cells. The details of the protocol are described by Coumans and Terstappen (2015). The performance of the system is extensively described in 2004 by Allard et al. (2004), Prevalence of CTC was determined in blood from healthy donors, patients with nonmalignant disease, and in samples from 964 patients with metastatic carcinomas. The control population contained practical no CTC, whereas 36% of the blood samples of cancer patients contained >2 CTC in 7.5 mL, with a broad range of 0–23,618 CTC per 7.5 mL.

The first clinical studies conducted with the CellSearch system showed that CTC are clearly associated with poor prognosis in metastatic breast (Cristofanilli et al., 2005, 2004), colorectal (Cohen et al., 2008) and castration-resistant prostate cancer (De Bono et al., 2008). Later studies showed this for small cell lung cancer (Hiltermann et al., 2012; Hou et al., 2012) and non-small cell lung cancer (NSCLC) (Krebs et al., 2011), bladder cancer (Gazzaniga et al., 2012), pancreas cancer (Kurihara et al., 2008; Negin et al., 2010), head and neck cancer (Nichols et al., 2012), ovarian cancer (Poveda et al., 2011), neuroendocrine cancer (Khan et al., 2011; Oberstein and Saif, 2012), and hepatocellular cancer (Schulze et al., 2013; Zee et al., 2007). This association holds

true in pre- and on-treatment patient blood samples. For these studies patients were assigned to a favorable group (<5 CTCs/7.5 mL of blood) or unfavorable group (≥5 CTCs/7.5 mL of blood). Transition from the unfavorable group to the favorable group improves survival and can therefore be used as a predictive factor for treatment response (Cohen et al., 2008; Cristofanilli et al., 2004; De Bono et al., 2008; Hayes et al., 2006). In actuality, the larger the CTC count the worse the prognosis as is illustrated by Kaplan–Meier plots in Figure 3. In the plots, patients are divided into categories with 0 CTC, 1–4 CTC, 5–24 CTC, and >24 CTC and CTC counts in 7.5 mL blood samples taken before a new line of therapy was initiated. The difference in survival curves increases after the first cycles of therapy, as the CTC in those patients are eliminated by successful therapy. Interpretation of changes in CTC counts is described elsewhere (Coumans et al., 2012). In short for a treatment to be effective and prolong survival of the patient it is clear that all CTC will need to be eliminated.

