Circulating tumor cells

counts and characteristics

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CIRCULATING TUMOR CELLS

counts and characteristics

Circulerende tumorcellen, tellen en typeren

PROEFSCHRIFT

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Chapter 1

General introduction and outline of the thesis

1.1 CIRCULATING TUMOR CELLS

In recent years, many new anti-cancer agents have been developed and introduced into clinical care. While these new agents have led to substantial gains in response rates and life expectancies, they have also increased the need for tools to select those patients benefitting from said therapies. Once patients develop metastatic disease, treatment is aimed at improving quality of life and prolonging life expectancy, but is always a trade-off against the side-effects that are inevitably associated with anti-tumor therapy, underscoring the need to select only those patients who are likely to respond to a particular drug. However, there is still an unmet need for such an array of reliable predictive factors, a need that can be met by designing studies in which patient subgroups are defined and stratified based on rational, biology-driven but feasible tumor characteristics. An increasing number of studies is being designed in which, for example, only patients with a specific gain-of-function mutation are subjected to a monoclonal antibody therapy aimed at the activated pathway this gene is involved in. While substantial progress is being made with this approach[1-2], patient selection has thus far been far from perfect. Even a powerful predictor such as a *KRAS* mutation for EGFR-inhibiting therapy results in a response in just 20% of patients who are deemed sensitive based on their *KRAS* wild-type status[3-4]. One of the reasons for the disappointing performance of predictive factors could be the fact that they are most often based on primary tumor characteristics, while at the time of metastatic disease, a patients' prognosis is determined by their metastatic tumor load and its biological phenotype. Through processes such as clonal selection and the inherent genomic instability of the tumor or as a consequence of therapy pressure, metastatic tumor cells can differ substantially and vitally from primary tumor cells[5-7]. Analysis of metastatic tissue would thus probably be better indicative of the actual tumor load and its underlying biology, and lead to better response prediction. Unfortunately, repetitive metastatic biopsies are invasive and painful, understandably limiting their use in clinical practice. Circulating tumor cells (CTCs) provide a very promising solution for this problem, as they can be obtained and characterized repetitively and non-invasively through venipunctures, and thus serve as a surrogate 'liquid biopsy' of metastases.

CTCs are cells that circulate in the peripheral blood of cancer patients, originating from either primary tumor or metastases. Since their discovery in 1869, CTCs have been regarded as a very promising field of research due to the opportunities that lie within the ability to easily and repeatedly access tumor cells. Only since recent years, this promise has started to be fulfilled, as the detection of CTCs turned out be extremely challenging.

CTC detection methods

CTCs are extremely low-frequent in the circulation, with a median CTC count of 3 – 5 per 7.5 mL of blood in metastatic cancer patients[8]. The scarcity of CTCs and the abundance of leukocytes amongst which they are present[9] demand extreme sensitivity and specificity from their detection methods. Additionally, tumors, and thus probably also CTCs, are heterogeneous within and between patients[6-7], and the search for the perfect CTC detection marker is by no means finished. It is this heterogeneity, caused by the existence of CTC subpopulations with distinct biological roles or simply reflecting the heterogeneity that is also present in solid tumors, which has hampered the development of an assay that is able to detect all types of CTCs in every patient. Moreover, because most assays rely on the expression of epithelial markers on CTCs, cells that have undergone epithelial-to-mesenchymal transition (EMT) upon entering the bloodstream could remain undetected. The detection of CTCs lacking epithelial markers may however be of crucial clinical importance, as EMT is thought to be mandatory for haematogenous metastasis and is associated with poor prognosis[10-12]. Importantly, it is currently not known whether all CTCs are equal, or if most are passively-shed 'by-stander' CTCs accompanied by a few aggressive counterparts that have the ability to grow out into new metastatic lesions.

In an effort to obtain the highest and most relevant CTC count, many different CTC detection methods have been developed, ranging from flow cytometry-based methods[13-15] and immunocytochemistry[16-18] to microfluidic chips[19-20] and quantitative reverse transcriptase PCR (qRT-PCR)[21-24].

CTC enumeration

While advances in CTC detection methods are crucial to increase their clinical value, an FDA-approved CTC detection method is already available. Enumeration of CTCs with this CellSearch® assay (Veridex™ LLC, Raritan, NJ) has repeatedly been proven to be prognostic in an expanding number of tumor types such as metastatic breast[25-28], colorectal[28-29], lung[30] and prostate cancer[28,31]. Moreover, CTC change during the first cycle of chemotherapy is a reliable predictor of therapy response[25], performing at least as good as but earlier than conventional radiological assessment[32] or PSA measurement[31,33] do. In the primary setting, CTCs are even less frequent and lower in numbers than in the metastatic setting, but the subgroup of patients with detectable CTCs, usually comprising 10 – 20% of patients, does have a considerably worse prognosis than those without CTCs[34-35].

Because of this low CTC frequency in patients with primary cancer and the fact that even in metastatic cancer patients, CTCs cannot be detected in 40 – 60% of patients[8], CTC enumeration is far from ready to be employed as a cancer screening tool. With the introduction of more

sensitive assays, which could importantly lead to the detection of more CTCs in more patients, screening too could become a potential application of CTC enumeration.

CTC characterization

Characterization of CTCs is even more challenging than mere counting, as their low numbers and presence among, even after enrichment procedures, a substantial number of leukocytes[9] complicates techniques such as DNA sequencing and qRT-PCR. Despite these hurdles, DNA mutation[36] and methylation detection[37], mRNA and miRNA expression[38-39], FISH analysis[40-42] and immunocytochemistry (ICC)[43-44] have all proven to be feasible in these low-frequent cells. The potential clinical value of CTC characterization is seemingly endless as technology improves; analysis of drug targets such as *HER2*[45-48] and estrogen receptor (ER)[45,47,49] has already revealed clinically relevant discrepancies between primary tumor and CTCs, and besides drug targets, quantification of gammaH2AX expression on CTCs enables their use as a pharmacodynamic marker[50], while large-scale gene expression analysis of CTCs provides insight into their biology and heterogeneity[38-39].

Is there more to the circulation?

The studies described thus far have focused on the enumeration or characterization of CTCs in whole blood, but this is not the only compartment of the circulation from which tumor-derived information can be obtained. Blood plasma[51] and serum[52], but also peritoneal[53], pleural[54-55] and cerebrospinal fluid[56] can contain tumor-derived particles, and could provide a valuable addition to CTCs in blood. Also, whole tumor cells are not the only source of tumor-derived information and the presence and prognostic value of circulating DNA[57], mRNA[51,58-59,] miRNA[52,60], fragments[61] and exosomes[62-63] stemming from tumor cells has been shown in a number of studies. All these different approaches could add to the knowledge we are acquiring on the behavior and relevance of tumor cells in the circulation, and eventually to the identification of novel predictive factors and models, together contributing to a more personalized treatment approach of cancer patients.

1.2 AIMS AND OUTLINE OF THE THESIS

This thesis is aimed at optimizing the quantity and quality of predictive and prognostic information that can be obtained from CTCs, through improvement of CTC detection and characterization methods and by correlating CTC counts and properties with clinical outcome. The value of CTC enumeration in metastatic breast cancer patients is confirmed, and the possibilities of CTC characterization within this and other cancer populations are explored.

Since the first description of CTCs in 1869, many different CTC detection methods, both cytometry-based and nucleic acid-based, have been described. Depending on the choice of method for enrichment –capturing cells based on morphological properties or on the expression of tumor-specific markers–, and on the choice of method for detection –measuring mRNA, miRNA, DNA or protein expression–, varying degrees of success have been achieved. An overview of these different CTC detection methods is provided in **Chapter 2**.

The only currently FDA-approved CTC detection method is CellSearch, which captures CTCs based on anti-EpCAM enrichment followed by selection based on cytokeratin 8/18/19, DAPI and CD45. After having previously established that a subgroup of breast cancer cells lacks EpCAM-expression and are thus missed using the conventional CellSearch method[64], we describe the use of an alternative enrichment marker, CD146, in **Chapter 3**. The addition of CD146 to EpCAM enables detection of all breast cancer subtypes, and CD146-positive CTCs were identified in breast cancer patients.

Despite marked improvements in breast cancer detection by adding CD146 to the enrichment step in the CellSearch assay, some cytokeratin-negative cells were still missed. Attempting to further improve the CellSearch assay, we tested various CTC selection markers as an alternative to cytokeratin 8/18/19 in **Chapter 4**. CD49f was identified as being broadly expressed among all breast cancer subtypes, and the addition of CD49f to cytokeratin 8/18/19 led to improved detection of cytokeratin-negative breast cancer cells.

In addition to CTC enumeration, CTC characterization enables the assessment of prognostic and predictive factors, which are probably better reflective of tumor load and its biology at the time of metastatic disease than characteristics obtained from a primary tumor that was resected years before, and, importantly, before administration of systemic treatment. In **Chapter 5**, we show that a CTC-specific gene expression panel consisting of 55 mRNAs and 10 miRNAs can be reliably measured in CTCs of metastatic breast cancer patients. This panel is able to discriminate between healthy donors (HDs) and three patient subgroups, and the patient subgroups were characterized by differential expression of growth factor receptor-, estrogen receptor- and proliferation-associated genes. In addition, we describe clinically relevant discrepancies between CTC and primary tumor ER and HER2 expression.

After showing the feasibility of the measurement of our CTC-specific gene expression panel, we proceeded to establish its clinical relevance as a prognostic factor in metastatic breast cancer patients. In **Chapter 6**, we show that a 16-gene CTC gene expression profile identifies poor prognosis patients among those with <5 counted CTCs, while a separate 9-gene CTC profile further discriminates patients with ≥5 CTCs, identifying those patients with treatment switch or death within 9 months after start of 1st line systemic therapy versus those with a more favorable outcome. These CTC profiles provide additional prognostic information on top

of a CTC count, and thus allow for better patient selection and stratification.

Chapter 7 describes the generation of a CTC-specific gene expression profile in metastatic colorectal cancer patients, whose CTCs were isolated before resection of their liver metastases. In this patient group too, like in metastatic breast cancer, we were able to identify a panel of CTC-specific mRNAs and miRNAs capable of discriminating HDs from patients with ≥3 CTCs. In patients without detectable CTCs according to the CellSearch technique, a subgroup was identified whose molecular profile was clearly distinct from HDs, and expression of various epithelial markers suggested the presence of circulating tumor load in their blood.

Besides mRNA and miRNA expression, mutation analysis on CTCs also provides great opportunities for the improvement of personalized cancer treatments. In recent years, *KRAS* and *BRAF* mutations have been established as crucial predictive factors in colorectal cancer patients treated with EGFR-inhibitors. As with gene expression, mutation analysis of CTCs at the time of metastatic disease will probably lead to a more reliable identification of those patients susceptible to EGFR-inhibiting treatment than analysis of the primary tumor. In **Chapter 8**, we describe the *KRAS* and *BRAF* mutation analysis of matched CTCs, primary tumors and liver metastases of 42 colorectal cancer patients, and show that clinically relevant discrepancies are present between these tumor compartments.

MiRNAs are an important new topic in cancer research, as these small RNA fragments are directly involved in regulating a large array of mRNAs. MiRNAs too can be measured in CTCs or, presumably because of their profound stability compared to MiRNAs, exosome-bound or cell-free in the circulation. In **Chapter 9**, we review the diagnostic applications of cell-free and CTC-associated miRNAs in various tumor types, and provide our opinion on the technical specifications and validations needed to establish these tests as reliable prognostic and predictive factors.

Chapter 2

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

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ABSTRACT

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

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

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

INTRODUCTION

The outcome of breast cancer largely depends on the development of metastases in the course of the disease. Given this vital importance of metastases, means to detect and monitor their existence are continuously sought for. The detection of circulating tumor cells (CTCs) is one field of research focusing on a new method to detect metastatic disease earlier, less invasive and more reliably than currently available conventional methods, such as clinical presentation, radiographic evaluation and serum tumor markers do. CTCs are defined as tumor cells circulating in the peripheral blood of patients, shed from either the primary tumor or its metastases. Numerous efforts have been made to reliably detect and quantify CTCs in peripheral blood, but development of a suitable assay has proven to be difficult. Unfortunately, there is not one specific feature that universally distinguishes CTCs from blood cells. Ideally, a specific marker would be identified, which is expressed in every cell of every breast cancer type. In reality, different histological and molecular types of tumors express different arrays of markers, and marked heterogeneity of expression exists even within one histological distinct tumor type. Another challenge regarding sensitivity of assays is the fact that CTCs are rare events, with numbers as low as one CTC in 10^6 - 10^7 leukocytes[65]. In spite of these challenging characteristics, the importance of detecting and enumerating CTCs in breast cancer has been established in several clinical studies, showing a correlation with decreased progression-free survival (PFS) and overall survival (OS)[66-67]. In addition to detection and enumeration, molecular characterization of CTCs provides a second much-anticipated application in oncology. Currently, we are dependent on the primary tumor for molecular characteristics in order to determine the type of therapy the patient will benefit from most. However, tumor genotype and/or phenotype may change in the course of treatment as indicated by therapy resistance. CTCs might function as a real-time biopsy of tumor load, and enable oncologists to make better-informed choices regarding therapy.

In addition to CTCs, disseminated tumor cells (DTCs), i.e., isolated tumor cells in bone marrow, are thought to reflect the metastatic potential of tumors. DTCs have also been correlated with prognosis[68-69], but for their detection an invasive diagnostic procedure, bone marrow aspiration, is necessary. This requirement makes their implementation in the clinic more troublesome. By contrast, CTCs have the advantage of being readily available in peripheral blood and given this, together with mounting evidence supporting their clinical feasibility as reviewed here, the detection of CTCs is anticipated to gain clinical relevance shortly. For a comprehensive review on DTCs and their complementary role to CTCs, we refer to the recent review by Riethdorf et al.[70].

Here we will discuss the principals and technical aspects of the different techniques available for detecting CTCs, in addition to the most frequently used markers in these techniques.

Furthermore, we will discuss clinical studies showing the utility of CTC detection in breast cancer patients with these techniques. Reviews focusing on the detection techniques of both DTCs and CTCs, as well as biological relevance, have been published recently[71-72].

DETECTION OF CIRCULATING TUMOR CELLS

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

As CTCs are rare events occurring at rates as low as 1 cell per 10^6- 10^7 leukocytes, enrichment is generally needed to increase sensitivity to an acceptable level. One type of enrichment relies upon the selection of target cells with tumor-specific markers (immunoseparation). Other methods for enrichment are based solely on morphologic criteria, such as cell size or density. Sensitivity and specificity is an issue with both techniques, due to heterogeneity of tumors in size, density and marker expression. Consequently, while enrichment is thought to be required, some tumor cell loss is likely to occur irrespective of the enrichment technique used. The extent of cell loss should be determined with recovery experiments for each technique to validate the results[73-74].

After enrichment, nucleic-acid based techniques like reverse transcriptase (RT)-PCR or cytometric methods are applied to detect CTCs through putative tumor-specific markers. Here we will discuss the most commonly used enrichment and detection techniques. Some techniques have combined their enrichment and detection steps and these will be presented separately. While a number of studies have compared the performance of different assays[16-17,75], not all techniques have been compared directly to each other. In an attempt to clarify the hierarchy in the various techniques, we have depicted their major advantages and disadvantages in **Tables 1** and **2**.

