METASTASIS AND CIRCULATING TUMOR CELLS

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ABSTRACT

Cancer is a prominent cause of death worldwide. In most cases, it is not the primary tumor which causes death, but the metastases. Metastatic tumors are spread over the entire human body and are more difficult to remove or treat than the primary tumor. In a patient with metastatic disease, circulating tumor cells (CTCs) can be found in venous blood. These circulating tumor cells are part of the metastatic cascade. Clinical studies have shown that these cells can be used to predict treatment response and their presence is strongly associated with poor survival prospects. Enumeration and characterization of CTCs is important as this can help clinicians make more informed decisions when choosing or evaluating treatment. CTC counts are being included in an increasing number of studies and thus are becoming a bigger part of disease diagnosis and therapy management. We present an overview of the most prominent CTC enumeration and characterization methods and discuss the assumptions made about the CTC phenotype.
Extensive CTC characterization of for example the DNA, RNA and antigen expression may lead to more understanding of the metastatic process.

INTRODUCTION

Cancer is the world's 3rd cause of death and the leading cause of death in economically developed countries[1]. In cancer in most cases it are the metastases and not the primary tumor which cause death. When a patient is diagnosed with cancer before it has spread outside of the primary tumor, it improves the chance of survival. But spotting a primary tumor before it has the chance to metastasis is difficult. Physical examination and traditional imaging methods such as MRI, PET, CT, X-ray or ultrasound have a detection limit which is not sufficient to detect smaller metastasis. For breast cancer this detection limit is for example 6mm or larger [2]. This makes it hard to spot small lesions or micro-metastases. It is still unclear exactly when and how the metastatic process begins and which factors drive the process, but it is known that tumor cells spread via the lymphatic system and subsequently into the blood circulation or are shed directly into the blood. These Circulating Tumor Cells (CTCs) are associated with poor progression free and overall survival [3-10]. CTC are rare and require multiple steps to enumerate, but we catch metastasizing cells in the act and thus they may increase our understanding of the metastatic process. CTC may also provide a way to monitor disease progression more directly than traditional imaging methods. Here we review the current state of the CTC detection field and the extra information these cells can provide us with now and in the future.

METASTATIC PROCESS

Cancer occurs after a cell is progressively genetically damaged and turns into a cell bearing a malignant phenotype. These cells are able to undergo uncontrolled abnormal mitosis, which leads to an increase of these cancerous cells at that location. In absence of regular control mechanisms a heterogeneous population of cells is created and these cancerous cells together form the primary tumor [11]. A tumor is considered benign if it lacks the ability to invade other tissue. When cells acquire the ability to penetrate and infiltrate surrounding normal tissues, the cancer is considered malignant and has the potential to metastasize. Before tumor cells can start to metastasize, they need to succeed in stimulating angiogenesis. In this way tumor cells gain direct access to the blood circulation. This leads to improved access to the nutrients and oxygen carried by the blood, but also an opportunity for the tumor cells to enter the blood stream. This process is shown in Figure 1. An alternative route for tumor cells to end up in the blood circulation is through the lymphatic system. Tumor cells circulating in the blood can reach in principle most sites of the body, but different kinds of cancer create metastasis at different sites. For example breast cancer generally creates metastases in liver, lung and bone while prostate cancer most often metastasizes in bone as illustrated in Figure 2. This preference is driven by two processes. The first is mechanical of nature, a large amount of CTC arrests in the first capillary bed they encounter. The second is more biological, the CTCs will form a metastasis in tissue only if they are able to extravasate out of the blood stream and the local environment is suitable for them to grow. This preference has been noted for the first time by Stephen Paget and is known as the seed and soil hypothesis. Tumor cells thus have a preference for a certain site, and this opens an interesting research field to identify the cell surface molecules on the tumor cells and the endothelial cells aligning the capillaries at the specific sites [12-15].