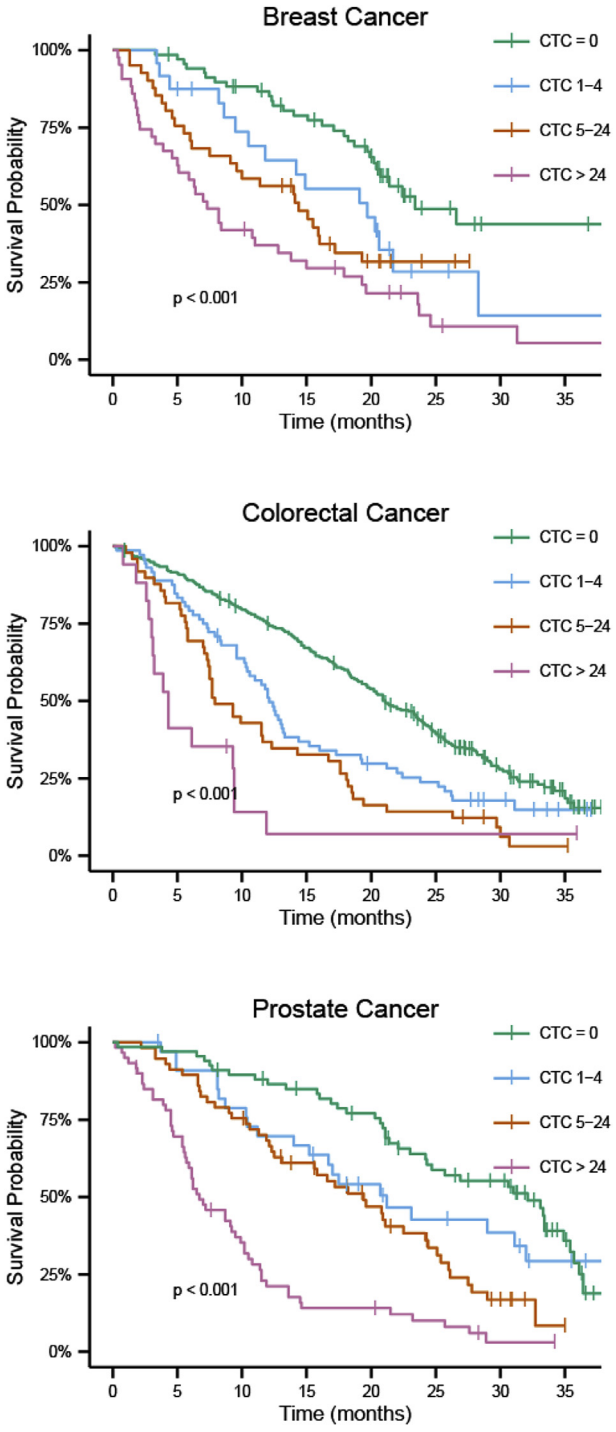

Figure 3 – CTC versus survival in breast, colon and prostate cancer. Kaplan–Meier plots of metastatic breast (top), colon (middle) and prostate (bottom) cancer patients with 0, 1–4, 5–24 and >24 CTC before initiation of a new line of therapy. Plots were generated using the data from the studies presented by (Cohen et al., 2008; Cristofanilli et al., 2004; de Bono et al., 2008).

Using CellSearch CTC are not only found in metastatic patients, several studies have reported on the presence of CTC before and after surgery for non-metastatic breast (Franken et al., 2012; Janni et al., in press; Lucci et al., 2012; Pierga et al., 2012, 2008; Rack et al., 2014; Serrano et al., 2012; Van Dalum et al., 2014), colorectal cancer (Gazzaniga et al., 2013; Hiraiwa et al., 2008; Sastre et al., 2008; Thorsteinsson et al., 2011; Van Dalum et al., 2015), esophagus cancer (Reeh et al., 2015) and bladder cancer (Karl et al., 2009).

The number of CTC per mL found in these studies is much lower than in metastatic patients. To increase the sensitivity of $EpCAM^+$, CK^+, $CD45^-$, $DAPI^+$ CTC as detected by CellSearch larger blood volumes will need to be tested. Extrapolation of the frequency of CTC detected in 7.5 mL of blood of prostate, breast and colorectal cancer patients to large blood volumes showed that in all patients CTC could have been detected in when 1 liter of blood would have been examined (Coumans et al., 2012). A model predicting the CTC frequency in patients with early breast disease suggested the presence of CTC at a frequency of 0.9 CTC/liter at the time of first metastasis in breast cancer (Coumans et al., 2013). One of the approaches taken to overcome this low frequency problem and test larger blood volumes is the use of leukapheresis. Fischer et al. (2013) introduced diagnostic leukapheresis (DLA), a shortened leukapheresis protocol which uses the conditions to harvest peripheral blood stem cells, but without stem cell mobilization. The CellSearch assay was adapted and up to 2×10^8 leukocytes (~60 mL of peripheral blood) could be processed while maintaining the ability to recover spiked tumor cells and increasing the number of CTC detected in cancer patients.

For cancer cells not of epithelial cell origin, EpCAM will not be expressed and other target antigens are needed to enrich the cancer cells. For example, for melanoma the CD146 antigen was chosen. After immunomagnetic enrichment of CD146 expressing cells the cells were stained with the nucleic acid dye 4′,6-diamidino-2-phenylindole (DAPI), antibodies recognizing CD45 & CD34 labeled with Allophycocyan (APC) and antibodies recognizing the high molecular weight melanoma-associated antigen (HMW-MAA) labeled with PE. The CD146 antigen is not only expressed on melanoma cells but also on endothelial cells, therefore there is a need for CD34 (expressed on hematopoietic progenitor cells and endothelial cells) in addition to CD45. The presence of circulating melanoma cells defined as $CD146^+$, $HMW\text{-}MAA^+$, $CD45^-$, $CD34^-$ cells was also associated with poor prognoses (Khoja et al., 2013; Rao et al., 2011).

By not using the staining reagents, the immunomagnetic enrichment of CD146 cells can be used for gene profiling of endothelial cells (Smirnov et al., 2006). Labeling CD146 enriched cells with CD45 and CD105 identifies endothelial cells that are present in blood of healthy donors but appear in higher frequencies in cancer patients (Rowand et al., 2007; Simkens et al., 2010; Strijbos et al., 2008).