INCREASING ASSAY SENSITICITY: ENRICHMENT TECHNIQUES

As mentioned before, enrichment can be based on morphologic cell characteristics, such as size or density, or on immunoseparation, using magnetic beads, ferrofluids or rosettes.

Morphology-based enrichment

ISET (Isolation by Size of Epithelial Tumor cells) isolates tumor cells individually by filtration based on their larger size (>8 μm) compared to leukocytes[76]. The *Nucleopore* assay (Whatman International Ltd, England) is based on the same assumption[77]. However, no validation

studies have been executed confirming that CTCs are indeed never smaller than 8 µm, leaving questions about the sensitivity of this method.

Density gradient-based techniques are techniques separating mononuclear cells based on their lower density compared to other blood compartments. Mononuclear cells and tumor cells are separated from blood cells and granulocytes using a density gradient of 1,077 g/mL. Similarly, *Oncoquick* (Greiner Bio One, Frickenhausen, Germany) is based on density gradient separation, but adds a porous barrier, which prevents the gradient-separated cells to be contaminated with the whole blood.

Immunomagnetic separation

The simplest separation can be done with immunomagnetically labeled monoclonal antibodies and a basic handhold magnet. Negative selection of a blood sample can be done with *magnetic beads* loaded with an anti-CD45 antibody, a pan-leukocyte marker, or against CD61 thereby removing megakaryocytes and platelets[78].

MACS® (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), or Magnetic Activated Cell Sorting system, is a dedicated instrument that captures cells by immunomagnetic labeling with microbeads. It does so by membrane or intracellular staining, the latter requiring a permeabilization and fixation step. Magnetic beads are available linked to anti-epithelial antibodies for positive selection through EpCAM, for example. EpCAM, or tumor-associated calcium signal transducer 1 (CD326), is a cell surface molecule involved in cell-to-cell adhesion and is highly expressed in most epithelial carcinomas[79]. Magnetic beads targeting the tumor-specific cell antigen epidermal growth factor receptor 2 (HER2) are also available.

The *AdnaTest* (AdnaGen AG, Laggenhagen, Germany) combines two epithelial and tumor associated antigens. Two antibodies against MUC1 and one antibody against EpCAM are conjugated to magnetic beads. As neither MUC1 nor EpCAM are present on all circulating tumor cells[80], cells expressing MUC1 and/or EpCAM should be isolated with this double-antibody method.

RARE™ (StemCell Technologies, Vancouver), i.e., RosetteSep-Applied imaging Rare Event, is a technique that combines a density gradient separation with an antibody-mediated enrichment step. Enrichment is done through negative selection, as $CD45^+$ cells are cross-linked to multiple red blood cells by bispecific tetrameric antibody complexes, forming rosettes. As the density of these unwanted cells then increases, the CD45-positive cells accumulate in the lower compartment after density gradient centrifugation. CD45-negative mononuclear cells are isolated between the separation medium and plasma[81]. A more extensive negative selection can be done with the same technique, by using a kit containing antibodies directed against CD2, CD16, CD19, CD36, CD38, CD45, CD66b and glycophorin A.

DETECTION TECHNIQUES

Cytometric methods

The presence of tumor cells in the bone marrow was first identified using conventional imaging techniques[82]. Building on this, detecting tumor cells in the circulation was attempted using simple hematoxylin and eosin staining[83]. This exhaustive method consisted of visually identifying large numbers of gradient-separated cells and comparing them with primary tumor cells morphologically. Nowadays, as previously mentioned, detection of CTCs occurs on a cytometric or a nucleic-acid basis.

Cytometric methods isolate and enumerate individual cells based on their antigen expression, using for example monoclonal antibodies directed against epithelium-specific antigens. The advantage of cytometric methods over nucleic-acid based methods is the possibility to further characterize the cells, as the target cells are not lysed in the procedure. This allows subsequent morphological identification and molecular characterization of CTCs. The major draw-back is the current lack of a tumor specific antibody. The commonly used Cytokeratin (CK) antibodies bind specifically and non-specifically to macrophages, plasma cells and nucleated hematopoietic cell precursors[84-85]. The same holds true for Mucin-1 (which binds nonspecifically to erythroid progenitors)[86]. This problem can be reduced significantly by counterstaining with CD45, a pan-leukocyte marker. Breast cancer specific markers have been used (i.e., HER2, anti-Mammaglobin), but as these are not present on all breast cancer tumors or on every cell of a particular tumor, false negatives are likely to occur. Advances in terms of sensitivity and specificity have been made using multimarker assays, which can overcome detection problems due to tumor heterogeneity[64].

To overcome the problem of high numbers of immunofluorescently labeled mononuclear cells having to be analyzed to identify rare CTCs, *FAST* was developed. This Fiber-optic Array Scanning Technology locates immunofluorescently labeled cells on glass substrates at rates 500 times higher than conventional automated digital microscopy. The key innovation is a light collection system that has a very large field of view (50 mm), which is large enough to enable continuous scanning without the need to analyze the sample in multiple steps. Because larger volumes of peripheral blood can be analyzed than using conventional microscopy in the same time, purification or enrichment steps are avoided, which reduces the risk of cell loss. In cell line spiking experiments, an average sensitivity of 98% was reached in colorectal and breast cancer after whole blood lysis[87-88].

Attempting to improve scanning of fluorescent cells, the *Laser Scanning Cytometer* (LSC) (Compucyte Corporation, Cambridge, MA) was developed. Following whole blood lysis and staining with anti-human epithelial antibody (HEA) in combination with CD45, this cytometer analyses fluorescence after the cells are contoured using forward scatter as a threshold

parameter. The cytometer determines background fluorescence dynamically to calculate peak and integral fluorescence on a per-cell basis. This calculation results in improved correction for background fluorescence variation. It is also possible to relocate the cells within the positive population, allowing for visual verification through the microscope[14,89]. In a recent study, three different combinations of techniques were compared; immunomagnetic separation and LSC vs. cell filtration and LSC vs. a multimarker quantitative RT-PCR assay. qRT-PCR was found to be the most sensitive. Samples from patients with metastatic breast cancer were significantly more likely to be positive for one or more of three markers (*CK19*, *mammaglobin*, and *PIP* (prolactin inducible protein) using RT-PCR than to be positive in LSC[75].

ACIS®[85] (Automated Cellular Imaging System) (DAKO, Glostrup, Denmark) and *ARIOL*®[90] (Applied Imaging Corp., San Jose, CA) are automated scanning microscopes enabling faster examination of slides. After initial automated scanning and analysis of slides in a manner that can be configured to the assay used, the investigator reviews the presented images and classifies them morphologically. Numerous other automated scanning systems have been used in the immunocytochemical detection of rare events[91-93].

Nucleic-acid based methods

CTCs may be identified through the detection of (epi)genetic alterations that are specific for cancer cells. Alterations in DNA such as mutations in proto-oncogenes or tumor suppressor genes, microsatellite instability and sequences of oncogenic viruses may be detected. Circulating free total DNA in the blood of cancer patients was detected for the first time in 1977 using a radioimmunoassay[94]. In later studies, circulating mitochondrial DNA[95] and amplification of *MYC-N* (a neuroblastoma-derived MYC oncogene) DNA[96-97] in neuroblastoma patients was detected in greater amounts in patients with cancer than in healthy individuals. Implementing DNA-based CTC detection in clinical practice is difficult however. DNA changes occur in merely dysplastic lesions as well as in full-blown neoplasm. Furthermore, there is uncertainty about the half-life of circulating cells and nucleic acids, which means that the presence of circulating free DNA may reflect merely the presence of nucleic acids, not tumor cells. As a result, the detection of free total DNA has not been implemented into clinical practice.

Detection of mRNA of factors that are overexpressed or mutated in breast cancer using RT-PCR is a more widely used alternative. As RNA disappears quickly from the blood after cell death, detection of RNA is likely due to the presence of a whole tumor cell, not cell fragments or free RNA. In RT-PCR, after cDNA synthesis, the gene of interest is amplified using oligonucleotide primers specific for this gene of interest. The sensitivity of RT-PCR

was higher than immunocytochemistry in several studies[16-17,98]. However, RT-PCR is prone to false-positivity, as sample contamination, expression of target genes in normal cells, and pseudo genes (genes without protein-coding abilities) can all occur. The problem of false-positivity was demonstrated very clearly in work on activated peripheral blood mononuclear cells (PBMCs)[99]. A multimarker RT-PCR assay was performed on healthy donors, stimulated PBMCs and unstimulated PBMCs from patients with immune thrombocytopenic purpura (ITP). While all markers (*SCCA* (secondary structure conserved A), *EGFR* (epidermal growth factor receptor), *hMAM* (mammaglobin), *SBEM* (small breast epithelial mucin) and *CA-9* (carbonic anhydrase 9)) were negative in healthy donors, 4 out of 5 (*SCCA, EGFR, hMAM, SBEM*) were positive in stimulated PBMCs and 3 of 5 (*SCCA, EGFR, SBEM*) were positive in patients suffering from ITP. In another study, it was revealed that CK19 and CEA (carcinoembryonic antigen) expression is present in lymphatics following cytokine stimulation, as well as in 50% of bone marrow samples of patients with chronic inflammatory disease[100]. As cancer can induce inflammatory responses[101], these inducible signals may be the cause of false-positive outcomes in CTC detection. Another possible source of false-positivity is the presence of free RNA or genomic DNA, which can be eliminated by adding a gradient separation step or genomic DNA elimination by DNAse, respectively[102].

In general, nucleic-acid based methods combine their higher sensitivity with a lower specificity, as background noise due to expression of markers in normal cells is hard to distinguish from a true positive signal. Quantitative RT-PCR provides a way of visualizing low and high expression of a chosen marker, increasing discrimination between mRNA expression of normal cells and tumor cells. Like in cytometric methods, in RT-PCR as well the absence of a true tissue-specific marker has been an issue with regard to specificity. RT-PCR outperformed immunocytochemistry in sensitivity (49.6 vs. 42% positive samples in 133 patients) in a study on CK19 detection. However, no data were provided on results in healthy donors[17]. The importance of the latter was underlined by the findings of another study comparing CK19 detection by immunocytochemistry vs. RT-PCR vs. Nucleic Acid Sequence-Based Amplification (NASBA). While RT-PCR was more sensitive than immunocytochemistry and NASBA, all three methods showed false-positive results in healthy donors[16], prompting the authors to deem CK19 an unsuitable marker. As these studies show, single-marker assays reach sufficient sensitivity but lack in specificity. Given the heterogeneity of breast cancer, the consistent presence of a specific tumor marker or fusion gene such as in Ewing tumors[103-104] seems unlikely. Instead, the use of multiple marker assays, combining several breast cancer-specific markers as well as leukocyte-specific markers, might at least in part resolve the issue of specificity.

COMBINED ENRICHMENT AND DETECTION TECHNIQUES

CellSearch® (Veridex™, Warren, PA) is a semi-automated technology by which whole blood is enriched for CTCs by adding ferrofluids loaded with antibodies directed towards EpCAM. Currently, *CellSearch* is the only FDA-approved assay for CTC detection. CTCs in the enriched population are stained with CK and DAPI using fluorescent antibodies, while hematopoietic cells are counterstained with CD45. The CK^+/$DAPI^+$/CD45- cells are then enumerated with an automated fluorescence microscope. The semi-automated character of this system enables samples to be analyzed rapidly and reproducibly. When CellSearch was compared directly to *Oncoquick* followed by labeling with CKs, EpCAM and DAPI, both methods reached 100% specificity in 15 healthy donors, but *CellSearch* detected more samples with >1 CTC in a group of 61 heterogeneous carcinoma patients (14 vs. 33 positive samples). The mean number of CTCs per sample was also higher using the CellSearch technique[105].

A technologically advanced and novel method to isolate CTCs is the '*CTC-chip*'. This chip consists of 78,000 microposts, each coated with EpCAM antibodies. As whole blood is pumped across the chip under controlled flow conditions, EpCAM-positive cells bind to the microposts, which are then detected by a camera based on their morphology, viability and tumor markers. This system uses CKs and DAPI for positive selection together with CD45 for negative selection[20,106]. The micropost system should prevent trapping of EpCAM positive cells among leukocytes. According to the developers, sensitivity was remarkably high with this method, as CTCs were detected in every patient, including those with localized disease[20].

Another new approach is the epithelial immunospot (*EPISPOT*) assay, an immunological assay based on the enzyme-linked immunosorbent assay (ELISPOT). The assay is preceded by immunomagnetic depletion of $CD45^+$-cells and enrichment for $CXCR4^+$-cells (a chemokine receptor involved in the homing of metastatic tumor cells[107]). *EPISPOT* detects specific proteins released by breast cancer CTCs, such as cathepsin-D (a cysteine protease) or Mucin-1, thus counting only viable, protein-excreting cells[108-109]. In theory, viable cells have more clinical relevance than apoptotic cells as they should still be capable of forming metastases. In a first study on breast cancer CTC detection, this assay was performed using Mucin-1 and CK19 as markers, showing high sensitivity and specificity[108].

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

MARKERS

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

CLINICAL APPLICATIONS OF CTC DETECTION

The presence of occult metastases cannot be deduced from the finding of CTCs alone, as CTCs must pass through several stages before forming a metastatic colony. Cells must extravasate from the circulation into target organs and subsequently proliferate whilst evading immunological response and overcoming metabolic difficulties. It has been estimated that only one in 10,000 CTCs is able to form a metastasis[110].

Despite all this, the clinical usefulness of CTC detection has been demonstrated in metastatic breast cancer[66,111], metastatic colorectal[29,112] and metastatic prostate cancer[31]. In addition, CTCs have been studied in pancreatic[113], gastric[114-117], bladder[118-121] and lung cancer[14,122-123], among others, with variable results. Of all tumor types, breast cancer is the tumor type in which CTCs have most strongly proven their value, and in which the largest variety of techniques has been applied.

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

CTC DETECTION IN LOCALIZED BREAST CANCER

Neoadjuvant setting

For patients presenting with locally advanced breast cancer, i.e., tumors presenting with extensive regional lymph node involvement, skin involvement or a large size (>5 cm), resection of the primary tumor is frequently either not possible or only at the cost of an amputation of the breast. Systemic therapy given prior to management of the primary tumor, also known as neoadjuvant therapy, aims to reduce tumor size thereby rendering the residual tumor amendable for a breast-conserving resection. In addition, neoadjuvant systemic therapy aims to eradicate micrometastases, which may otherwise have resulted in incurable, overt metastatic disease later on. Until now, the value of CTC detection in the neoadjuvant setting has not been extensively studied. Recently, CTCs were detected before and/or after neoadjuvant chemotherapy with *CellSearch* in 118 patients included in a phase II trial[34]. In 23% of the patients, one or more CTC per 7.5 mL blood was detected before the administration of neoadjuvant chemotherapy, while 17% had >1 CTC per 7,5 mL blood after neoadjuvant chemotherapy. The persistence of CTCs during neoadjuvant chemotherapy was not correlated with treatment response, but the presence of CTCs either at baseline or after neoadjuvant chemotherapy was an independent prognostic factor for distant metastasis-free survival[34]. In another study, CTCs were monitored by *Laser Scanning Cytometer (LSC)* before each of 3 therapy cycles in 30 patients[124]. CTCs were detected in all patients prior to the start of therapy, but the decrease in number of CTCs for different patients varied up to several hundred-fold. A strong correlation was however shown between a reduction in the number of CTCs and a favorable pathological response at surgery[124]. This correlation suggests that CTCs may serve as an early marker to assess response to neoadjuvant therapy. However, the remarkable high CTC detection rate in these primary breast cancer patients, as well as in another 30 patients treated in the adjuvant setting using the same technique as discussed below, has been questioned[125]. It was suggested that further characterization is needed to confirm that the cells assigned as CTC using this technique are tumor cells indeed. The authors explained their findings by stating that the lack of enrichment in their method accounts for less cell loss and, consequently, the high CTC counts[125].