CIRCULATING TUMOR CELLS

The first observation of tumor cells in blood was made by Thomas Ashworth in 1869 [16]. In subsequent reports CTCs where only observed in blood when present in high numbers [17-21]. As technology advanced it became possible to detect the presence of CTC in a much lower concentration. For example various PCR techniques can be used to detect CTC in blood but are less
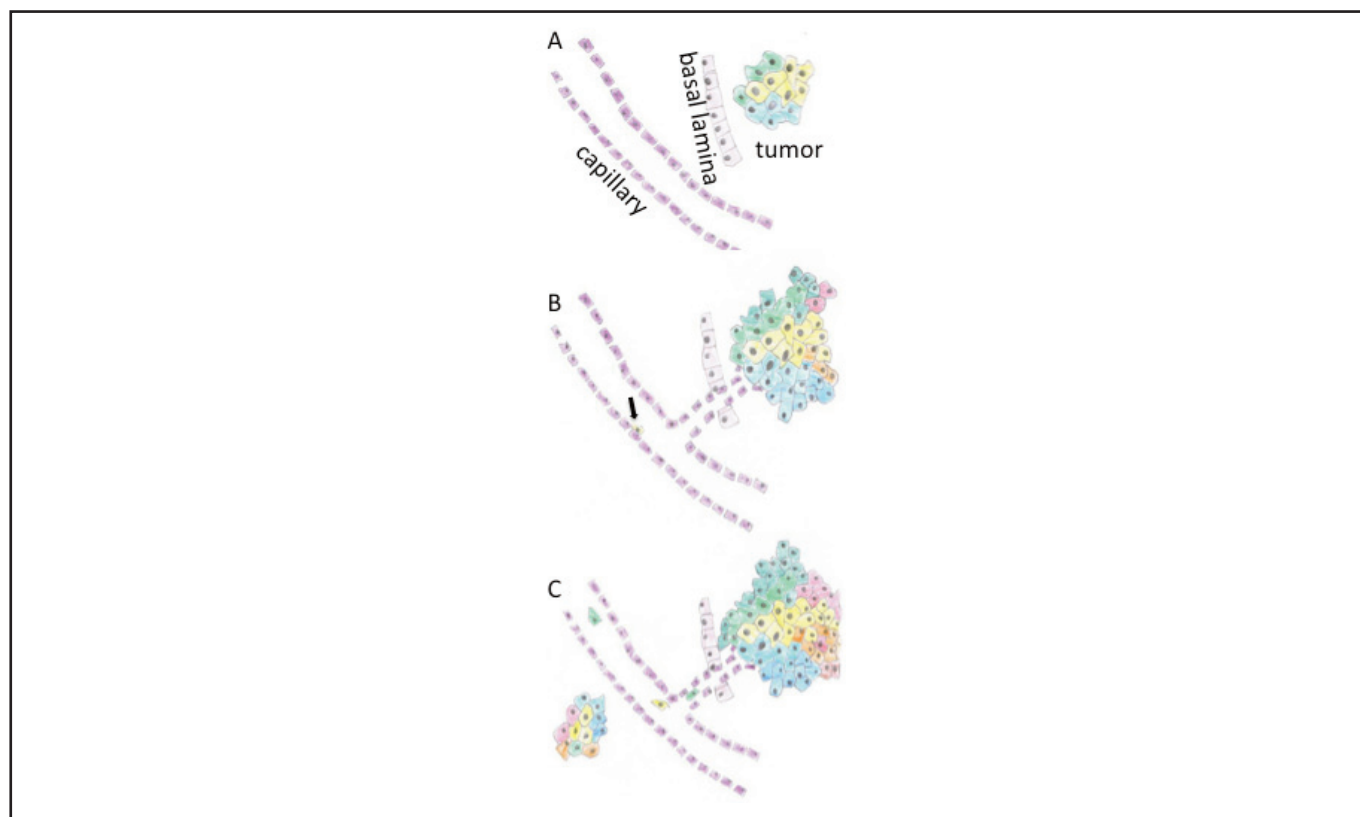
Figure 1
Formation of metastasis. Panel A shows a primary tumor after its inception. The heterogeneity of the tumor is indicated with different colors. Panel B shows blood vessels providing nutrients to the tumor (angiogenesis) leading to further growth and diversity. In this process either the tumor cells or the endothelial cells will need to penetrate the basal lamina. At this time tumor cells can enter the blood. The arrow depicts a CTC attached to the blood vessel wall of a distant organ. Panel C shows the formation of a metastasis after extravasation of the CTC. Only few CTC will have the characteristics necessary to create a metastasis.
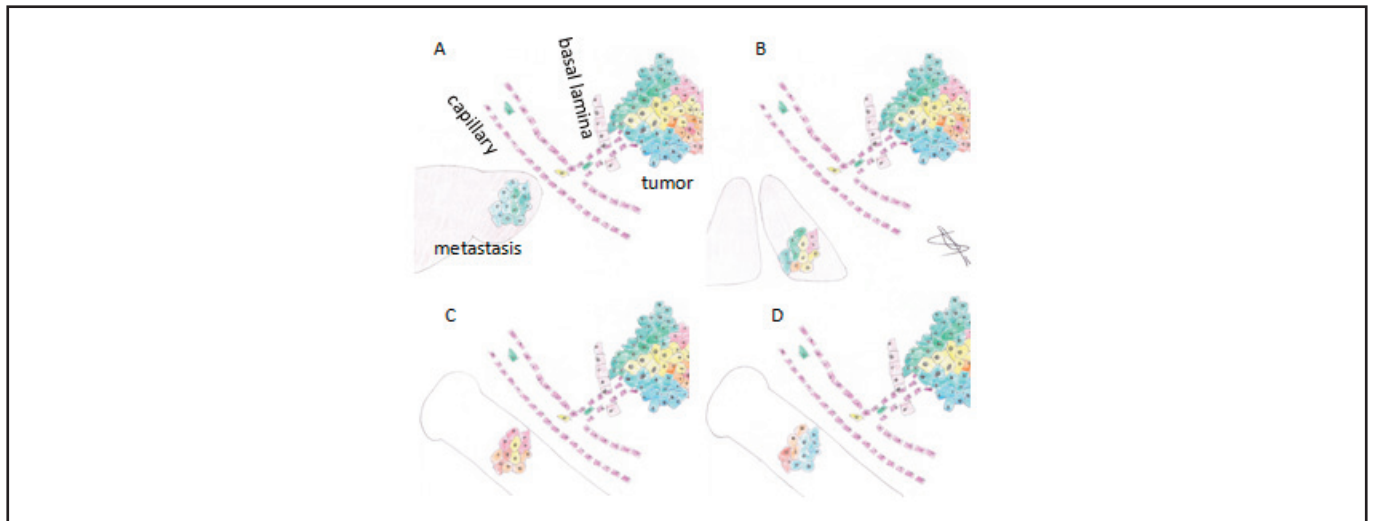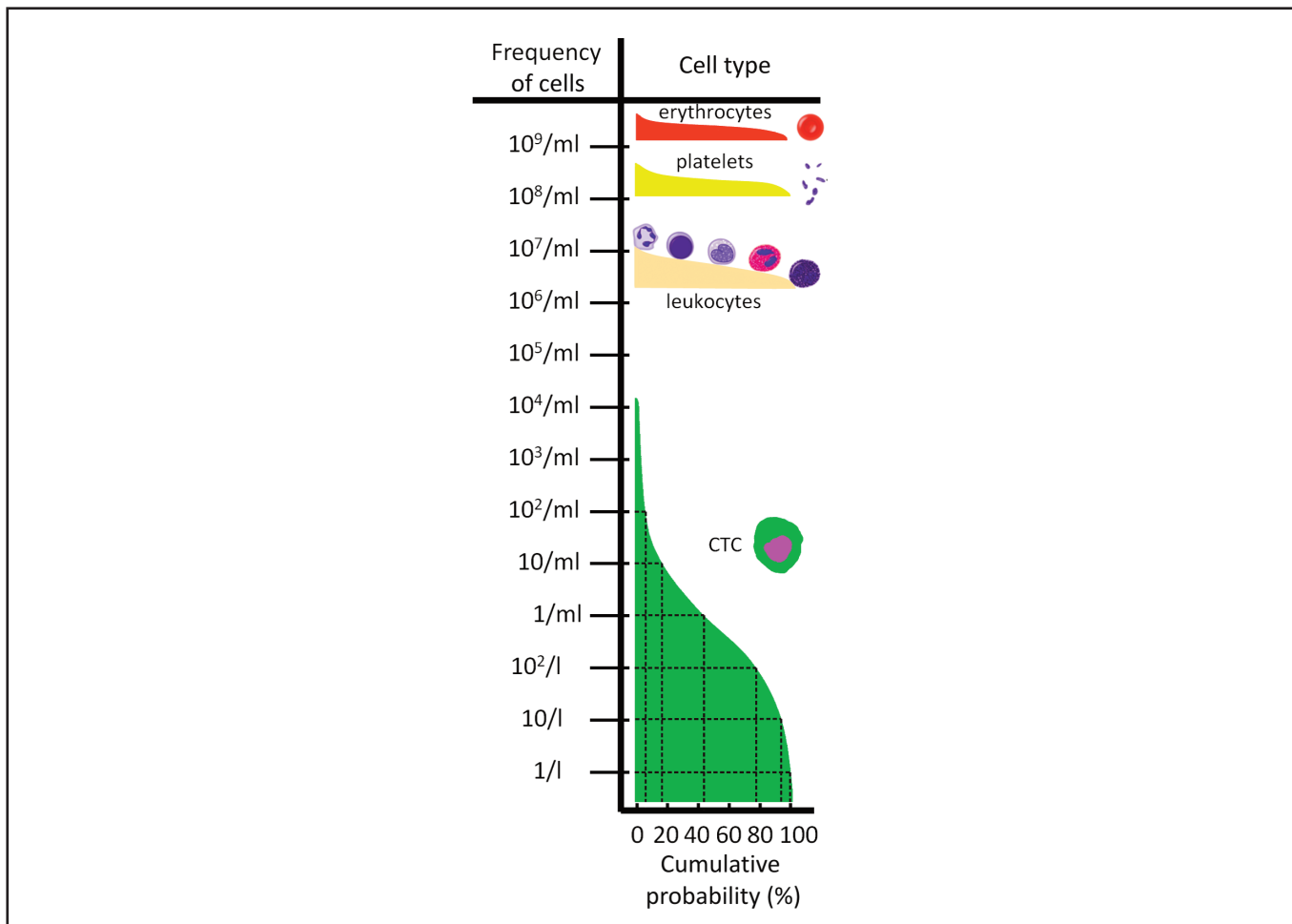
Figure 2
Differentiation into heterogeneous metastases. A primary tumor in the breast creates generally spreads to the liver (panel A), lung (panel B) or bone (panel C). Due to tumor heterogeneity some tumor cells have a preference for a certain organ indicated by their color. Panel D shows a primary tumor in the prostate which only spreads to bone.