To determine which antigens are expressed on CTC additional fluorescently labeled antibodies can be added, such as for example her2 (Ligthart et al., 2013). For antigens expressed at a low antigen density such as IGF-1R or bcl-2, FITC does not provide a sufficiently strong signal and the PE fluorochrome is preferred. In these cases the PE labeled antibodies recognizing cytokeratin are replaced by FITC antibodies (De Bono et al., 2007; Smerage et al., 2013).

In case viable tumor cells are needed, for example for the use in animal models or for molecular characterization, the CTC profile kit can be used. The EpCAM enriched CTC along with ~5000 leukocytes can be used for gene expression profiling (Smirnov et al., 2005) and these viable cells but also non-viable cells from the CTC kit can be immunofluorescently labeled and isolated as single tumor cells with various technologies for molecular analysis (Hodgkinson et al., 2014; Neves et al., 2014; Peeters et al., 2013; Swennenhuis et al., 2015, 2013).

5. CTC appearance and relation with survival

The current CellSearch system defines a CTC as an object that has a nucleus (stains positive for DAPI); stains positively for cytokeratin's, recognized by the antibodies C11 and A53-B/A2; does not stain for CD45; is more than 4 × 4 μm^2 in size and has cell like morphology and immunological features (Allard et al., 2004). When looking at the morphological features of CTC, a large heterogeneity within and between carcinoma patients is found (Allard et al., 2004; Larson et al., 2004). In a study by Coumans et al. (2010) these cytokeratin staining objects were divided in various categories, their frequency was determined as well as their relation to clinical outcome. Figure 4 shows images of these objects and their relative frequencies in patients with metastatic disease. The most abundant cytokeratin positive objects are small round vesicles. The frequency of such vesicles may well be underestimated as the fluorescent microscope used has a limit of ~1 μm, in addition the blood is spun down at 800 g and the plasma is aspirated before the blood is processed on the CellTracks Autoprep which will remove all extracellular vesicles with a lower density/size. The relevance of these extracellular vesicles, that also contain tumor derived exosomes, is explored by a large number of groups (Speicher and Pantel, 2014). Next in line, are small apoptotic cells or fragments of cells, proof that these indeed were undergoing apoptosis was obtained by staining with antibodies recognizing cleaved cytokeratin 18 (Larson et al., 2004). Larger cytokeratin$^+$ and CD45$^-$ cells are also observed, in these cells one clearly can discern the fragmentation of the cytokeratins strings that are clustered in small round vesicles within the cell. Intact CTC such as depicted in the figure only are a minority among the objects classified as CTC using the CellSearch criteria. Even less frequent are

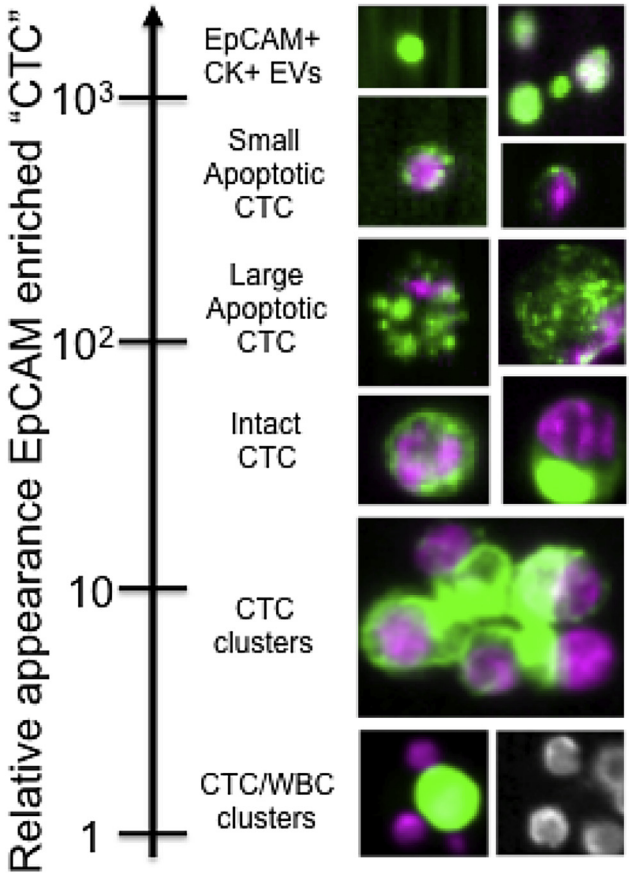

Figure 4 – Different appearances of CTC. From top to bottom: EpCAM$^+$, CK$^+$ extracellular vesicles; small apoptotic CTC; CellSearch CTC; large apoptotic CTC; clusters of CTC and lymphocytes attached to CTC. The relative frequencies are indicated on the y-axis.