Adjuvant setting

Adjuvant chemotherapy refers to systemic therapy after primary surgery for early stage breast cancer patients who are considered to have a high risk for metastatic disease developing from micrometastases that are already present at initial presentation. The intent of adjuvant therapy is to cure patients by eradicating these micrometastases. Currently, it is not possible to adequately identify patients who do not harbor micrometastases and therefore should

be spared from adjuvant therapy and the accompanying toxicities. On the other end of the spectrum, 20 - 30% of patients treated with adjuvant therapy will develop overt metastasis, in spite of the adjuvant therapy[126]. Detecting this population not cured by the administered adjuvant therapy could open the door for additional treatments with new drugs. Several studies have recently been conducted to establish whether or not CTC detection and enumeration may guide treatment in this setting.

In a study of 91 clinically non-metastatic primary breast cancer patients, CTCs were quantified by *LSC* before adjuvant therapy, before each new cycle and at the end of the chemotherapy[127]. There were 3 distinct patterns of response: 28 patients showed a decrease in cell number of 10-fold or more, 30 patients showed changes less than 10-fold in cell number and 33 patients had an increase of more than 10-fold. The pattern of CTC counts during therapy correlated significantly with relapse, and in multivariate analysis, an increasing CTC count of 10-fold or more at the end of therapy was associated with shorter relapse-free survival[127]. If confirmed, this group of patients may be candidate for additional therapy. As in another study of the same group as discussed before[124], the remarkably high rate of CTC positivity in the current study[127] has been subject of discussion[125].

The prognostic relevance of the detection of CTCs with *RT-PCR* has recently been demonstrated in 444 early-stage breast cancer patients[128]. After a median follow-up of 53.5 months, patients with *CK19* mRNA-positive CTCs experienced significantly reduced disease-free survival (DFS) and OS compared to those without CTCs. In multivariate analysis as well, the detection of CTCs was associated with decreased DFS and OS[128]. It should be noted, as discussed before, that using only *CK19* as a marker does raise concerns about specificity[16].

As *HER2* has become an important target for therapy since the introduction of trastuzumab (Herceptin®), the determination of *HER2* expression on CTCs has caught the interest of research groups. Apostolaki et al. showed that the detection of *HER2* mRNA-positive cells with *RT-PCR* after the administration of adjuvant chemotherapy was correlated with shorter disease-free interval (DFI) in 214 stage I and stage II breast cancer patients[21]. However, this prognostic value of *HER2* on CTCs could not be reproduced in a more recent multimarker *qRT-PCR* based study[59]. Simultaneously studying mammaglobin A (*MGB1*), *HER2* and *CK19* in 175 patients with stage I-II breast cancer after primary surgery and before adjuvant therapy, marked heterogeneity was seen in the CTC phenotypes inter- and intra-individually. In multivariate analysis, *CK19* mRNA+ and *MGB1* mRNA+ cells were independent adverse prognostic factors, whereas *HER2* mRNA+ cells were not[59]. This apparent difference in significance of *HER2* might be due to the fact that the presence of *HER2* mRNA+ cells after adjuvant therapy, which was only determined in the first study, could reflect resistance to chemotherapy and therefore be stronger associated with prognosis.

In a single-marker assay, using *CK7* as a marker for *qRT-PCR*, CTCs were detected in 37 of 206 primary breast cancer patients[129]. Ninety-eight patients were followed up 24 months after primary surgery. Of those, the *CK7*-negative group showed significantly longer DFS than the *CK7*-positives. This difference was even more profound in 61 lymph node-negative patients observed over 24 months after surgery. This suggests that *CK7+* CTCs are a prognostic marker for early recurrence after primary surgery[129].

Employing the *CellSearch* technique, a large trial is currently being conducted to assess the value of CTCs in the adjuvant setting[130]. This SUCCESS trial has enrolled 1767 primary breast cancer patients. Preliminary results show detection of >1 CTCs per 7.5 mL blood with *CellSearch* in 10% of 1500 patients before the start of systemic therapy. Of those, 10% remained positive after chemotherapy. Persistence of CTCs after chemotherapy was associated with decreased PFS and OS. As follow-up of this trial is ongoing, the prognostic value of these promising data cannot yet be determined.

Oncoquick was combined with *immunocytochemical* staining with anti-cytokeratin, CD45 and Ki-67, the latter being a proliferation marker, to detect CTCs in 60 primary and 63 metastatic breast cancer patients[131] CTCs were detected in 8.3% of primary and 39.7% of metastatic breast cancer patients, but this didn't correlate with prognosis or tumor characteristics. Remarkably, in a subset of 47 randomly chosen patients, none of the 9 CTC-positive patients expressed Ki-67 on their CTCs, suggesting that CTCs are at the very least rarely proliferative.

Studying 341 primary breast cancer patients included within 3 years after primary surgery, CTCs could be detected by *immunocytochemical* staining with anti-cytokeratin following enrichment with Ficoll and immunomagnetic CD45-depletion in 10% of the patients[132]. While the presence of CTCs was correlated with DFS and breast cancer specific survival in the whole group of patients, when 23 patients who had had a breast cancer-related event prior to the collection of peripheral blood were excluded, CTC detection no longer correlated with DFS.

CTC DETECTION IN METASTATIC BREAST CANCER

In metastatic disease, the intention of treatment is essentially palliative, striving to optimize quality rather than duration of life. Assessing prognosis in patients with metastatic breast cancer with CTCs can be helpful in the individualized management of these patients. In a multicenter, prospective study conducted by Cristofanilli et al[25], CTC count was assessed using *CellSearch* in 177 progressive metastatic breast cancer patients who were to start a new line of systemic therapy. CTCs were enumerated before the start of new treatment and at first follow-up visit. Patients with a level of CTCs before the start of treatment of ≥5 cells per 7.5 mL blood had a shorter median PFS (2.7 vs. 7.0 months) and shorter OS (10.1 vs.

>18 months). Maybe even more interestingly, patients with initially elevated CTC levels at baseline that had declined below 5 cells per 7.5 mL blood at first follow-up after the first administration of therapy had a PFS and OS similar to the patients with low levels of CTCs at baseline and follow up. These results indicate the potential role of CTCs as a prognostic marker, and as a marker establishing at an early stage whether a patient benefits from anti-tumor therapy[133]. Recently, the patients in this cohort whose CTC levels were determined at the time of newly diagnosed recurrent or de novo metastatic disease, and therefore being treatment-naïve, were analyzed retrospectively[134]. Also in these patients, it could be confirmed that a CTC level of ≥5 was an independent prognostic factor for death (HR 3.64), and median OS was 28.3 vs. 15 months in patients with CTCs <5 vs.≥5.

Even more so than in localized disease, in metastatic breast cancer the clinician should carefully evaluate the effects of chemotherapy taking into account its side-effects. It is important to assess response or lack thereof as early as possible in order to avoid exposure of the patient to unnecessary toxicity. The current methods to evaluate this response, namely clinical presentation and radiographic imaging, are suboptimal, as they are often only helpful late in the disease process. Ideally, CTCs could predict response to therapy after one or two cycles of therapy, and do so more reliable than traditional parameters. Budd et al[32] compared the prognostic value of the presence of ≥5 CTCs per 7.5 mL blood with radiologic response on OS in 138 metastatic breast cancer patients in the same cohort as Cristofanilli et al[25]. Radiologic evaluation was conducted 10 weeks after initiation of therapy, CTC counts were determined with the *CellSearch* system 4 weeks after initiation of therapy. CTC determination showed lower inter-reader variability than radiologic evaluation (0.7% vs. 15.2%, respectively). In patients who were non-progressive according to radiological evaluation, the median OS was significantly shorter for patients with ≥5 CTCs than for patients with <5 CTCs per 7.5 mL blood (15.3 versus 26.9 months). In patients with radiological progressive disease, a similar significant difference in median OS was objectified in patients with ≥5 CTCs versus patients with <5 CTCs per 7.5 mL blood (6.4 versus 19.9 months)[32]. This study strongly suggests that CTCs are a good tool to evaluate tumor response to therapy, and in fact better than radiologic evaluation.

The study of multiple rather than single markers to increase sensitivity of CTC detection assays, studying multiple markers seems promising. Combining *CK19, p1B, PS2* and *EGP2*, CTCs were detected by *qRT-PCR* in 94 metastatic breast cancer patients. After combining the four expression levels into a single discriminant value, a positive value correlated with a significantly worse PFS and OS[111].

DISCUSSION

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

Studies have shown remarkably varying CTC counts, ranging from <5 to thousands per mL in the same patient category. Whether these differences are caused by cancer biology or varying sensitivity of the techniques used, awaits clarification. Limited studies have been performed comparing the sensitivity and specificity of the different detection techniques. The ultimate goal is to set up an assay that generates inter-individually interpretable results for each individual patient, underlining the necessity for consensus on the exact technique for enrichment and detection that should be applied and on the markers that should be used. Given the heterogeneity between breast cancer subtypes, it is anticipated that assays cannot rely on a single, universally expressed and specific marker. Therefore, efforts should be made to develop a marker set, as multiple markers probably do more justice to the heterogeneity of breast cancer. EpCAM seems to insufficiently detect some molecular subtypes of breast cancer[64] prompting the need for a combination of cellular markers by which all breast cancer subtypes can be detected.

Despite these challenges, enumeration of CTCs has shown to bear prognostic information, and may inform oncologists about response to systemic therapy shortly after its start. Building on this, it would be of great benefit if the increase or decrease in CTC count after the first cycle of systemic treatment would be a better guideline for continuation or switch of therapy than conventional (radiologic) evaluation after 2-3 cycles. Currently, a study is being conducted by the Southwest Oncology Group, measuring CTCs at baseline and after the first cycle of chemotherapy. Patients with ≥5 CTCs per 7.5 mL blood at baseline who remain at ≥5 CTCs per 7.5 mL blood after completing one course of chemotherapy are randomized to either continue their current chemotherapy or to switch to a different regimen. The results of this trial (www.clinicaltrials.gov search for NCT003820128[135]) could prove a big step forward in the clinical field.

Another interesting possibility in the CTC field is their molecular characterization. Klein et al. have applied comparative genomic hybridization and discovered remarkable heterogeneity between individual tumor cells in patients treated in the adjuvant setting over time as well as between patients[136]. In these patients, only a few *TP53* mutations were revealed. In patients treated in the metastatic setting, genetic heterogeneity over time was less marked, but *TP53* mutations were encountered more frequently[136]. Smirnov et al. were the first to generate

global gene expression profiles of CTCs from 3 cancer patients, resulting in 35 cancer- and CTC-specific genes[39]. The expression of these genes was subsequently confirmed by *qRT-PCR* in 74 metastatic breast cancer patients and 50 healthy donors, ultimately generating 16 genes (such as *AGR2* and *FABP1*), which may be useful to distinguish individuals without cancer from cancer patients, as well as distinguishing breast cancer from colon cancer and prostate cancer patients[39].

Molecular characterization of primary tumor tissue by gene expression profiling has shown to yield prognostic and predictive models in breast cancer[137-139]. The level of expression of various genes is determined, which results in a number of differentially expressed genes, which can classify patients into a poor-prognosis and good-prognosis group. Gene expression profiles have also shown a correlation with response to therapy. However it is likely that in the metastatic setting, molecular characterization of CTCs better represents tumor genetics than of primary tumors. Studies have shown that expression of clinically relevant markers such as ER, PR and *HER2* can differ between the primary tumor and its metastases[80,140]. For *HER2*, nearly one-third of patients whose primary tumor was *HER2* negative, had amplified *HER2* on CTCs[80]. Furthermore, tumor characteristics may change over time under pressure of therapy. In a preliminary report, 27% of patients with *HER2*-negative primary tumors acquired *HER2* overexpression during the course of chemotherapy[41]. Among 23 patients obtaining less than a pathologic complete response on neoadjuvant chemotherapy with concomitant trastuzumab, 7 (30.4%) had *HER2*-negative residual tumors at surgery[141]. It is likely that when the metastatic tumor cells gain or lose such important markers, treatment should change accordingly. Prospective studies should clarify whether it is indeed of benefit, for example, to start trastuzumab therapy when the CTCs of a prior *HER2*-negative primary tumor do express *HER2*. A predictive and prognostic gene expression model for CTCs could be of great help to the oncologist in making treatment decisions as the disease progresses. In addition, comparison of molecular profiles of primary tumors to CTCs may provide better insight into those mechanisms involved in dissemination. Tumor cells are thought to lose and/or gain specific gene expression as they evolve to enter the circulation and proliferate in a target organ. Identifying these changes in gene expression for each point in the evolution toward overt metastasis could improve our understanding of the metastatic cascade.