suitable for enumeration[22]. The combination of flow cytometry with a magnetic enrichment step allowed for CTC enumeration down to 1 cell per ml [23]. CTC in peripheral blood of patients with metastatic disease turned out to be very rare, and range from 0 to 10000 CTCs per mL of whole blood [24]. Their frequency compared to other cells present in blood is shown in Figure 3. Prospective clinical studies in breast, colon and prostate cancer[3-5]showed that the presence of CTCs in 7.5 ml of blood strongly correlated with progression free and overall survival. The relation between the number of CTC in 7,5 ml of blood from 294 metastatic breast and prostate cancer patients and survival is illustrated in a Kaplan-Meier plot in Figure 4. The blood samples were taken after the patients received the first cycle of chemo therapy. The figure clearly shows the relation between the CTC load and survival. Intuitively the difference between <1CTC and 1-4 CTC should make a larger difference, but because not the complete volume of blood is measured the concentration of CTC contains a poison error due to sampling. This together with the chance of a false positive makes it hard to distinguish between none and a few CTC. If error free detection would be possible even one CTC in 7,5 ml would already lead to a worse prognosis[25]. Lower concentrations of CTC can be found in patients when the analysis of a higher volume is possible. If the concentration of CTC currently found in patients is plotted as a cumulative distribution function a distribution can be plotted through it to extrapolate the lower concentrations[26]. The result is a distribution such as seen in Figure 3, where 99% of the patients with metastatic disease have at least 1 CTC in 5L of blood before initiation of therapy. The combination of low numbers and the risk of identifying a false positive makes the selection of a good threshold for the separation of patients important. The definition of a CTC also has a strong influence on this threshold. For example in immunofluorescent microscopy a more loose definition of what is a CTC will result in higher counts in patients and in healthy controls, but also in a lower distinguishing power[27, 28]. It is important to only select the CTC or objects which influence outcome. Comparisons between CTC and other predictors of prognosis or response to therapy such as serum tumor markers and imaging modalities have shown that CTC perform well and are independent predictors of outcome [4, 5, 29-33].