clusters of CTC observed with CellSearch (Allard et al., 2004). Before initiation of the clinical studies a definition of a CTC needed to be set and we decided to count a cluster of CTC as one CTC. One of the reasons being that it is often very difficult to assess how many cells are within a tumor cell cluster. The earlier versions of the image analysis software had the tools incorporated to enumerate tumor cell clusters, but to simplify matters it was omitted from the commercial versions of the software. These tumor cell clusters are also observed using other platforms (Aceto et al., 2014; Adebayo Awe et al., 2013; Hosokawa et al., 2013; Molnar et al., 2001; Sarioglu et al., 2015; Werner et al., 2015) but also for these platforms no clear guidance is provided what is and what is not a cluster. A minority of these clusters are composed of tumor cells and lymphocytes of which an example is shown in the figure. In this example, 4 lymphocytes are attached to the CTC suggesting that these lymphocytes recognize and "attack" the tumor cell. This phenomenon suggests an active role of the immune system and a boost of this response may represent alternative strategies for therapies. In case enrichment strategies are used based on depletion of leukocytes this phenomenon cannot be observed.

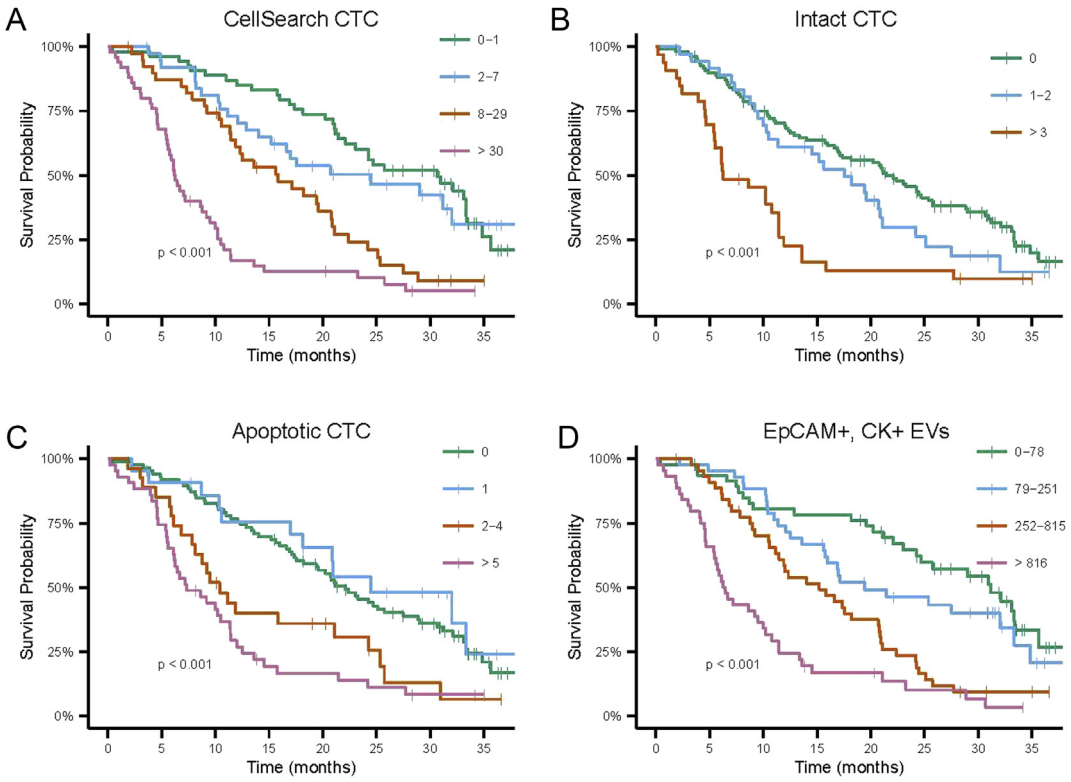

Figure 5 – Survival versus CTC definition. Kaplan–Meier plots showing the relation with survival of objects, from metastatic prostate cancer patients, subdivided into different groups. A) cells classified according to the CellSearch criteria; B) cells classified as intact CTC; C) cells classified as apoptotic and D) the EpCAM$^+$, CK$^+$ extracellular vehicles (EVs). Plots were generated using the data from the studies presented by (Coumans et al., 2010).