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

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

Table 1

Merits and demerits of CTC enrichment techniques

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

Table 2

Merits and demerits of CTC detection techniques

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

Table 3

Markers for enrichment and detection in cytometric techniques

	Techniques								
	ARIOL	**ICC**	**IF**	**Flow cytometry**	**ELSIPOT**	**CellSearch**	**FAST**	**CTC-chip**	**LSC**
Enrichment	IMS-CKs[90]	Density gradient[16,98,105,1]	IMS-EpCAM[146]	Density gradient[147]	IMS-CD45[109]	EpCAM[79,90,105,148-149]			Density gradient[75]
	IMS-EpCAM[90]	IMS-CKs[142,150-151]		IMS-CKs[152]	IMS-CXCR4[109]				IMS-EpCAM[75]
		IMS-EpCAM[140,153-154]		IMS-EpCAM[155]					
		IMS-CD45[145]							
Detection	CKs[90]	CKs[16,98,105,131,140,142-145,150-151,153-]	CKs[146]	CKs[147,152,155]	Cath-D [109]	CKs[79,90,105,148]	CKs[87]	EpCAM[20][20]	EpCAM[14,89,124]
		EpCAM[105]	HER2[146]		MUC1 [109]	IGF-IR[149]			CKs[75]
		HER2[140,142]	uPAR[146]						

ICC; Immunocytochemistry, *IF*; Immunofluorescence, *FAST*; Fiber-optic Array Scanning Technology, *LSC*; Laser Scan Cytometry, *IMS*; Immunomagnetic separation, *CKs*; Cytokeratins, *EpCAM*; epithelial cell adhesion molecule, *HER2*; c-erbB-2, *uPAR*; Plasminogen activator receptor, *Cath-D*; Cathespin-D, *MUC1*; Mucin 1, *IGF-IR*; Insulin-like Growth Factor I Receptor

Table 4

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

	Techniques			
	RT-PCR		**qRT-PCR**	
Enrichment	Density gradient[23,51,143,156-165]		Density gradient[24,98,166-168]	
	Adnatest[140]		Adnatest[169]	
			IMS-EpCAM[170]	
Detection	*ß-HCG*[58,159,171-172]	*HER2* [140,173-175]	*ANKRD30A*[176-177]	*Mammaglobin* [75,168-169,176-183]
	c-MET[58,184-185]	*MAGE-A3*[58-59,186-187]	*B305D*[176-177]	*Maspin*[179,182,187-188]
	CD44[189]	*Mammaglobin*[51,160,165,172,178,185,187,190-193]	*Bmi-1*[129,194]	*MUC1*[111,170,180-181]
	CEA[188,190,194-197]	*Maspin*[23,169,188,190,194,198]	*CEA*[180]	*MUCL1*[183]
	CK7[167,185,199-200]	*MUC1*[140,163,189-190,195-197]	*CK7*[98,111,166-167]	*p1B*[111]
	CK19[16,51,143,156-158,161,164,171-172,178,182,188-190,195-198,201-204]	*PTHrP*[163,205]	*CK19* [24,75,98,111,177-178,180-181,183,199,206-210]	*PIP*[75,180-181]
	CK20[162,171,190,195,202]	*Survivin*[211]	*EGFR*[178]	*Secretoglobin*[180-181,183,187]
	EGFR[169,178,190,202,212-213]	*Telomerase*[214]	*EGP2*[169]	*SPDEF*[181]
	EpCAM[58,195]		*EpCAM*[24,169,206]	*TTF1*[24,206,215]
	GalNAc-T[58-59,140,169]		*GABRP*[176-177]	*TTF3*[24,206]
			HER2[179-180,206,216]	*uPAR*[217]

ANKRD30A; Ankyrin repeat domain 30A, *B305D*; antigen B305D, *ß-HCG*; Chorionic gonadotrophin, *Bmi-1*; B lymphoma Mo-MLV insertion region 1 homolog, *c-MET*; Proto-oncogene met, *CEA*; Carcino-embryonic antigen, CK; Cytokeratin, *EGFR*; Epidermal growth factor receptor, *EGP2*; Epithelial glycoprotein 2, *EpCAM*; epithelial cell adhesion molecule, *GABRP*; GABA A receptor pi, *GalNAc-T*; UDP-N-acetyl-D-galactosamine, *HER2*; c-erbB-2, *IMS*; immunomagnetic separation, *MAGE-A3*; Melanoma antigen family A subtype 3, *MUC1*; Mucin 1, *MUCL2*; Mucin-like 2, *PIP*; Prolactin-induced protein, *PTHrP*; Parathyroid Hormone Receptor Protein, *SPDEF*; SAM pointed domain containing ets transcription factor, *TTF1*; Trefoil factor 1,*TTF3*; Trefoil factor 3, *uPAR*; Plasminogen activator receptor

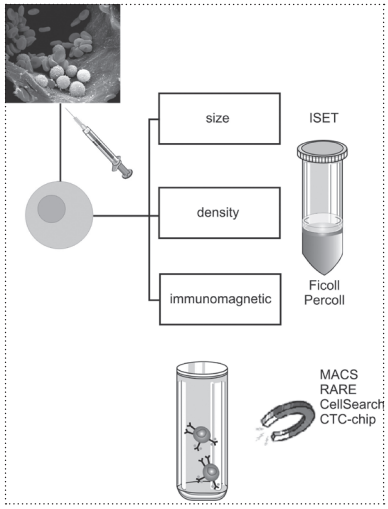

Figure 1: see section 'Color figures'

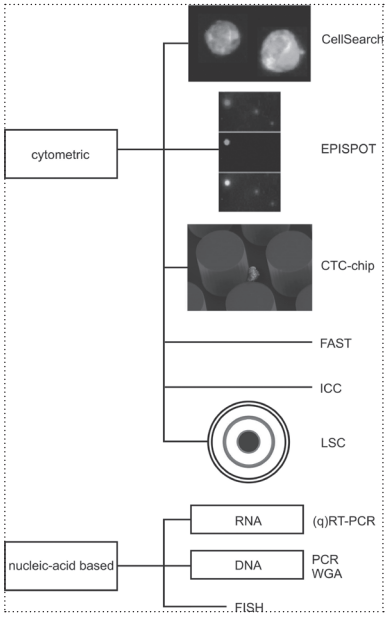

Figure 2: see section 'Color figures'

Chapter 3

Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146

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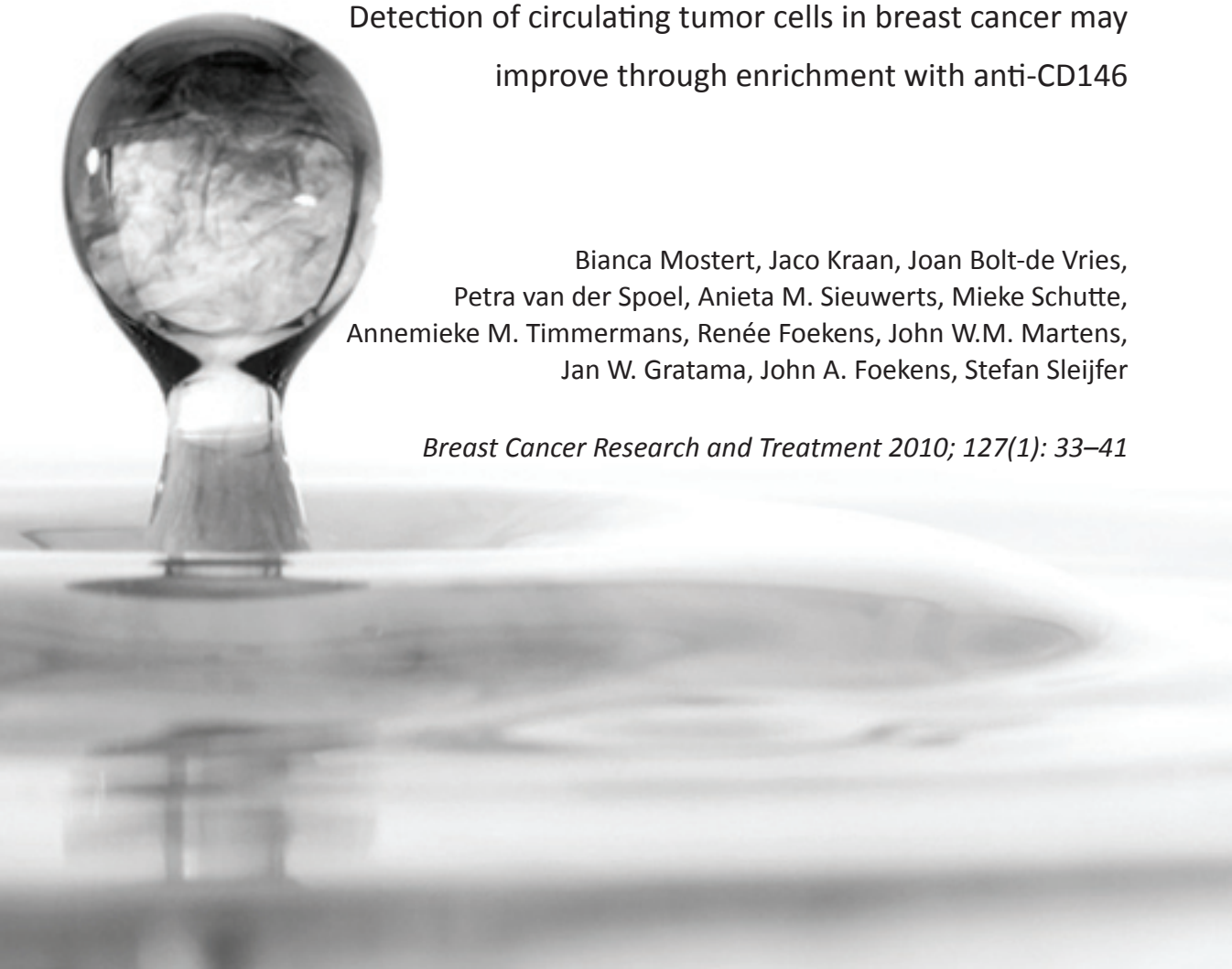


ABSTRACT

Most assays to detect circulating tumor cells (CTCs) rely on EpCAM expression on tumor cells. Recently, our group reported that in contrast to other molecular breast cancer subtypes, "normal-like" cell lines lack EpCAM expression and are thus missed when CTCs are captured with EpCAM-based technology[64]. Here, the use of CD146 is introduced to detect EpCAM-negative CTCs, thereby improving CTC detection.

CD146 and EpCAM expression were assessed in our panel of 41 breast cancer cell lines. Cells from 14 cell lines, 9 of which normal-like, were spiked into healthy donor blood. Using CellSearch® technology, 7.5 mL whole blood was enriched for CTCs by adding ferrofluids loaded with antibodies against EpCAM and/or CD146 followed by staining for Cytokeratin and DAPI. Hematopoietic cells and circulating endothelial cells (CECs) were counterstained with CD45 and CD34, respectively. A similar approach was applied for blood samples of 20 advanced breast cancer patients.

Eight of 9 normal-like breast cancer cell lines lacked EpCAM expression but did express CD146. Five of these 8 could be adequately recovered by anti-CD146 ferrofluids. Of 20 advanced breast cancer patients whose CTCs were enumerated with anti-EpCAM and anti-CD146 ferrofluids, 9 had CD146+ CTCs.

Cells from breast cancer cell lines that lack EpCAM expression frequently express CD146 and can be recovered by anti-CD146 ferrofluids. CD146+ CTCs are present in the peripheral blood of breast cancer patients with advanced disease. Combined use of anti-CD146 and anti-EpCAM is likely to improve CTC detection in breast cancer patients.

INTRODUCTION

The enumeration of circulating tumor cells (CTCs) in the peripheral blood of cancer patients is a promising tool to detect and monitor cancer earlier and less invasively than conventional methods[218-219]. In recent years, several assays to detect CTCs have been introduced[219]. CTCs measured with the FDA-approved CellSearch technique (Veridex™, Raritan, NJ) have prognostic value in metastatic breast cancer[25-26] and changes in CTC counts during systemic therapy can serve as early marker for response[25-26,35,67].

Like most CTC detection assays in breast cancer, the CellSearch technique relies on EpCAM (epithelial cell adhesion molecule, CD326) expression on tumor cells. However, despite its robustness and proven clinical relevance, CellSearch identifies CTCs in only 60% of metastatic breast cancer patients[25]. There are two possible explanations for this; CTCs are simply not present, or they cannot be detected by this assay.

In breast cancer, five different molecular subtypes have been identified[220]. Recently, our group reported that in contrast to the other molecular subtypes, most normal-like breast cancer cell lines lack EpCAM expression and are missed using CellSearch technology[64]. Normal-like breast cancer accounted for 7.8% of all breast cancers in 344 breast cancer samples[221] thus forming a substantial breast cancer subgroup. In accordance with our preclinical findings[222], Spizzo *et al.*, showed that 10.3% of 1715 invasive breast cancer patient samples lacked EpCAM expression[223].

Using the lack of EpCAM expression on a subset of breast cancer cells as a model for EpCAM-negative CTCs, we embarked on a search for additional markers, ultimately aiming to improve CTC detection in all breast cancer patients, irrespective of the molecular subtype of their primary tumor. As a first attempt, CD146 was our marker of choice, based on our gene expression data[64], availability of anti-CD146 ferrofluids, and a recent study by Zabouo et al[224,] who showed that CD146 is expressed in a subset of primary breast cancers and is correlated with poor prognosis.

METHODS

Breast cancer cell lines

In order to identify an additional marker expressed on EpCAM-negative breast cancer cells, we used our well-defined panel of 41 human breast cancer cell lines[225-226]. The intrinsic subtype of these cell lines has been determined by gene expression profiling previously[64].

CD146 mRNA expression levels

The transcript levels of the 41 cell lines were determined with Affymetrix GeneChip Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wooburn Green, UK) as described previously[226]. Additionally, expression of *CD146* was determined by real-time reverse transcriptase PCR. RNA was isolated from breast cancer cell lines with the RNeasy (Micro) kit (Qiagen, Valencia, CA). cDNA was prepared with the Superscript II RNase H-kit (Invitrogen, Carlsbad, CA). The resulting cDNA preparations were analyzed by real-time PCR in a 20 µL reaction volume in a MX3000P™ Real-Time PCR System (Agilent/Stratagene, Amsterdam, The Netherlands), using TaqMan™ Gene Expression Assays in combination with TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Levels of hydroxymethylbilane (*HMBS*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and glucuronidase, beta (*GUSB*) were used to control sample loading and RNA quality.

CD146 protein expression levels

Cells from cultured human breast cancer cell lines were incubated with fluorochrome-conjugated monoclonal antibodies and presence of antigens was assessed as described before[64]. In brief, cells were incubated with CD34 conjugated with FITC (clone 8G12; BD Biosciences, San Jose, CA), CD146 conjugated with PE (clone P1H12; BD Biosciences) and anti-EpCAM conjugated with FITC (clone EBA-1; BD Biosciences). Cells were then analyzed on a FACSCanto flow cytometer (BD Biosciences). Unstained cells were used as a negative control.
Tissue microarrays (TMAs) were prepared in duplicate from blocks of 37 formalin-fixed, paraffin-embedded (FFPE) breast cancer cell line cells that were cultured to near confluence in complete growth medium, and from blocks of 206 FFPE primary breast cancer specimens. These blocks and 23 FFPE normal-like primary breast cancer specimens were sectioned at 4 µm, mounted on StarFrost slides (Waldemar Knittel–GmbH, Braunschweig, Germany), dried, deparaffinized in xylene and rehydrated in graded solutions of ethanol and distilled water. Specimens were pretreated with retrieval buffer (DAKO, Glostrup, Denmark) at pH 6.0 for CD146 and pH 9.0 for CD34 for 40 min at 95-99°C in a water bath, cooled to room temperature for 20 min and rinsed in phosphate-buffered NaCL solution (PBS). This was followed by a 10-min blocking step with a 0.3% peroxide PBS solution and a 30-min blocking step with a 5% bovine serum albumin PBS solution. Slides were stained with monoclonal antibodies against CD146 (Abcam, Cambridge, UK, N1238) and CD34 (Neomarkers, Fremont, CA, clone QBE) and counterstained with the peroxidase-conjugated Envision technique (DAKO EnVision™+ System, HRP). Each core was scored by a well-trained technician and positive staining was recorded as 100%, 50 - 99%, 25 - 50%, 10 - 25%, 0 - 10% or 0% number of positive cells.

Enumeration of epithelial cells spiked in whole blood

Blood samples containing EDTA (7.5 mL aliquots of blood) from a single healthy male donor were obtained from CellSave Preservative Tubes (Veridex). To each sample, a predefined amount (50 - 500) of cultured human breast cancer cells was added. To determine the actual viable cell number, a 100-µL aliquot of the cultured cells was incubated with 10 µL of 7AAD (Sigma-Aldrich, St. Louis, MO) and 100 µL of fluorescent beads (Beckman-Coulter, Miami, FL). After 15 min incubation at room temperature, 2 mL PBS was added, and samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences). At least 10,000 beads were acquired to estimate the number of 7AAD-negative (viable) cells. The efficiency of retrieving the tumor cells was controlled by counting the number of viable cells that were drawn in triplicate by light microscopy after serial dilution.