CURRENT CTC DETECTION METHODS

Of all the methods to enumerate CTC most have some form of enrichment to make the number of cells that have to be analyzed manageable. But what exactly is the definition of a circulating tumor cell? A nucleated cell of non-hematopoietic origin, a nucleated cell of epithelial cell origin, or a cell with an aberrant genotype? To find CTC all sample preparation methods make certain assumptions, for erythrocyte lysis the assumption is made that CTC are not lysed, for separation based on density, size or flexibility that they have the chosen properties, for depletion of the hematopoietic cells that they are not aberrantly expressing the hematopoietic antigens or are bound to the hematopoietic cells and for enrichment based on antigen expression that they bear the antigens chosen for separation. To identify CTC in the enriched sample similar assumptions will have to be made. A PCR method on the CTC enriched sample targeting for example cytokeratins assumes that the expression of these keratins is confined to tumor cells. Moreover a large heterogeneity of the number of cytokeratin copies is present in cells prohibiting enumeration of the CTC. Detection of extracellular and intracellular antigens by means of fluorescence labeled antibodies is the most frequently used method. The combination of antigens and the sensitivity by which the antigens are detected will greatly

Figure 3
The frequency of erythrocytes, platelets, leukocytes and circulating tumor cells in blood of metastatic carcinoma patients and their cumulative probability[74].
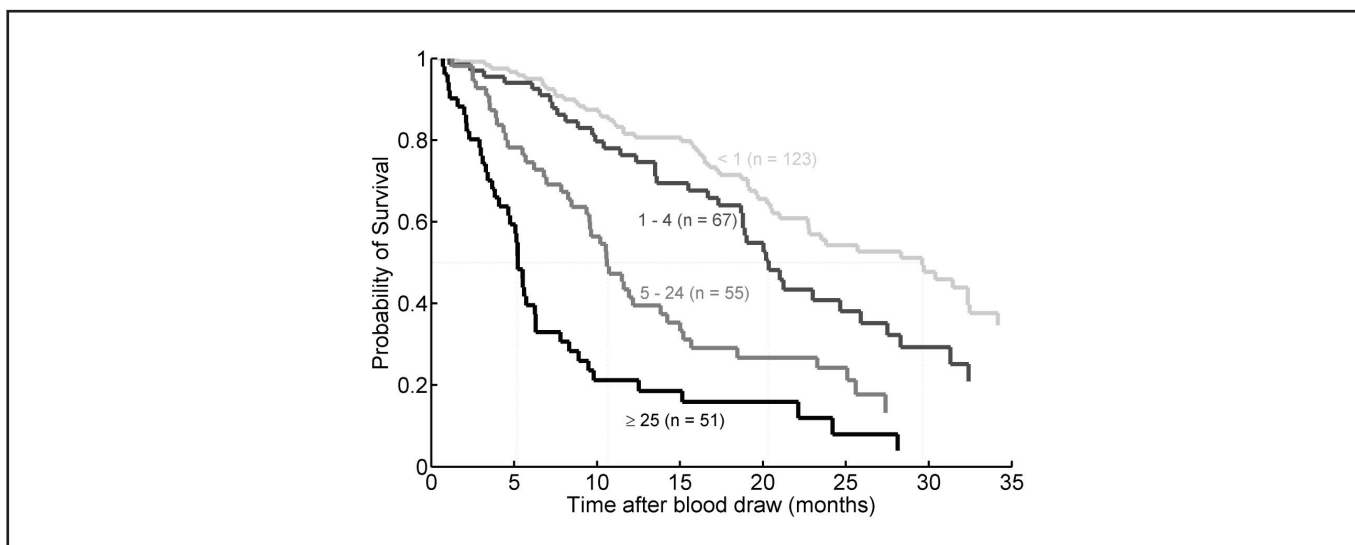
Figure 4
The overall survival time of metastatic breast and prostate cancer patients was calculated from the date of the first follow-up blood draw after initiation of a new line of therapy. Kaplan-meier plots of the probability of overall survival for 123 patients with <1 CTC, 67 patients with 1–4 CTC, 55 patients with 5–24 CTC and 51 patients with >25 CTCs is shown. [74]