Demonstration that the presence of EpCAM$^+$, cytokeratin$^+$, CD45$^-$ objects subdivided into different groups based on morphological appearance also related to clinical outcome was shown by Coumans et al. (2010). Figure 5 shows the relation with survival of metastatic prostate cancer patients and cells classified according to the CellSearch criteria, cells classified as intact CTC, cells classified as apoptotic and the EpCAM$^+$, CK$^+$ extracellular vehicles (EVs) which were called tumor micro particles in the original publication. Although the relation between EpCAM$^+$, CK$^+$, CD45$^-$ EVs and clinical outcome strongly suggests that they are derived from the tumor, proof can only be obtained by subsequent molecular analysis which is feasible on cells in which the nucleic acids are accessible (Attard et al., 2009; Fehm et al., 2002; Gasch et al., 2013; Meng et al., 2006, 2004a; Swennenhuis et al., 2009) but not on EVs that do not contain nucleic acids.

6. Need for uniform definition and ultimately automated classification of CTC

The number of CTC reported vary widely between different platforms urging the need for uniform definitions and at least a clear definition of what the criteria are for assigning an object as a CTC. Various validation studies have been performed using the CellSearch system (Cummings et al., 2013; Kraan et al., 2011; Riethdorf et al., 2007) and show a reasonable concordance. Still the large variety in the appearance of the cells will always result in different assignments of objects with manual review of the images by different operators. Figure 6 illustrated seven areas in which the CellTracks software identified objects stained with cytokeratin as well as DAPI. These areas are presented to the reviewer as thumbnails to identify CTC. To the right the decision tree is provided that the reviewer follows to sore the objects. The thumbnail images shown by the CellSearch system have been adapted to increase contrast and reduce storage space. The image processing in CellSearch is described in more detail by Coumans and Terstappen (2015). To visualize the effect of scaling of the images thumbnails of the CK staining as presented by CellTracks software (Normalized CK) and a thumbnail that is not scaled (Unscaled CK) is shown beside the DNA, CD45 and CK/DNA overlay. The unscaled image in Figure 6 is a reconstruction of the original image where 0 and 255 are mapped to the full camera range. The top left corner of the Unscaled CK thumbnail shows a 4 × 4 μm box used as a minimum size criteria of a CTC. All seven objects fit the size criteria. All reviewers likely will score the object in row 1 as a CTC. In row 2 four nuclei can be observed one clearly staining for CK, but is it staining with CD45? Actual measurement of the signal and comparison with that of leukocytes could