To establish the number of recovered CTCs following spiking into blood from a healthy donor, samples were processed on the CellTracks AutoPrep analyzer (Veridex) with the CellSearch Epithelial Cell kit (Veridex). Briefly, ferrofluids coated with antibodies directed towards EpCAM were added to a blood sample, and CTCs bound to anti-EpCAM coupled ferrofluid were isolated from whole blood by magnets. Unbound cells and the remaining plasma were aspirated, followed by staining of the isolated cells with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI), anti-cytokeratin 8, 18 and 19 antibodies labeled with PE and CD45 antibodies labeled with APC. After incubation, CTCs were separated magnetically once more and unbound staining reagents removed. Finally, the cells were resuspended in a MagNest Cell Presentation Device (Veridex). In this device, labeled cells are oriented by two magnets for analysis in the CellSpotter Analyzer (Veridex) after which the number of CTCs are enumerated according to the manufacturer's instructions.

For the detection of cells expressing CD146, anti-CD146 ferrofluid from the CellSearch Circulating Endothelial Cell kit (Veridex) was used in a volume of 150 µL per sample plus an excess volume of 300 µL. The sample was enriched for CD146-positive cells and further characterized by staining for the presence or absence of DAPI, cytokeratin 8, 18 or 19 and CD45 as provided in the CellSearch Epithelial Cell kit. As CD146 enriches for circulating endothelial cells (CECs)[227], and CECs can express cytokeratin 18[228], a marker to exclude the cytokeratin 18-expressing subset of CECs was needed. As CD34 is a pan-endothelial marker[229], CD34 conjugated with FITC (clone 8G12; BD Biosciences) was added to the CellSearch Epithelial Cell kit, in a volume of 150 µL per sample plus an excess volume of 300 µL. The number of CTCs was determined on the CellSpotter Analyzer (Veridex) according to the manufacturer's instructions. CD146+ CTCs were defined as DAPI+/CK+/CD45-/CD34-.

For combined anti-EpCAM and anti-CD146 enrichment, anti-EpCAM ferrofluids from the CellSearch Circulating Epithelial Cell kit and anti-CD146 ferrofluids from the CellSearch

Circulating Endothelial Cell kit were combined in even parts to a total volume of 150 µL per sample plus an excess volume of 300 µL. The number of CTCs in samples enriched for CD146-expressing and EpCAM-expressing events was determined and defined as mentioned above.

The pivotal differences between the classic CellSearch CTC enumeration assay and the new combined anti-EpCAM/anti-CD146 assay are highlighted in **Figure 1**.

Blood samples

Blood samples were obtained from a laboratory volunteer and patients with metastatic breast cancer seen at the outpatient clinic. For each patient, 22.5 mL of blood was drawn into CellSave tubes and CTCs were enumerated after enrichment using anti-EpCAM, anti-CD146 and mixed anti-EpCAM/anti-CD146 ferrofluids, using 7.5 mL of blood for each enrichment method.

Additionally, 10 healthy female donors (age 23 - 56) were tested for CD146+ events by enumerating CTCs in 7.5 mL blood after enrichment with anti-CD146 ferrofluids.

This study was approved by the Erasmus MC Institutional Review Board (METC protocol 2006-248), and all donors and patients gave their written informed consent.

RESULTS

Breast cancer cell lines

As previously reported, our cell line panel consists of 10 normal-like, 5 basal-like, 5 erbb2 and 21 luminal breast cancers as determined by gene expression profiling according to the intrinsic subtypes of Perou and Sorlie[64,226]. As the MDA-MB-435 normal-like cell line has been the subject of debate in literature recently with doubts about its origin[230-232], we excluded this cell line from our experiments.

Candidate markers

We have previously shown that 8 out of these 9 cell lines lack EpCAM-expression[64]. Using these normal-like cell lines as a model for non-EpCAM expressing breast cancer, several candidate markers for the assessment of EpCAM-negative CTCs were identified by comparing gene expression data between molecular subtypes. Candidate markers required membrane expression of the proteins on the EpCAM-negative breast cancer cells and absence on hematopoietic cells, or, if present, hematopoietic cells expressing the marker should be easily excluded using additional markers. Several markers fulfilled these criteria, amongst which *CAV1*[64], *MUC1*[64] and *MCAM* (CD146). Given the availability of ferrofluids coated with CD146

antibodies[233], and the possibility to exclude interference of expression by hematopoietic cells (by CD45-counterstaining) and CECs (by CD34-counterstaining), we chose to further explore this marker.

CD146 and EpCAM expression

Based on Affymetrix micro-array and qRT-PCR data, 7 out of the 9 normal-like cell lines expressed high levels of *CD146* mRNA. In addition, one basal-like cell line, SUM149PT, expressed *CD146*, resulting in a total of 8 cell lines expressing *CD146* mRNA (**Table 1**).

As previously reported[64], we confirmed that cells from only 1 of the 9 normal-like cell lines (MDA-MB-157) expressed EpCAM (**Table 1** and **Figure 2a**). Evaluation of CD146 expression by flow cytometry showed that 8 of the 9 normal-like cell lines had CD146 membrane expression at a level likely to be detected using CellSearch technology (i.e., a signal to noise ratio >5) (**Table 1** and **Figure 2a**). Remarkably, cells from an early passage of the MDA-MB-157 cell line were CD146–positive, while cells from a later passage showed a CD146-positive and a CD146-negative population. In addition to these normal-like cell lines, two of 5 basal-like cell lines (SUM149 and SUM229) expressed both CD146 and EpCAM (**Table 1** and **Figure 2b**).

To obtain an overview of CD146 expression across all different breast cancer subtypes, CD146 expression was determined by immunohistochemistry on a TMA of 37 breast cancer cell lines. A total of 17 cell lines stained positive for CD146, among which 8 were normal-likes, 2 basal-like, 4 luminal and 3 erbb2 cell lines. Most cell lines staining positive for CD146 showed a heterogeneous expression pattern (**Figure 3a**).

CD146 staining was also performed in a set of 23 normal-like primary breast tumors, 7 of which stained positive for CD146 on epithelial cells (**Figure 3b**).

CD34 to distinguish CTCs from CECs

The expression of CD146 on activated T-lymphocytes[234] and CECs[235] might interfere with the detection of CD146-positive CTCs. While leukocytes can be excluded with CD45, which is already incorporated in the standard CellSearch Circulating Epithelial Cell kit, we assessed whether CECs could be excluded with CD34, a pan-endothelial marker[227,229]. CD34 proved to be a suitable marker to distinguish CTCs from CECs, as none of the normal-like cell lines expressed CD34 as determined by flow cytometry (**Table 1**). In addition, CD34 staining was completely negative on a TMA containing 37 breast cancer cell lines (data not shown). CD34 staining on 206 primary breast tumors showed less than 10% CD34-positivity in only one tumor specimen, all other tumors were entirely CD34 negative.

EpCAM-negative breast cancer cells detected with anti-CD146 ferrofluids

To test whether EpCAM-negative breast cancer cells could be detected in healthy donor blood using CellSearch with anti-CD146 ferrofluids, a fixed number of cells from normal-like and basal-like cell lines was spiked into 7.5 mL healthy donor blood of the same donor (**Figure 4b**). As a control, a fixed number of luminal cells (CAMA-1) was spiked into 7.5 mL healthy donor blood (**Figure 4b**, bottom bar).

As previously reported[64], cells from 7 out of 9 normal-like cell lines could not be recovered with anti-EpCAM ferrofluids while cells from 1 of the other 2 normal-like cell lines (MDA-MB-231, **Figure 4b**) were partially detected (11%). Cells from the remaining cell line, MDA-MB-157, could be adequately recovered with anti-EpCAM ferrofluids, consistent with its EpCAM-expression. Of the 8 cell lines insufficiently recovered with anti-EpCAM, 5 could be detected with anti-CD146 (45 - 88% recovery). Additionally, the EpCAM-expressing cell line MDA-MB-157 was partially detected by anti-CD146 (11% recovery). After enrichment with mixed anti-EpCAM/anti-CD146 ferrofluids, cells from 6/9 normal-like cell lines were recovered at rates of 46 to 100%. Despite CD146 expression, cells from 3 normal-like cell lines could not be recovered (**Figure 4b**).

Cells of the luminal cell line CAMA-1 were detected with a recovery approaching 100% using anti-EpCAM ferrofluids. Enrichment with anti-CD146 ferrofluids alone did not result in recovery of CAMA-1 cells. The combined use of anti-CD146 and anti-EpCAM ferrofluids still enabled detection of all spiked CAMA-1 cells (**Figure 4b**), suggesting that mixing both ferrofluids does not greatly compromise enrichment. In accordance, 2 basal-like cell lines expressing EpCAM and CD146 could be detected with both anti-EpCAM and anti-CD146 ferrofluids as well as with a mixture of both (**Figure 4b**).

CD146+ cells in healthy donors

In all blood samples from 10 healthy female donors and a healthy male donor enriched with anti-CD146 ferrofluids, either in mixture or alone, a small number of CD146+/CK+/DAPI+/CD45-/CD34+ cells was identified (median 1, range 0 - 7), most likely accounting for a subset of CECs from the healthy donors[236]. In none of the healthy donors, CD146-expressing tumor cells, i.e., CD146+/CK+/DAPI+/CD34- cells, could be identified (data not shown).

CD146+ CTCs in breast cancer patients

To assess whether CD146-expressing CTCs do occur in breast cancer, CTCs were enumerated in 20 advanced breast cancer patients using anti-EpCAM, anti-CD146 and mixed ferrofluids. Of these 20 patients, 9 had CD146+ CTCs (**Table 2**). As expected, given the heterogeneity of breast cancer and the fact that some breast cancer cell lines express both EpCAM and CD146,

8 of these 9 CD146+ patients also had EpCAM+ CTCs. Enrichment with mixed ferrofluids yielded a higher CTC number compared to EpCAM enrichment alone in 8 patients (**Table 2**, patients 1, 2, 7, 9, 12. 13, 14 and 20). In six patients (**Table 2**, patients 4, 5, 15, 17, 18 and 19), the combined enrichment for CD146 and EpCAM resulted in a lower recovery of CTCs. In this small series of patients, CD146+ CTCs were present in patients with both hormone receptor positive and hormone receptor negative primary tumors.

DISCUSSION

CTC detection with the CellSearch assay has proven its value as a prognostic marker in metastatic breast cancer[25-26]. Additionally, a change in CTC numbers at first follow-up of therapy is associated with outcome[32,34-35,237]. Nevertheless, CTCs can be detected in only 60% of the metastatic breast cancer patients, suggesting that there is room for improvement in the CellSearch assay. As enrichment for EpCAM is the limiting first step in this and many other CTC enumeration assays, EpCAM expression on tumor cells is pivotal. However, evidence is accumulating that EpCAM is not a perfect marker for breast cancer CTCs[64,221-222].

In this study we demonstrated that CD146 is frequently present on EpCAM-negative breast cancer cell lines. CD146 is ubiquitously expressed on endothelial cells[238] and melanoma cells[239]. A correlation with poor prognosis has recently been found in breast cancer; of 635 primary breast tumors, 7% were CD146-positive, and CD146 expression was associated with poor prognosis[224]. Our finding that CD146 is present on EpCAM-negative breast cancer cell lines renders it a putative marker to detect EpCAM-negative CTCs. Expression of CD146 by other cells in blood such as CECs could hinder detection of CTCs. We found that the exclusion of CK+/DAPI+/CD45-/CD34+ events after anti-CD146 enrichment resulted in CTC-negative test results in healthy donors. Hematopoietic cells expressing CD146, such as activated T-lymphocytes[240] and NK-cells[241], can be identified according to their bright expression of CD45.

Using the definition of CD146+/CK+/DAPI+/CD45-/CD34- for CD146-positive CTCs we were able to detect cells from five out of eight normal-like cell lines when spiked in healthy blood. For the remaining three cell lines, for which detection is limited despite CD146 expression, the assay may need further improvement, for instance by adding an additional enrichment or positive selection marker, such as different cytokeratins.

In the clinical setting, both EpCAM-positive and EpCAM-negative tumor cells are likely to be present in the blood of patients, given the heterogeneity in terms of EpCAM expression within primary tumors[222]. As a consequence, one can imagine EpCAM-negative CTCs in patients with a primary tumor that is predominantly comprised of EpCAM-positive cells and

vice versa. Although we mostly observed CD146 expression in breast cancer cell lines with a normal-like subtype, the added value of CD146 is probably not limited to patients with normal-like primary tumors. CD146+ CTCs can be present in other patients too, as shown by the detection of CD146+ CTCs in patients with hormone receptor positive primary tumors, due to tumor heterogeneity or clonal selection during progression. Improved CTC detection in breast cancer using combined anti-CD146 and anti-EpCAM enrichment may be of value irrespective of the molecular subtype of the primary tumor.

To determine whether CD146-expressing CTCs are present in breast cancer patients, CTCs were captured with anti-EpCAM, anti-CD146 alone and a mixture of ferrofluids in twenty patients with advanced disease. Besides EpCAM-positive CTCs, CD146-positive CTCs were detected in 9 patients. In contrast, no CD146-expressing CTCs could be detected in healthy female controls. Although in a small series of patients, in line with the assumption that the combination of anti-EpCAM and anti-CD146 enrichment might improve CTC detection, a higher CTC count was found in 8 out of 20 patients when combining anti-EpCAM and anti-CD146 ferrofluids. However, a lower recovery was found in 6 patients using this approach. Whether this is due to technical reasons, such as suboptimal concentrations of the ferrofluids, should be evaluated before implementation into clinical studies. Importantly, the application of CD146 as an additional marker to detect CTCs is not restricted to the CellSearch assay alone, but may be useful for all assays depending on EpCAM-expression on the target cells.

Although based on a relatively small series of patients, the EpCAM-positive CTCs seem to outnumber the CD146-expressing CTCs. Nonetheless, even if CD146+ CTCs occur in only a small subset of patients, and may be of limited clinical relevance, minimizing false negative results is crucial for diagnostic purposes. Studies are being initiated to determine the exact incidence of CD146-positive CTCs in breast cancer patients and to reveal their clinical relevance.

In conclusion, we have shown that the majority of EpCAM-negative breast cancer cell lines can be detected using anti-CD146 ferrofluids. Furthermore, CD146-positive CTCs are present in breast cancer patients. It must be stressed that the clinical relevance of CD146-positive CTCs remains to be established, and whether the prognostic value of CD146+ CTCs is equivalent to that of EpCAM+ CTCs will be determined in a large clinical study. Nevertheless, the detection of CTCs, although already well established in metastatic breast cancer, can become even more relevant when detecting EpCAM-positive as well as CD146-positive CTCs.

Table 1

CD146 mRNA and CD146, CD34 and EpCAM protein expression in normal-like and basal-like breast cancer cell lines.