influence the number of cells that are identified as CTC. A more strict criterion will result in more specific but less sensitive detection method. The choice of fluorochromes and the method to detect them will also influence the sensitivity and specificity of the assay. Whereas flowcytometry is in general more sensitive to detect fluorescence as compared to mercury arc based fluorescence microscopy the measurement of light scatter properties by flowcytometry will not provide the same level of confidence on cell morphology as microscopic images provide. The ideal CTC enumeration assay has: high sensitivity, high specificity, is reproducible and is somewhat independent of sample lead time. For CTC characterization it would be ideal to have viable CTC with intact morphology and preserved cellular content to detect extracellular and intracellular antigens, RNA and DNA.

ANTIBODY BASED CTC ENRICHMENT

Although there are many different detection assays described in literature only one is cleared by de FDA, this is the CellSearch system from Veridex. The CellSearch system uses anti-EpCAM antibodies conjugated to coated ferrofluids in combination with a strong magnetic field to selectively enrich cells expressing the EpCAM antigen as is illustrated in Figure 5A. After enrichment the cells are permeabilised and stained with DAPI, anti-CD45-APC and anti-CK-PE (CK 8, 18 and 19). The stained cells are then placed in a sample holder with a magnetic field that uniformly distributes the cells across the imaging area. The sample is then imaged with an automated fluorescent microscope and an image processing algorithm identifies possible CTC. These candidates are displayed in a gallery of thumbnails as illustrated in Figure 6A for review by an operator[34, 35]. When looking into the CellSearch CTC definition it stipulates that: a cell should be sufficiently large (> 16um2), with an intact intracellular nucleus, does not stain with CD45-APC and does stain with keratins 8, 18 and 19 enveloping the nucleus. Expression of EpCAM is likely as the cells were immunomagnetically pulled to the surface, cells of hematopoietic origin will however also be present.

A method that samples a much larger volume is the functionalized and structured medical wire [36]. One end of the wire is functionalized with human anti-EpCAM antibody. A schematic representation is illustrated in Figure 5B. The wire is inserted into the cubital vein of a patient for 30 minutes, so cells can be captured in vivo. After which it is removed and stained for DNA, EpCAM, cytokeratins 4, 5, 8, 11 ,18 and CD45. Imaging is done using conventional fluorescence microscopy. The frequency of EpCAM positive epithelial cells among the captured cells has not been reported yet. As a larger blood volume is sampled the method has the potential to be more sensitive as compared to the CellSearch system[26] .

The Epispot method uses an antibody coated surface to capture the proteins secreted by a circulating tumor cell [37, 38]. Cells are deposited in a petri dish and incubated to permit the cells to secrete proteins. The proteins secreted by the cells are selectively bound to the surface, after the cells are washed of the target proteins are stained with a second antibody targeting the same protein. This method is only sensitive to the target antigens of the spotted antibodies that are secreted by live cells and does not allow direct interaction with the CTC as they have been washed away.