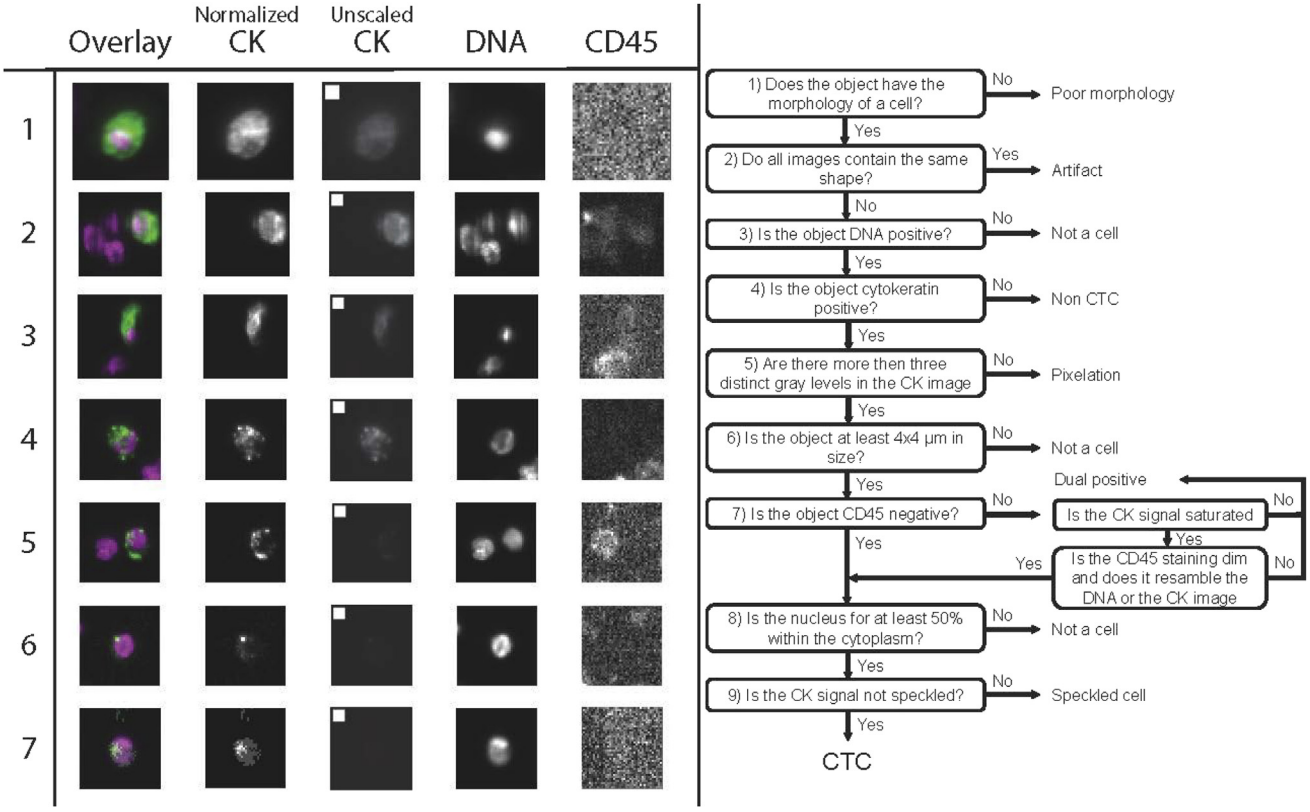

Figure 6 – What is and what isn't a CTC. Left part of the figure shows seven areas with objects in which the CellTracks software identified cytokeratin (CK) as well as DNA staining. Shown are, thumbnails of the CK staining as presented by CellTracks software (Normalized CK); CK staining that is not scaled (Unscaled CK); DNA; CD45 and a CK/DNA overlay. To the right the decision tree is provided that the reviewer follows to sore the objects. All seven objects fit the size criteria.

provide a quantitative assessment. In row 3 two objects can be discerned the lower one can be assigned as a leukocyte, the other object stains with CK, but is its morphology consistent with that of a cell and is the nucleus for at least 50% within the cytoplasm? In row 4 also two objects are present one can be assigned as a leukocyte and the other stains with CK, has a clear nucleus, but at least two speckles of CK are present so do we assign it as a CTC? In row 5, 6 & 7 nuclei can be observed of which only one faintly stains with CD45, in the unscaled CK images no signal is observed and none of the objects would be assigned as a CTC. In the normalized CK images a CK signal is observed and one might assign one of them as a CTC. This illustrates that it is not that simple to identify CTC even within one platform and not only highlights the need for uniforms criteria such as outlined in the decision tree of Figure 6, but also the need for a more quantitative approach or preferably introduce automation in the classification of CTC (Ligthart et al., 2011).

Effort to arrive at a CTC definition consensus across platforms have been started in the EU funded CANCER-ID program and ACCEPT an Open Source Computer program has been made available to identify CTC in images obtained from various platforms. The program can be downloaded free of charge from www.CANCER-ID.eu. The program is continuously being improved using input of the experts in the field that participate in this program.

7. Concluding remarks

Here we discussed the challenges faced during the development of the CellSearch system and the difficulties in assigning objects as CTC. In order to use CTC as a liquid biopsy, it would be ideal to detect as many CTC as possible in as many cancer patients as possible. However, the low frequency of CTC together with the heterogeneity observed in CTC makes the detection very difficult. This demands for a technique both very sensitive as well targeting a broad range and variety of CTC. Criticism is often expressed on the fact that CellSearch only detects EpCAM^{+} cells, thereby missing CTC lacking EpCAM expression. Still the CellSearch system remains the gold standard for CTC enumeration and has set the bar quite high (Bidard et al., 2014; Mehra et al., 2015). Over the years several alternative technologies have been developed reviewed extensively elsewhere (Alix-Panabières and Pantel, 2014; Barradas and Terstappen, 2013; Krebs et al., 2014; Myung and Hong, 2015) and demonstrated to detect CTC both by targeting EpCAM and not targeting EpCAM but using

other characteristics such as physical properties or biological features. However, up until now it remains difficult to compare these technologies due to the lack of a uniform CTC definition. Also, new technologies need to be tested in multi-center clinical trials in order to compare their performance. For a new technology to become useful in clinical practice it is of utmost importance that a system went through a validation and can be used and accessed easily.

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