	EpCAM membrane expression	**CD146** mRNA expression	mRNA expression	membrane expression	membrane expression	**CD34** membrane expression
Cell line	FACS s/n[a]	Affymetrix[b]	qRT-PCR[c]	FACS s/n[a]	IHC	FACS s/n[a]
SUM102PT (n)	<5	569	0.6285	5-20	ND	<5
SK-BR-7 (n)	<5	708	0.2736	20-200	100	<5
MDA-MB-157 (n)	20-200	420	0.2300	5-20[d]	100	<5
Hs578T (n)	<5	235	0.1416	5-20	100	<5
SUM159PT (n)	<5	281	0.0526	20-200	100	<5
MDA-MB-231 (n)	<5	196	0.0238	20-200	100	<5
SUM1315MO2 (n)	<5	44	0.0315	20-200	<10	<5
MDA-MB-436 (n)	<5	65	0.0039	5-20	<10	<5
BT549 (n)	<5	59	0.0027	5-20	25-50	<5
SUM149PT (b)	5-20	301	0.0292	20-200	>50	<5
MDA-MB-468 (b)	20-200	60	0.0005	<5	<10	<5
BT20 (b)	20-200	53	0.0008	<5	0	<5
SUM229PE (b)	20-200	49	0.0063	20-200	0	<5
HCC1937 (b)	20-200	31	0.0010	<5	0	<5

[a]Data are values of one representative experiment performed in duplicate, s/n; signal-to-noise ratio, s/n > 5 considered detectable expression, [b]values >100 considered detectable expression; [c]qRT-PCR; quantitative real-time PCR, values >0.001, *i.e.,* requiring ≥10 cycles to pass the threshold compared with the number of cycles required for the reference gene set, considered detectable expression, IHC; immunohistochemistry, [d]late passage of cell line, ND; not determined, (n); normal-like subtype, (b); basal-like subtype

Table 2

CTC counts with anti-EpCAM, anti-CD146 and mixed anti-EpCAM/CD146 ferrofluids for 10 advanced breast cancer patients, and their primary tumor characteristics

	Primary tumor characteristics			CTC count		
Patient number	ER[a]	PR[b]	Her2	EpCAM+	CD146+	EpCAM/ CD146+
1	+	+	-	15	1	17
2	+	-	+	19	1	25
3	+	-	-	0	4	1
4	+	+	-	38	5	20
5	+	+	-	47	2	30
6	+	+	NA	2	1	1
7	-	+	-	28	5	40
8	+	NA	+	0	0	0
9	+	-	-	54	0	75
10	+	+	-	0	0	0
11	+	+	-	0	0	0
12	+	+	-	0	0	1
13	+	+	-	0	0	1
14	+	+	-	1	0	2
15	+	+	NA	2	0	1
16	-	-	NA	0	0	0
17	+	+	-	19	0	11
18	+	+	-	155	24	148
19	+	+	-	107	3	94
20	+	+	-	0	0	1

[a]ER; estrogen receptor, [b]PR; progesterone receptor, NA; not available

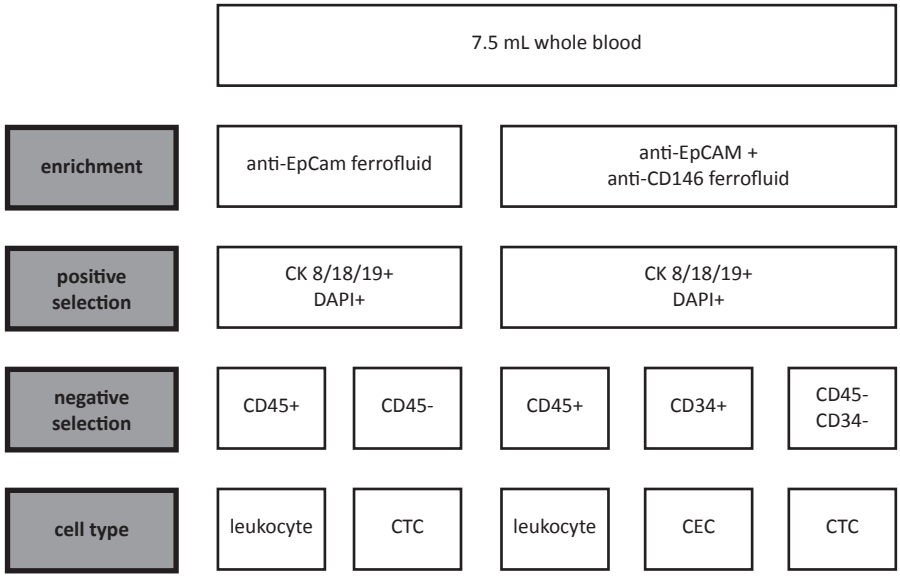


Figure 1

Setup of the CellSearch CTC enumeration assay (left column) versus the new anti-EpCAM/anti-CD146 assay (right column). Description of the makers used in each assay for enrichment, positive selection and negative selection, and the cell type associated with these selection criteria. CK; cytokeratin

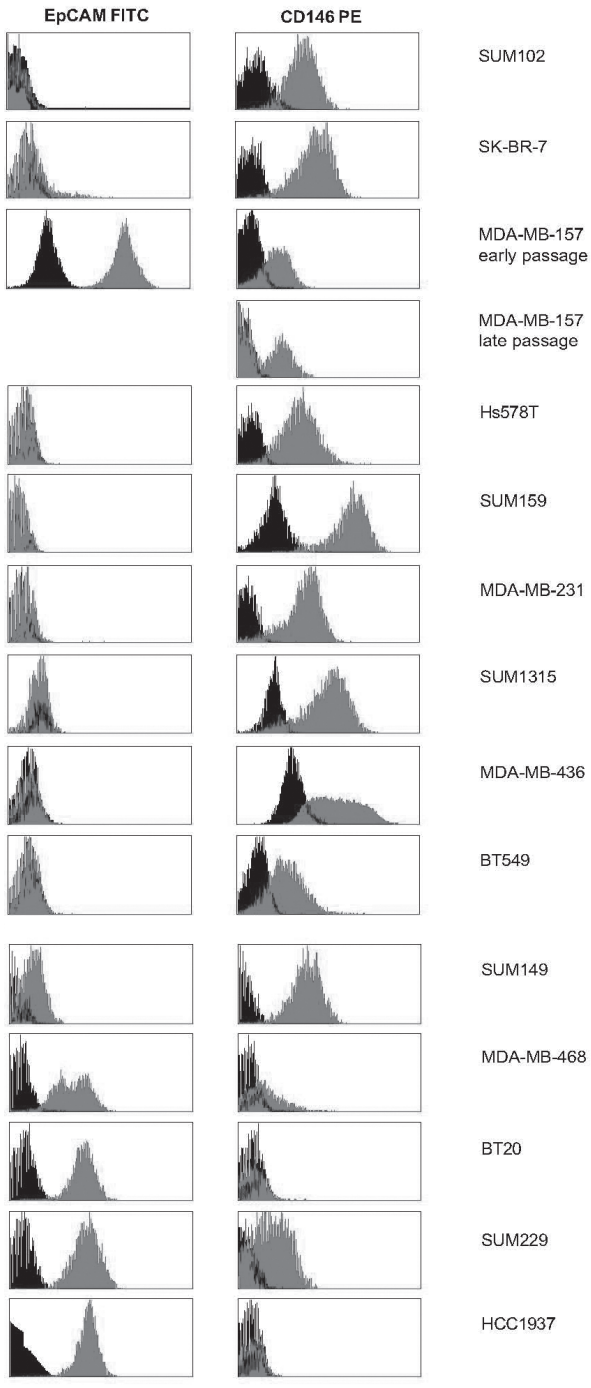


Figure 2

EpCAM and CD146 membrane expression as assessed by flowcytometry. Black histograms show unstained cells, grey histograms show expression on EpCAM-stained cells (left panel) and CD146 stained cells (right panel) of normal-like a and basal-like b cell lines. X-axis; fluorescence intensity; Y-axis; number of counted cells

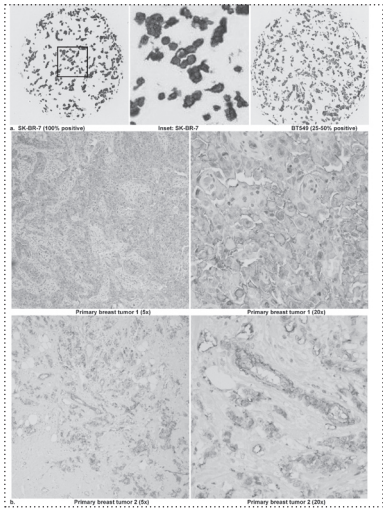
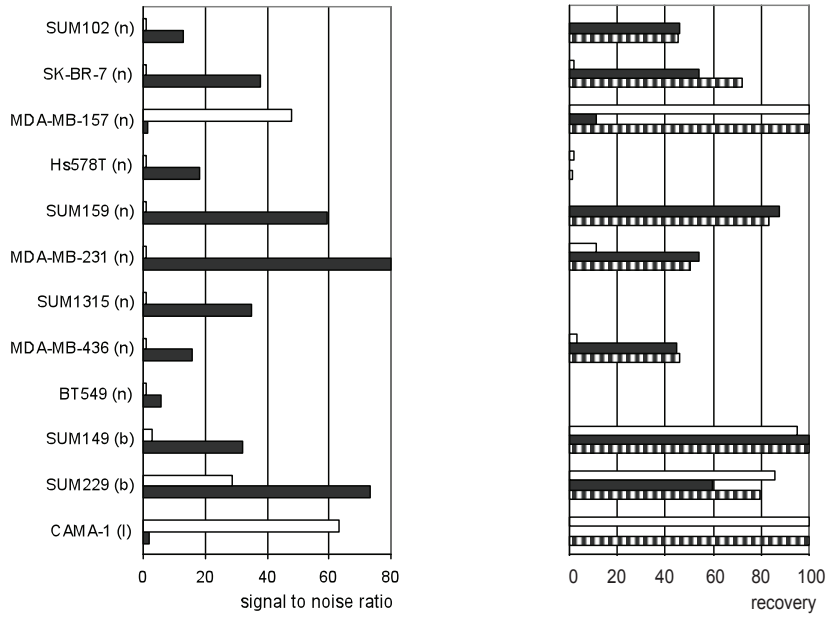


Figure 3: see section 'Color figures'



a. **b.**

Figure 4

EpCAM and CD146 membrane expression in normal-like n, basal b and luminal l cell lines a, and recovery of these cell lines b. a Open bar EpCAM membrane expression, closed bar CD146 membrane expression, s/n; signal-to-noise ratio, s/n >5 considered detectable expression. b Open bar recovery with anti-EpCAM ferrofluids, closed bar recovery with anti-CD146 ferrofluids, hatched bar recovery with anti-EpCAM/anti-CD146 ferrofluids

Chapter 4

CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer intrinsic subtypes

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ABSTRACT

Circulating tumor cells (CTCs) can be enumerated using CellSearch, but not all breast cancer subtypes, specifically those with epithelial-mesenchymal transition (EMT) characteristics, sufficiently express the enrichment (EpCAM) and selection (CK8/18/19) markers used in this method. While CD146 can detect EpCAM-negative CTCs, we here evaluated the value of various cytokeratins and CD49f to detect CK8/18/19-negative CTCs. The tested cytokeratins provided no substantial benefit, but adding CD49f to CK8/18/19 as a selection marker resulted in improved recovery of normal-like cell lines.

Combined staining of CK8/18/19 and CD49f after CD146/EpCAM enrichment is likely to further improve CTC detection in breast cancer.

INTRODUCTION

In recent years, numerous assays for the detection of circulating tumor cells (CTCs) have been described[219]. One of these is the CellSearch® technique (Veridex™ LCC, Raritan, NJ), the only FDA-approved method to detect CTCs based on their prognostic value in metastatic breast[25,] colorectal[29] and prostate cancer[31]. The CellSearch technique consists of an EpCAM-based immunomagnetic enrichment step, followed by selection of CTCs based on cytokeratin (CK) 8/18/19 and 4,6-diamidino-2-phenylindole (DAPI) nuclear expression, and absence of the pan-leukocyte marker CD45. While CTCs according to this definition are present in 60% of metastatic breast cancer patients[8], we have recently shown that breast cancer cell lines of the normal-like type - one of the five intrinsic subtypes of breast cancer[242] - lack EpCAM expression. Consequently, CTCs of this subtype are missed when using the standard CellSearch method[64]. The normal-like subtype has been the subject of heavy debates regarding its characteristics, and has recently also been described as Claudin-low, characterized by low expression of the epithelial markers CD24 and EpCAM, and high expression of CD44 and CD49f[243]. Epithelial markers have been shown to be down regulated in circulating and disseminated tumor cells in the context of EMT, a process which tumor cells are thought to undergo upon entering the blood stream[11,244]. Evidence of EMT in CTCs, showing stem cell-like features rendering them resistant to chemotherapy, has recently been reported[245-249]. This loss of epithelial markers has been associated with the process of metastasis and poor prognosis[10-12], and tumors harboring more mesenchymal features frequently exhibit drug resistance. The detection of CTCs lacking epithelial markers may therefore be of crucial clinical importance.

Following up on the subtype-specific expression of EpCAM in vitro[64], we identified CD146 to be highly expressed on normal-like breast cancer cell lines, and showed that the combined enrichment of CTCs with anti-EpCAM and anti-CD146 ferrofluids improves breast cancer cell detection[250]. Additionally, CD146-positive CTCs were identified in the blood of metastatic breast cancer patients[250], and we are currently undertaking a clinical study in primary breast cancer patients in whom both EpCAM and CD146-positive CTCs are enumerated to establish the clinical relevance of these cells. Despite high CD146 expression in most cell lines with EMT features, a subset of cell lines was still not optimally recovered after combined CD146/EpCAM enrichment followed by selection based on CK8/18/19 expression[250]. We hypothesized this insufficient recovery to be due to lack of CK8/18/19 expression, which is a prerequisite for identification of CTCs in CellSearch, but also in other CTC detection methods[59,131]. CK expression is known to be heterogeneous among breast cancer subtypes; basal-likes commonly express CK5, 6, 14 and 17, luminals CK8, 18 and 19, and normal-likes often have low expression of all CKs[220,251-252].

In addition to examining the added value of selecting cancer cells with a broader array of

CKs, we tested CD49f (*ITGA6*; integrin, alpha 6) as an alternative selection marker. CD49f is an integral cell-surface protein involved in cell adhesion which has also been described as a stem cell marker in breast cancer[253-256], making it a candidate to select EMT-like breast cancer cells with stem cell-like features.

In this study, we set out to identify a new additional marker to be used after capturing of breast cancer cells with combined anti-CD146/anti-EpCAM ferrofluids, in order to detect those cancer cells that lack CK8/18/19 expression.

MATERIALS & METHODS

Breast cancer cell lines

Expression of putative markers was assessed on the 34 cell lines of our well-defined human breast cancer cell line panel[225,257]. These cell lines have been analyzed for their global gene expression to reveal their intrinsic subtype as described previously[258]. The normal-like cell line MDA-MB-435s has been the subject of debate on its origin[231], some researchers suggesting that it is in fact a melanoma cell line. Recently however, compelling evidence has been presented on its breast cancer origin[232,259], justifying its presence in our cell line panel.

CD49f and CK mRNA expression levels

Cell line *CK* and *CD49f* transcript levels were determined with Affymetrix GeneChip Human Exon 1.0 ST Arrays (Affymetrix UK Ltd., Wooburn Green, UK) and confirmed by qRT-PCR. Total RNA was isolated using the Qiagen RNeasy kit and quality was assessed using the Agilent Bioanalyser, requiring RNA integrity >7.0. All further processing of the samples was performed according to the Affymetrix GeneChip Whole Transcript (WT) Sense Target Labeling Assay as described before[258]. Briefly, Affymetrix GeneChip Human Exon 1.0 ST Arrays were used to determine the expression levels of virtually all exons present in the human genome (1.4 million probe sets covering >1 million exon clusters). For this study, we used expression data of the core probe sets of the transcript clusters as mentioned in **Supplementary Table 1** that are supported by putative full-length mRNA from e.g. the RefSeq database (Geo dataset accession number GSE9385). Signal processing was performed as described before, followed by an additional normalization on the average of the core probes of three reference genes, *HBMS*, *HPRT1* and *GUSB*.