Microfluidic devices have been described [39-42] with a functionalized surface to capture CTC. The cells collide with EpCAM functionalized surface to enrich CTC. After the cells are captured in the microfluidic device they are stained while they remain on the chip. The ability to do further analysis of the captured CTC in a microfluidic device is one of the main advantages of using the lab on a chip approach[43]. The main disadvantage of microfluidic approaches is that all devices presented so far are limited in the amount of fluid that can be processed. The time for processing one milliliter of whole blood per hour [40] will make the analysis of larger blood volumes unpractical. The reports of higher number of CTC detected with these systems is likely due to a lesser stringency of the criteria used to assign objects as CTC. Comparison of CellSearch CTC detection with detection by flowcytometry using EpCAM-PE and CD45 PercP and a nucleic acid dye after erythrocyte lysis showed a maximum of 3 fold increase in sensitivity [26].
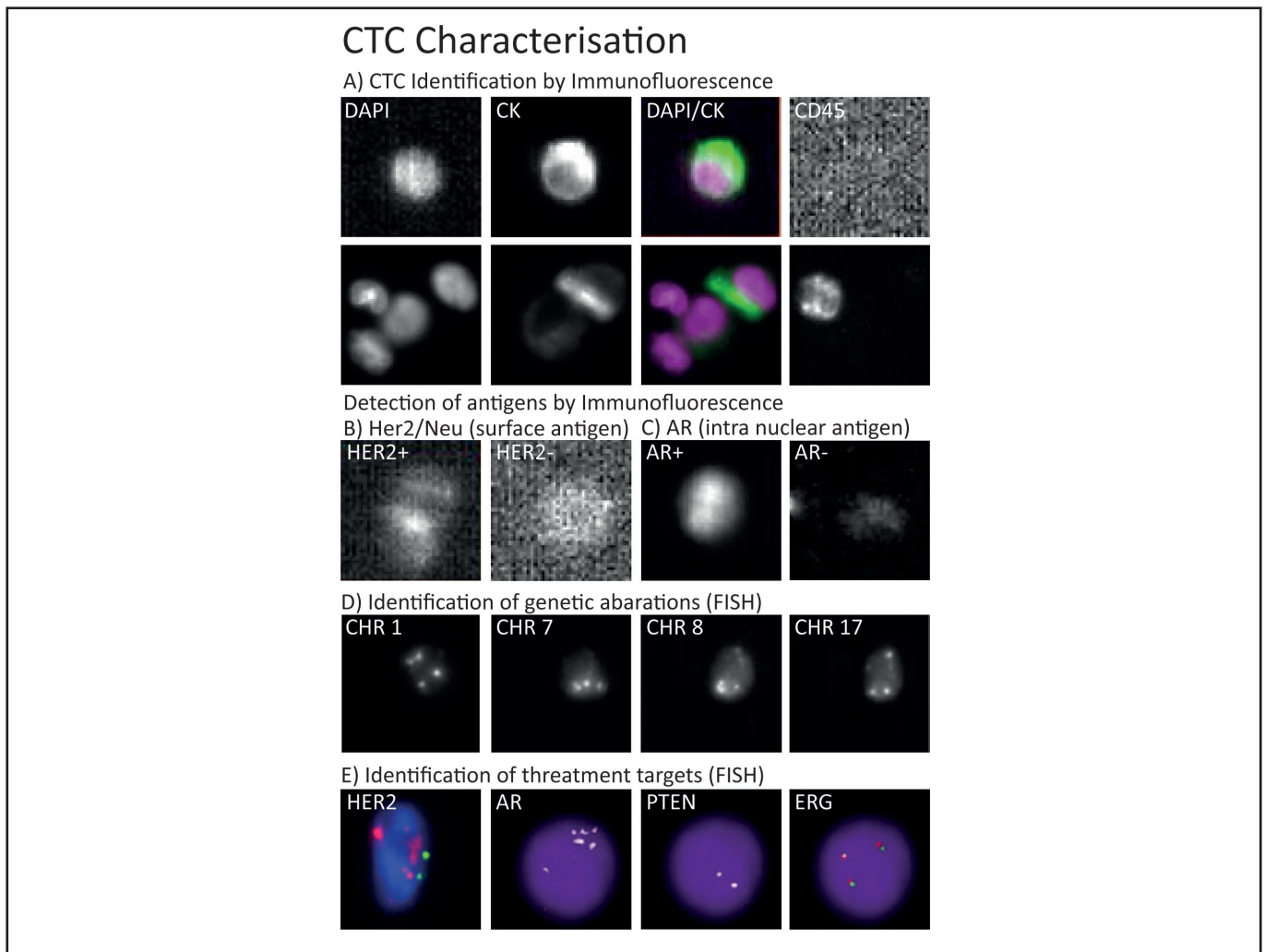
DENSITY BASED ENRICHMENT

Traditionally density based enrichment techniques such as Ficoll with its density of 1.077 is used to separate the mononuclear cell fraction from blood. It also has been used to enrich CTC although the range of densities in which CTC appear is not known. Other density based separation such as Percoll or Oncoquick permit a well defined range of densities, The Rosettesep method uses antibodies in combination with density separation for the depletion of CD45 positive cells as illustrated in Figure 5C. The antibodies present form aggregates of red blood cells and the CD45 positive cells, thus increasing the density of all white blood cells. After a density separation using Ficoll, the mononuclear cell population can be cytospinned on a glass and stained or run on a flow cytometer. Although 62% of cells derived from tumor cell lines can be recovered after spiking in whole blood using Rosettesep the implicit assumption is that CTC will have a similar density as peripheral blood mononuclear cells and cells derived from cell lines [44]. A novel density based method is RareCyte which use a floater. The floater has the same density as the mononuclear cells. The floater is designed such that there is only a small gap of a few um between the tube and the floater. This causes a thin layer of nucleated cells to be formed near the surface of the custom tube so all the nucleated cells can be imaged[45]. In general when comparing traditional density separating methods with EpCAM specific methods the latter are more sensitive, but might miss the EpCAM negative CTC[46-48].
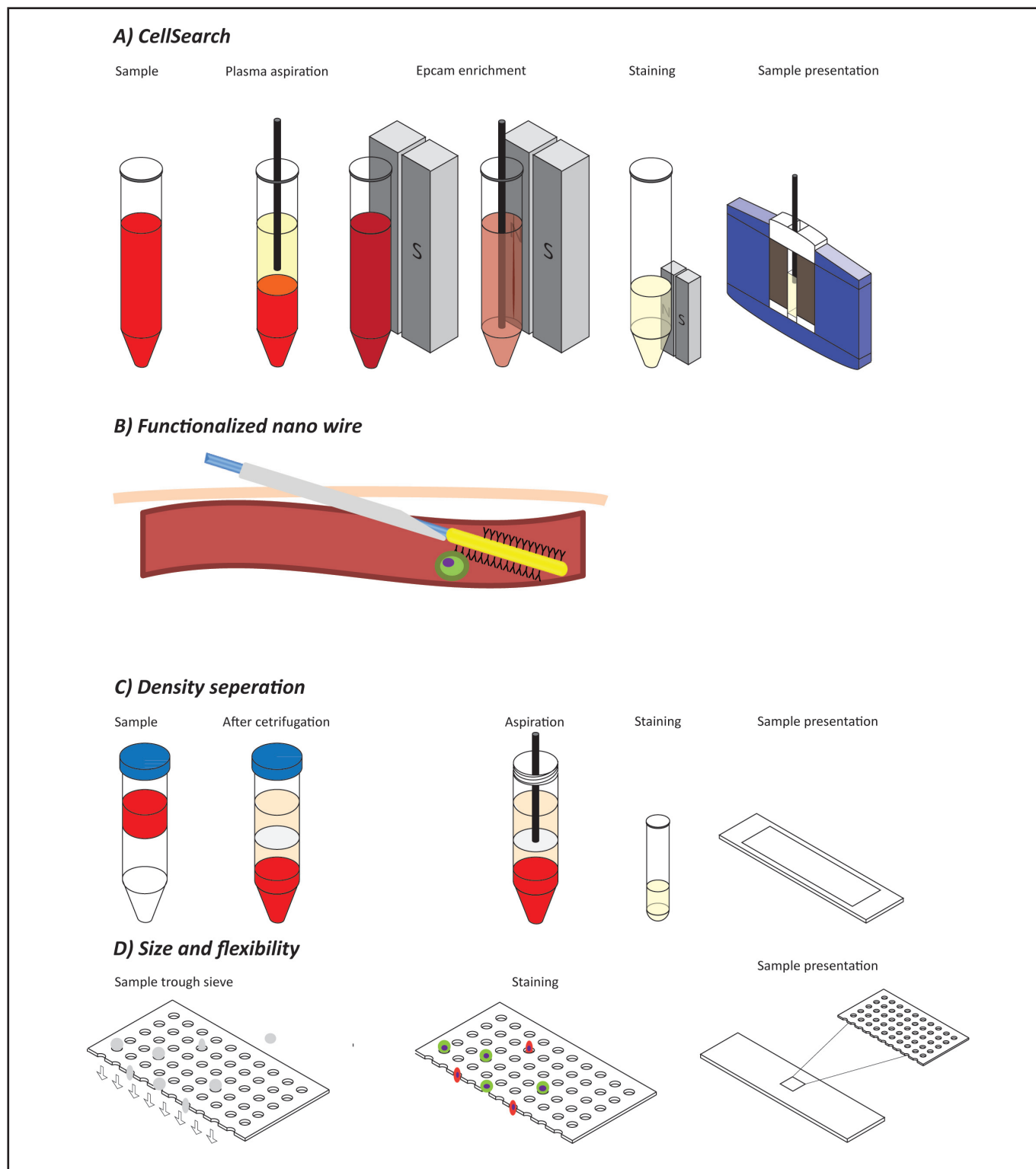
Figure 5
Examples of different CTC enrichment methods: Panel A) CellSearch which uses iron particles coupled to anti-EpCAM antibodies to enrich cells from epithelial origin. Cells are kept in a magnetic field during wash steps. After staining they are presented in a MagNest to pull all EpCAM positive cells to the imaging area. Panel B) Functionalized nano wire, here an EpCAM functionalized probe is placed directly in the blood stream to capture CTC. After 30 minutes the probe is removed from the blood and the attached cells are stained and fixed. The cells are investigated while still on the probe. Panel C) Rosettesep, here white blood cell depletion is achieved by forming aggregates between erythrocytes and CD45 positive cells. After which the aggregates are separated from the CTC using density separation. Panel D) passing whole blood through a sieve with 8 um pores. The erythrocytes and white blood cells will pass the pores while some of the bigger cells will stay on the membrane surface. The cells are fixed and stained on the filter.

CTC Characterisation

A) CTC Identification by Immunofluorescence

Detection of antigens by Immunofluorescence
B) Her2/Neu (surface antigen) C) AR (intra nuclear antigen)

D) Identification of genetic abarations (FISH)

E) Identification of threatment targets (FISH)

Figure 6
Panel A, thumbnail images of CTC detected in metastatic cancer patient using CellSearch Row 1 one DAPI+ cell, CK+, CD45- => CTC. Row 2 four DAPI+ cells, one CK+, CD45- CTC, one CKdim, CD45- CTC, one CK-, CD45+ leukocyte and one CK-, CD45- with no proof of origin. Panel B and C, detection of treatment targets Her2 and AR on CTC using immunofluorescence. Panel B, a Her2+ and Her2- CTC, Panel C, an AR+ and AR- CTC. For weak signals such as the androgen receptor a bright fluorophore is needed in this case the CK antibody is labeled with FITC and the AR labeled antibody with Phycoerythrin. Panels D and E, CTC can be restained for FISH to asses chromosomal abnormalities[71], panel D, or specific genes HER2, AR, PTEN, ERG [68].