CD49f and CK protein expression levels

Cells from cultured human breast cancer cell lines were incubated with fluorochrome-conjugated monoclonal antibodies as described before[64]. In brief, cells were incubated with

CD49f conjugated with PE (clone GoH3, BD Pharmingen, San Jose, CA), CK5 (Clone XM26 [Monosan, Uden, Netherlands]), CK7 (clone OV-TL 12/30 [Millipore, Billerica, MA]), CK14 (clone LL002 [Monosan]), panCK conjugated with PE (clone C-11 [Abcam, Cambridge, UK]) and a mixture as provided by the manufacturer of CK8/18 (clone C11) and CK19 (clone A53-B/A2) conjugated with PE (Veridex). For CK5, CK7 and CK14, a goat anti-mouse Ig labeled with PE was added as a second step. CK staining was preceded by a fixation and permeabilization step, using the fixation and permeabilization reagents as provided in the CellSearch Epithelial Cell kit. Cells were then analyzed on a FACSCanto flow cytometer (BD Biosciences). Unstained cells were used as a negative control.

Tissue microarrays (TMAs) were prepared in duplicate from blocks of formalin-fixed, paraffin-embedded (FFPE) breast cancer cell line cells that were cultured to near confluence, as described before[226]. These blocks were sectioned at 4 µm, mounted on StarFrost slides (Waldemar Knittel–GmbH, Braunschweig, Germany), dried, deparaffinized in xylene, and rehydrated in graded solutions of ethanol and distilled water. Specimens were pre-treated with retrieval buffer (DAKO, Glostrup, Denmark) at pH 6.0 (S1699) for CD49f, CK5, CK14 and pH 9 (S2367) for CK8/18 and CK19 for 40 min at 95–99°C in a water bath, cooled to room temperature for 20 min and rinsed in phosphate buffered NaCl solution (PBS). This was followed by a 10-min blocking step with a 0.3% peroxide PBS solution and a 30-min blocking step with a 5% bovine serum albumin PBS solution. Slides were stained with monoclonal antibodies against CK5 (1:100, clone XM26 [Monosan, Uden, Netherlands]), CK8/18 (1:100, clone 5D3 [Thermo Fisher Scientific, Waltham, MA]), CK14 (1:20, clone LL002 [Monosan]), CK19 (1:50, clone RCK108 [DAKO]) and CD49f (1:500, clone GoH3 [Abcam]) and stained with the peroxidase-conjugated Envision technique (DAKO EnVision™ System, HRP). Nuclei were counterstained with Haematoxylin. Each core was scored as 50 – 100% (++++), 25 – 50% (+++), 10 – 25% (++), 0 – 10% (+), or 0% (-) of positive cells.

Enumeration of cell line cells spiked in whole blood

Blood samples containing EDTA (7.5 mL aliquots of blood) from healthy donors were obtained from CellSave Preservative Tubes (Veridex). A predefined number of 500 cultured human breast cancer cells was added to each sample. In order to determine the actual viable cell number, a 100 µL aliquot of the cultured cells was incubated with 10 µL of 7-Aminoactinomycin D (7AAD) (Sigma-Aldrich, St. Louis, MO) and 100 µL of fluorescent beads (Beckman-Coulter, Miami, FL). After 15 min incubation at room temperature, 2 mL PBS was added, and samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences) or a FACSCanto flow cytometer (BD Biosciences). At least 10,000 beads were acquired to estimate the number of 7AAD-negative (viable) cells. The efficiency of retrieving tumor cells was controlled by counting the exact

number of viable cells that were drawn in triplicate by light microscopy after serial dilution. In order to establish the number of recovered breast cancer cells following spiking into blood from a healthy donor, samples were processed on the CellTracks AutoPrep analyzer (Veridex) with the CellSearch Epithelial Cell kit (Veridex), modified as described previously[250]. Briefly, for combined CD146 and EpCAM enrichment, EpCAM ferrofluids from the CellSearch Circulating Epithelial Cell kit and CD146 ferrofluids from the CellSearch Circulating Endothelial Cell kit were combined in even parts to a total volume of 150 µL per sample plus a standard excess volume of 300 µL. Ferrofluids coated with antibodies directed toward CD146 and EpCAM were added to the blood sample, and cancer cells bound to CD146/EpCAM coupled ferrofluid were isolated from whole blood by magnets. Unbound cells and remaining plasma were aspirated, followed by staining of the isolated cells with the nuclear dye DAPI, CK8, 18, and 19 antibodies labeled with PE and CD45 antibodies labeled with APC. As CD146 enriches for circulating endothelial cells (CECs)[227], and CECs can express CK18[236], a marker to exclude the CK18-expressing subset of CECs was needed. As CD34 is a pan-endothelial marker[229], CD34 conjugated with FITC (clone 8G12; BD Biosciences) was added to the CellSearch Epithelial Cell kit, in a volume of 150 µL per sample plus a standard excess volume of 300 µL. After incubation, cancer cells were separated magnetically once more in order to remove unbound staining reagents. Finally, the cells were resuspended in a MagNest Cell Presentation Device (Veridex), after which the number of cancer cells is enumerated according to the manufacturer's instructions.

For the detection of cells expressing CD49f, equal parts of staining reagent containing anti-CK8/18/19 conjugated to PE and CD45 conjugated to APC, and CD49f conjugated to PE were combined to a total volume of 150 µL per sample plus a standard excess volume of 300 µL. The sample was enriched for CD146 and/or EpCAM-positive cells and further characterized by staining for the presence or absence of DAPI, CK8/18/19 and CD45 as provided in the CellSearch Epithelial Cell kit and CD49f and CD34 as described above. Cancer cells were defined as DAPI+, CK8/18/19+/CD49f+, CD45-, CD34-.

Blood samples

Blood samples for spiking experiments were obtained from laboratory volunteers. Additionally, six healthy donors (age 28 – 58) were tested for the presence of CD146/EpCAM+, CD49f+ events in 7.5 mL blood. This study was approved by the Erasmus MC Institutional Review Board (METC protocol 2007-333), and all healthy donors gave their written informed consent.

RESULTS

CK8/18/19 expression

mRNA expression of *KRT8* (CK8), *KRT18* (CK18) and *KRT19* (CK19) was determined in all 34 cell lines in our well-defined breast cancer cell line panel using exon array (**Table 1** and **Supplementary Table 1**). Generally, *KRT8*, *KRT18* and *KRT19* mRNA was expressed at the highest level in luminal cell lines, while the normal-like cell lines showed the lowest expression level of particularly *KRT19*.

Additionally, CK8/18/19 protein expression was determined by flowcytometry and immunohistochemistry (**Table 1** and **Figure 1**). For flowcytometry, the standard mixture of CK8/18 and CK19 antibodies as provided as part of the CellSearch Epithelial Cell kit was used, whereas a separate anti-CK19 monoclonal antibody and a mixture of anti-CK8/18 monoclonal antibodies were used for immunohistochemistry. According to flowcytometry, normal- and basal-like cell lines showed weak to no expression of CK8/19/19 protein. Immunohistochemistry showed that, similar to mRNA expression data, the majority of normal-like cell lines particularly lacked CK19 protein expression and expressed variable CK8/18 levels.

CK5, 7 and 14 expression

According to literature and based on mRNA expression levels (**Supplementary Table 1**), CK5 (*KRT5*), 7 (*KRT7*) and 14 (*KRT14*) are putative alternative CKs for cancer cells lacking CK8/18/19 protein expression. However, the CK5, 7 and 14 protein expression assessed by flowcytometry and by immunohistochemistry was largely negative among normal- and basal-like cell lines (**Table 1** and **Supplementary Figure 1**).

PanCK expression and spiking experiments

PanCK is a monoclonal antibody targeted against CKs 4, 5, 6, 8, 10, 13 and 18. This combination of CKs provides coverage of all subtypes when looking at mRNA expression levels; normal- and basal-like cell lines express *KRT5* (CK5), *KRT6* (CK6) and *KRT10* (CK10) and luminal and erbb2 cell lines express *KRT8* (CK8) and *KRT18* (CK18) (**Supplementary Table 1**). PanCK protein expression was indeed positive in five out of nine normal-like and all basal-like cell lines. Four cell lines, the normal-like MDA-MB-436, SUM159PT and MDA-MB-231 and the basal-like SUM149PT, which all lacked CK8/18/19 protein expression, stained positive for panCK (**Table 1** and **Figure 1**). Of these four CK8/18/19-negative cell lines, three (MDA-MB-436, SUM159PT and MDA-MB-231) were previously not completely detected with CK8/18/19 as a selection marker in the CellSearch assay[250].

We proceeded to directly compare the recovery of various cell lines using CK8/18/19 versus panCK as a selection marker (**Figure 2**, left and middle panel). Recovery results of tested

normal-like and control (MDA-MB-468) cell lines are depicted in **Figure 3**. Cell lines were selected based on their previous low recovery with CK8/18/19 as a selection marker after CD146/EpCAM enrichment[250]. No improvement in recovery rates was seen in any of the cell lines; BT549 and Hs578T were still almost completely undetected (0.4 vs. 0 and 0.6 vs. 1.2%, respectively), while SUM1315 and SUM159 remained at low detection rates of about 20% (18.6 vs. 15.6% and 18 vs. 17.6%, respectively). The positive control, cell line MDA-MB-468, was fully recovered with both CK8/18/19 and panCK. Because of the observed lack of benefit from panCK staining, not all CK8/18/19-negative cell lines were tested for their recovery with panCK.

CD49f expression and spiking experiments

We next evaluated the value of CD49f, as an alternative to CK8/18/19. According to our Affymetrix data, CD49f (*ITGA6*) is highly expressed in the normal- and basal-like subtypes (**Supplementary Table 1**). Flowcytometry confirmed a high protein CD49f expression in all basal- and normal-like cell lines (**Figure 1** and **Table 1**)., CD49f was expressed on all cell lines with varying staining intensity among subtypes and cell lines using immunohistochemistry (**Table 1** and **Supplementary Table 1**). Discrepancies can be seen between flowcytometric and immunohistochemical CD49f expression (**Table 1**), especially in terms of intensity of staining. However, all cell lines classified as positive by flowcytometry were also positive by immunohistochemistry. The discrepancies can probably be explained by differences in staining techniques, for instance by the use of fixative in the immunohistochemistry procedure.

We then selected the cell lines that were not at all or suboptimally detected when selected based on CK8/18/19 after combined CD146/EpCAM enrichment[250]. All these seven cell lines belong to the normal-like subtype and showed moderate to strong CD49f and CD146 expression (**Figure 4** panel b). When using combined CD49f and CK8/18/19 selection after combined CD146/EpCAM enrichment in the modified CellSearch assay (**Figure 2**, middle and right panel), BT549, SUM1315, SUM102 and MDA-MB-435, which were only partially detected with CK8/18/19, showed substantial gain in recovery (i.e., 0.4 vs. 26.8% of 500 spiked cell line cells with CK8/18/19 vs. CD49f/CK8/18/19, 0.2 vs. 79.6%, 17 vs. 72.4% and 0.4 vs. 72.4%, respectively) (**Figure 4** panel a). No improvement in recovery was seen in the cell lines Hs578T (0 vs. 1.8%), MDA-MB-436 (17.2 vs. 17.2%) and MDA-MB-231 (54.6 vs. 45.6%). The luminal cell line CAMA1 was included as a positive control, and was completely recovered with both marker combinations.

To ensure the specificity of this new modification of the CellSearch assay, we tested six healthy donors (age 28 – 58) for the presence of CK8/18/19/CD49f+, CD45-, CD34- events

after combined EpCAM/CD146 enrichment. Importantly, occasional false positive events were detected (median 1.5, range 0 - 4). Because we also observed numerous CD49f positive, CD34 negative events with a very weak CD45 expression (CD45^{dim}), we suspected these CD45 negative events to be granulocytes with a CD45 expression too low to be detected in the CellSearch system. We proceeded to evaluate two healthy donor blood samples with the more sensitive Imagestream technique, which combines the scanning sensitivity of conventional flowcytometry with the capability to produce high-resolution images (Amnis, Seattle, WA)[260-261] and compared these results with those obtained using the CellSearch scanning technique. Indeed, no false positive (CK/CD49f positive, CD45/CD34 negative) events were seen using Imagestream, while the same two samples did both show one such false positive event when scanned and analyzed using CellSearch. To confirm the existence of a CD49f+/CD45^{dim} population in healthy donor blood, we stained healthy donor peripheral blood mononuclear cells (PBMCs) with CD49f, CD45 and two granulocyte markers (CD13 and CD66). Indeed, a clear cell population of CD49f+/CD45^{dim} granulocytes was confirmed to be both CD13 and CD66 positive, in line with our hypothesis that these false-positive events are in fact CD45^{dim} granulocytes (data not shown).

DISCUSSION

CTCs have proven their value as a prognostic factor in metastatic breast cancer. However, CellSearch, the only currently available FDA-approved method, detects CTCs in only 60% of metastatic breast cancer patients[25]. In the other 40%, CTCs are either not present at the time of blood draw, or CTCs remain undetected. Previously, we have shown that a subset of breast cancer cells lacks EpCAM expression and is therefore missed using the CellSearch assay[64]. These EpCAM-negative cells could be of pivotal importance in the dissemination process of breast cancer, possibly representing tumor cells that have undergone EMT. The presence of EMT-features has been associated with poor prognosis[10-12] and chemotherapy resistance[245-246]. To detect these EpCAM-negative cells, we identified CD146 as an alternative enrichment marker, and have shown that CD146+ CTCs can be detected in metastatic breast cancer patients[250].
In spite of this improvement, not all breast cancer cell lines could be detected by this modified CellSearch approach, despite apparent sufficient CD146 expression. These cell lines, both normal- and basal-like, are characterized by low CK8/18/19 expression, which is needed for the pivotal selection step in the CellSearch assay after CTC enrichment. Thus, we set out to identify an alternative marker to be used in combination with CK8/18/19 that would lead to the detection of all breast cancer subtypes. Because of the known heterogeneity in CK expression among breast cancer subtypes[220,251-252], we postulated that a broader array of

CKs could improve CTC detection. However, no single CK showed sufficient expression across normal- and basal-like subtypes. PanCK could theoretically cover all breast cancer subtypes and was indeed expressed on all basal-like and half of normal-like cell lines, suggesting an added recovery benefit when adding panCK as a selection marker. However, four normal-like cell lines still remained partially or completely undetected with combined panCK and CK8/18/19. This lack of observed benefit might be due to suboptimal fixation and permeabilization procedures in the CellSearch kit for this purpose. Indeed, we observed a stronger panCK staining on cell lines when fixed using formaldehyde and permeabilized using saponin than when using the fixation and permeabilization reagents as provided in the CellSearch Epithelial cell kit (data not shown).