SIZE OR FLEXIBILITY BASED ENRICHMENT

CTC are slightly larger than white blood cells [49] and this property is leveraged by several methods which select on size or flexibility. The whole or lysed blood is filtered through a membrane with distinct pore sizes varying from 5 to 8 um such as illustrated in Figure 5D. The premises is that CTC stay on the filter while white blood cells pass the pore. There are different fabrication methods for such membranes. Track etched membranes are made of polycarbonate with a random pore distribution and used as in several systems such as ISET and ScreenCell [50, 51] or directly[52]. Micro fabricated membranes from parylene [53-55], silicon[49, 56] or other materials [57] have a regular pore spacing and well defined pore dimensions. They can also have a more complex topology. In for example the ISET system the sample is first mixed a buffer that lyses the erythrocytes and fixes all other cells. The sample is loaded in a disposable with a filter membrane on the bottom. The sample flows through the membrane in 3 minutes [51]. After filtration the sample is stained and enumerated.   The different filtration methods all perform well using cells derived from cell lines with relatively large size and are able to catch EpCAM negative CTC. The parameters under which CTC filtration is done however varies greatly between method with variation in fixation, erythrocyte lysis, sample dilution and operating pressure[49]. One of the main advantages of filtration is that the CTC remain quite easily accessible which facilitates both reanalysis of the whole sample and individual cells. It is also possible to filter unfixed cells which results in viable enriched cells. Enriching CTC based on size has also been applied in a micro fluidic device[58, 59]. Here 900 cups consisting of three posts

placed in a triangle position with a 5µm gap in between the posts. The gaps allow normal cells to pass and capture CTC. The triangles are spaced 20um from each other so that cells can flow past them if a cup is occupied. There unique capturing structure allows for the release of captured cells from the chip. So far this chip by Clearbridge biomedics is the only commercial available microfluidic CTC isolation device.

CTC DETECTION AFTER ERYTHROCYTE LYSIS

CTCs can be detected by flowcytometry [60, 61] or laser scanning cytometry [62] after erythrocyte lysis and staining with fluorescently labeled antibodies. The combination of EpCAM-PE, CD45 PerCP and a nucleic acid indeed can be used to identify CTC in 100 µl of blood [60] increasing the blood volume however results in a large background in healthy controls [60, 61]. Using this flow cytometry approach the maximum increase in yield CTC was estimated at 3.3 fold as compared with the number of CTC found in CellSearch[26]. This increase is however flawed as by flow cytometry no detailed information about morphology is provided resulting in events falsely identified as CTC.
A technology introduced by epic sciences uses specially prepared slides to place the blood after erythrocyte lysis. The cells are allowed to settle to create an evenly distributed mono layer of cells. This allows for the imaging of all non-lysed cells present in blood. Identification of CTC is done using a dedicated image analyzer with a dedicated image processing technique[63] making the analysis of large amount of cells possible. Having no enrichment is advantageous for cell loss but will make the technique more sensitive to unspecific immunofluorescent staining.

COMPARING DIFFERENT ENUMERATION METHODS

Because of the low numbers of CTC found in patient samples new methods are being explored to increase sensitivity. Each CTC detection and isolation technique however makes its own assumptions about what constitutes a CTC. This is important to keep in mind when comparing techniques, especially considering the fact that changing the definition a CTC in the same technique can already have a large impact on the prognostic power of the assay[28]. Finding more CTC does not automatically imply that the distinguishing power of the assay increases. The recovery of spiked cells gives some information about the efficiency of a technique. For patient samples the biggest hurdle is that the number of CTC that are present is unknown and new techniques are frequently compared with CellSearch. When drawing conclusions from this comparison it is important to note that the phenotype of the cells found with the new method might not be the same as found by CellSearch. When the same phenotype is measured a correlation between the two techniques could indicate that the same prognostic value might apply to the CTC found. Ultimately real conclusions about their prognostic value prospective clinical studies are needed. These studies would not only provide interesting data about the enrichment method, but will also give an idea of which CTC phenotype is the most important for the prognosis of the patient.

DETECTION OF TREATMENT TARGETS ON CTC

Besides enumeration, CTC can also serve as a means to detect the presence or absence of treatment targets. For example in breast cancer the Her2/neu expression is of importance when considering Herceptin therapy and can be assessed on CTC both at the protein and gene level as illustrated in Figure 6B and 6E, [64-67]. In prostate cancer the expression of the androgen receptor, PTEN and ERG is important and can also be detected on CTC Figure 6C and 6E [68]. Clear advantage of CTC above traditional biopsies is that it can provide a real time assessment of the tumor to be treated. CTC give a more recent and thus sometimes different picture then the information from biopsies[65, 66, 69, 70]. Clinical studies will however need to be conducted to proof that the detection of treatment targets on CTC is more beneficial to the treatment outcome as the assessment of treatment targets on a biopsy. Most important is that the event detected is indeed a tumor cell and can be assessed by for example ploidy status as illustrated on a CTC in Figure 6D [71].
Ideally CTC should be used as a tool to guide therapy: if a patient's CTC are not eliminated after the first cycles of therapy a switch to another therapy should be considered as the current one is not working[4, 5, 72, 73]. The use of CTC in this manner is currently being investigated in a multicenter trial for breast cancer by the south west oncology group (SWOG 0500 - NCT00382018). The type of therapy to be administered can be obtained from the detected treatment targets on CTC as is currently investigated in the DETECT III trial (NCT01619111).
The future
For CTC not only enumeration is of importance, but also the ability to examine them for the absence or presence of treatment targets. To achieve this goal proteins, RNA and DNA that contain information pertinent to treatment should be preserved in CTC and most important we should be able to isolate and characterize them in all patients with metastatic disease whether detected with present technology or not. Giving the fact that in approximately 50% of patients with metastatic disease can be detected with the current available clinically validated CellSearch system and in a significantly lower portion of patients with primary disease with a risk of recurrence, it will be quite a challenge to develop technology that can benefit all cancer patients.

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