CD49f or alpha-6-integrin is a cell-surface protein implicated as a promoter of metastasis[262] and increased tumorigenicity[263-264]. In prostate cancer, CD49f has recently been described as the driver of a androgen receptor-regulated survival pathway that is independent of PI3K[265]. In hepatocellular carcinoma CD49f has been associated with EMT[266], and CD49f is regarded as a stem cell marker for prostate and bladder cancer[267]. It is also regarded as a stem cell marker in breast cancer[253-256]. On a cell line level however, CD49f expression is universal among normal- and basal-like subtypes, but is less pronounced in luminal and erbb2 cell lines. This expression pattern may reflect the stem cell-like phenotype of the two former breast cancer subtypes[268]. CD49f staining indeed enabled the detection of four cell lines which otherwise would have been partially or completely undetected. The recovery of three additional cell lines did not improve with combined CD49f and CK8/18/19 staining. This incomplete recovery can be caused by a number of reasons; insufficient CD146 or CD49f expression to enable detection or modification of antigen availability by for instance the fixation and permeabilization step used in the CellSearch method.

With any new marker for rare event detection, specificity is a key issue, so we assessed healthy donors for the presence of false positive CD49f+/CD45- cells. Using the CellTracks scanning microscope, up to four CD45- events per 7.5 mL of whole blood were seen. Analyzing these samples with the more sensitive Imagestream technology, no such CD45-negative events were seen, pointing to a difference in ability to detect weak CD45 signals between these techniques. Additionally, flowcytometry confirmed our hypothesis that these false-positive events are CD49f+/CD45^{dim} granulocytes. When implementing CD49f into clinical practice, it should be assured that the sensitivity of CD45 staining is high enough to prevent the detection of false positives in healthy donors, for instance by using a more sensitive fluorescence detection method. Alternatively, an additional marker to exclude granulocytes, such as CD13 could be added to the extra channel in CellSearch. CTCs would then have to be negative for CD45 to exclude leukocytes, negative for CD34 to exclude CECs and negative for CD13 to exclude

granulocytes.

After having improved breast cancer CTC detection with the use of CD146 as an additional enrichment marker, we have now shown that further benefit can be obtained by adding CD49f as a selection marker. Specifically the detection of those cell lines showing EMT-features was improved by adding CD49f, possibly enhancing the detection of CTCs involved in EMT-associated processes such as drug resistance and metastasis. While still no complete coverage of all CTC subtypes can be expected, a higher sensitivity of CTC detection is likely to improve the clinical value of this assay in breast cancer patients.

Table 1
mRNA expression and protein expression of CKs and CD49f in normal-like and basal-like cell lines

	CK5		CK7	CK14		CK18 (*KRT18*)	CK8-18	CK19 (*KRT19*)		CK8/18/19	panCK	CD49f (*ITGA6*)		
Cell lines	Protein expr FACS (s/n)[a]	Protein expr IHC[b]	Protein expr FACS (s/n)[a]	Protein expr FACS (s/n)[a]	Protein expr IHC[b]	mRNA expr Exon array[c]	Protein expr IHC[b]	mRNA expr Exon array[c]	Protein expr IHC[b]	Protein expr FACS (s/n)[a]	Protein expr FACS (s/n)[a]	mRNA expr Exon array[c]	Protein expr FACS (s/n)[a]	Protein expr IHC[b]
Normal-like														
SUM1315MO2	ND	+	<5	<5	-	0.68	ND	0.24	ND	<5	<5	1.30	20-200	+
MDA-MB-436	<5	-	<5	<5	-	4.68	+++	0.10	-	<5	5-20	0.38	5-20	++++
SUM159PT	<5	-	<5	<5	-	5.14	-	0.14	-	<5	5-20	1.78	5-20	+++
SUM102PT	<5	ND	<5	<5	-	ND	ND	ND	ND	<5	<5	ND	20-200	ND
SK-BR-7	<5	-	5-20	<5	-	27.28	++++	0.20	-	5-20	20-200	1.69	20-200	++++
MDA-MB-231	ND	-	<5	<5	-	4.70	+++	1.85	+	<5	5-20	2.67	5-20	+++
Hs578T	<5	+	<5	<5	-	3.72	+	0.17	-	<5	<5	0.15	20-200	+++
BT549	<5	-	<5	<5	-	0.31	-	0.13	-	<5	<5	1.02	5-20	++++
MDA-MB-435s	<5	-	ND	<5	-	3.03	+	0.15	-	<5	<5	5.085	20-200	++++
Basal														
SUM149PT	5-20	++++	ND	<5	-	11.62	++++	4.64	++	<5	20-200	2.35	20-200	+
MDA-MB-468	<5	++++	ND	<5	-	11.96	++++	11.11	++	5-20	5-20	0.73	5-20	++++
BT20	20-200	++++	ND	<5	-	14.52	++++	4.59	+++	5-20	20-200	0.65	20-200	+
Hcc1937	<5	+	ND	<5	-	6.83	++++	0.14	ND	5-20	20-200	1.26	>200	+++
SUM229PE	5-20	+++	ND	<5	-	11.66	++++	12.91	++	5-20	>200	3.11	20-200	+

[a]Data are values of one representative flow cytometric (FACS) experiment performed in duplicate. Expr; expression, s/n; signal-to-noise ratio, in which 5 or greater is considered detectable expression, [b]IHC; immunohistochemistry, -; 0% staining, +; 0 - 10% staining, ++; 10 - 25% staining, +++; 25 - 50% staining, ++++; 50 - 100% staining, [c]qRT-PCR; quantitative real-time PCR, in which values >0.001, i.e., requiring ≤10 cycles to pass the threshold compared with the numbers of cycles required for the reference gene set, are considered detectable expression, ND; not determined, CK; cytokeratin

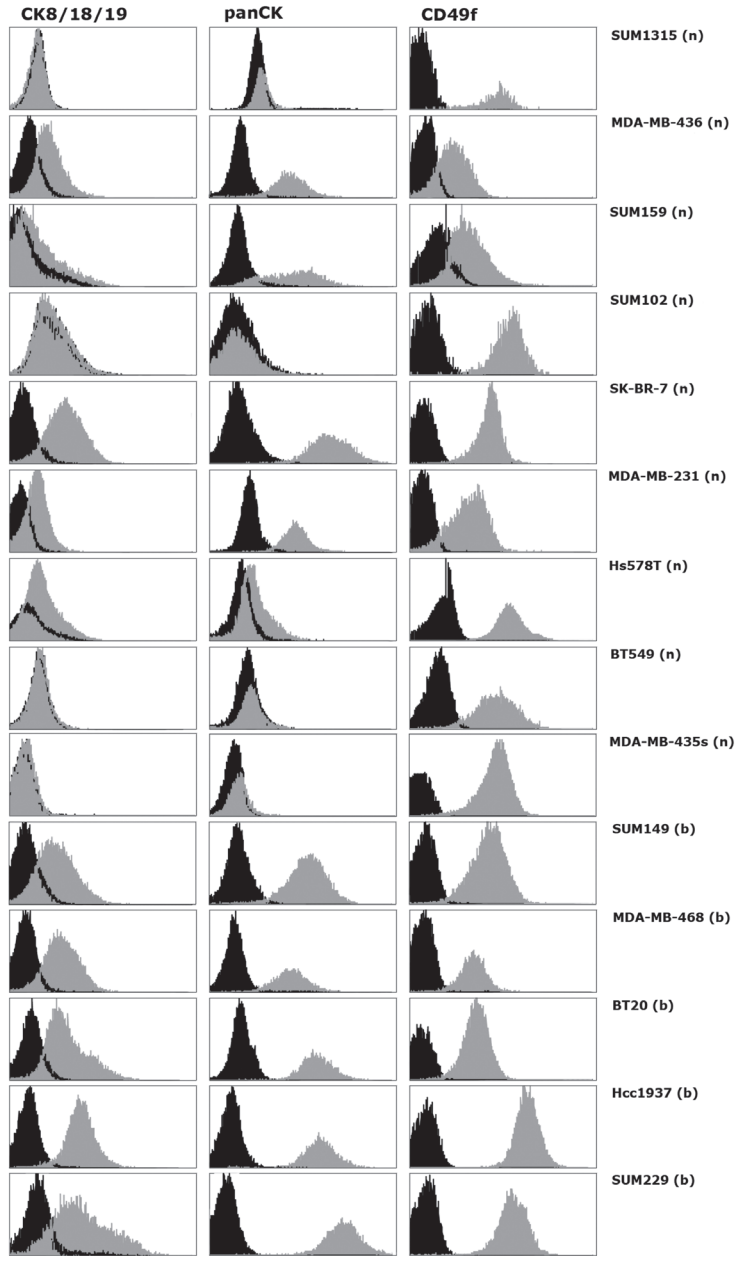

Figure 1

CK8/18/19 and panCK cytoplasmatic and CD49f membrane expression as assessed by flow cytometry. Black histograms show unstained cells, grey histograms show expression of CK8/18/19 (left panel), panCK (middle panel) and CD49f (right panel) stained cells of normal-like (n) and basal-like (b) cell lines. X-axis; fluorescence intensity; Y-axis; number of counted cells

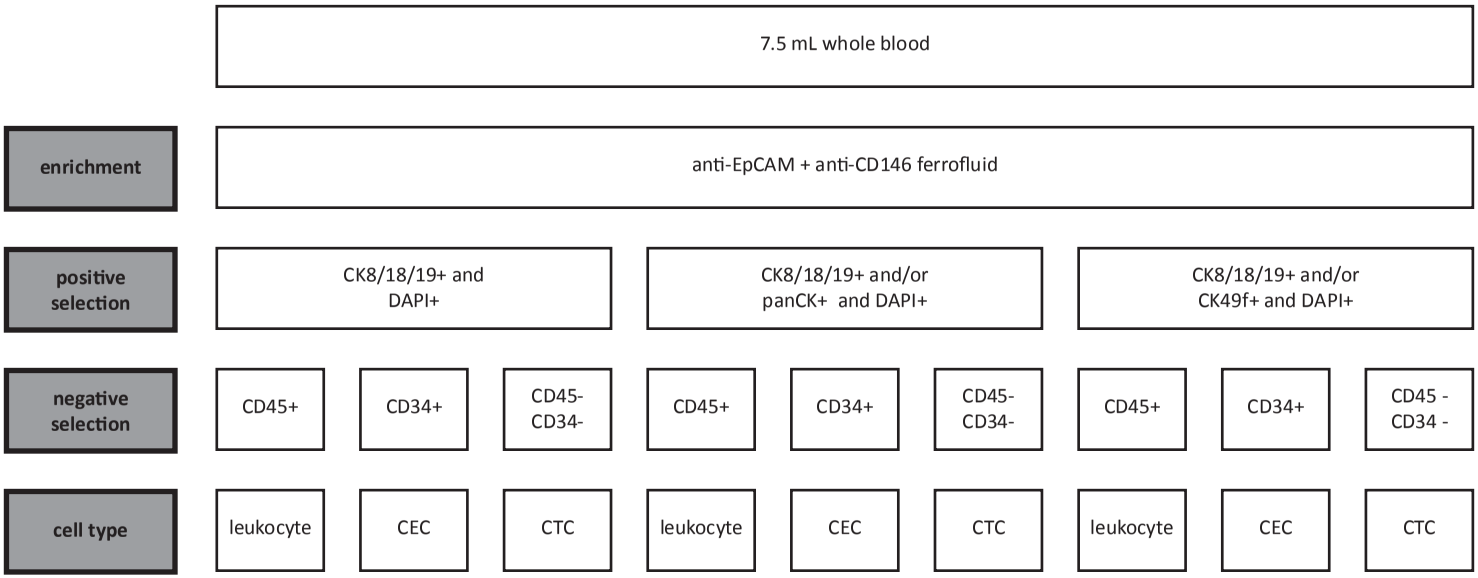

Figure 2

Setup of the modified CellSearch CTC enumeration assay using combined anti-EpCAM/anti-CD146 enrichment (left panel) versus the two described new modifications; positive selection based on CK8/18/19 and/or panCK (middle panel) and positive selection based on CK8/18/19 and/or CD49f (right panel). Description of the makers used in each assay for enrichment, positive selection and negative selection, and the cell type associated with these selection criteria. CK; cytokeratin, CEC; circulating endothelial cell, CTC; circulating tumor cell

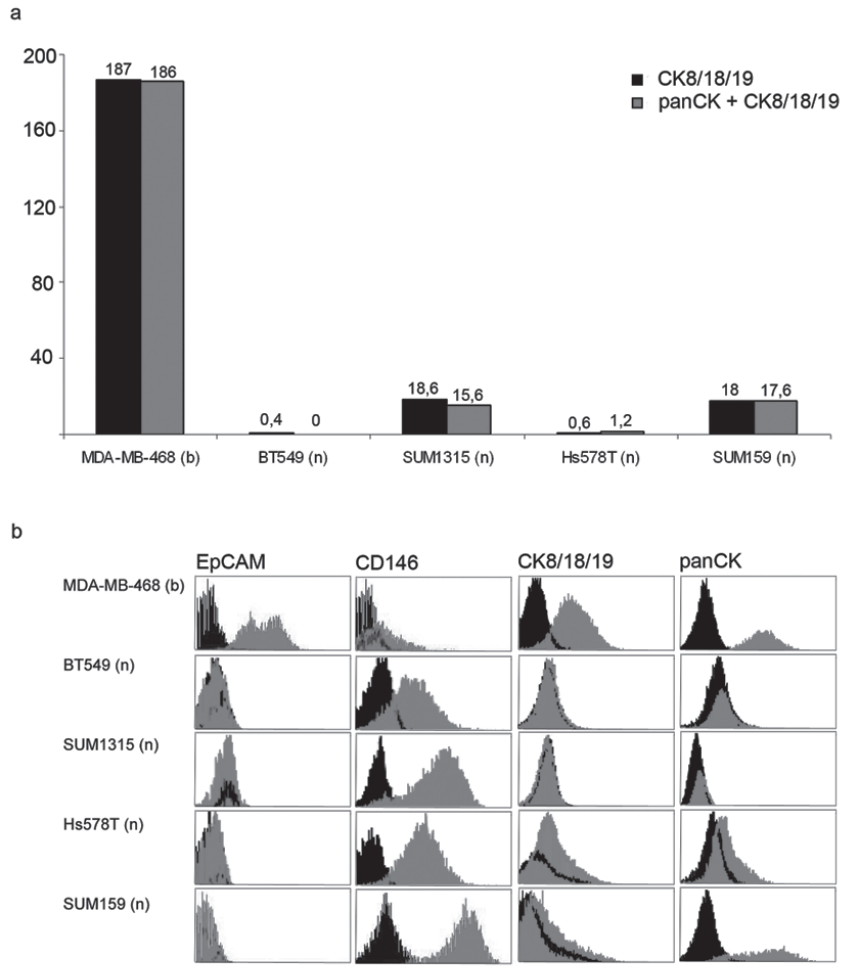

Figure 3

Recovery of basal-like (b) (MDA-MB-468, used here as a positive control) and normal-like (n) cell lines with CK8/18/19 (black bar) and panCK (grey bar) after combined CD146 and EpCAM enrichment (**a**). EpCAM and CD146 membrane expression and CK8/18/19 and panCK cytoplasmatic expression in basal-like (**b**) and normal-like (n) cell lines (b). Black histograms show unstained cells, grey histograms show expression on EpCAM (first panel), CD146 (second panel), CK8/18/19 (third panel) and panCK (fourth panel) stained cells of normal-like (n) and basal-like (b) cell lines. X-axis; fluorescence intensity; Y-axis; number of counted cells

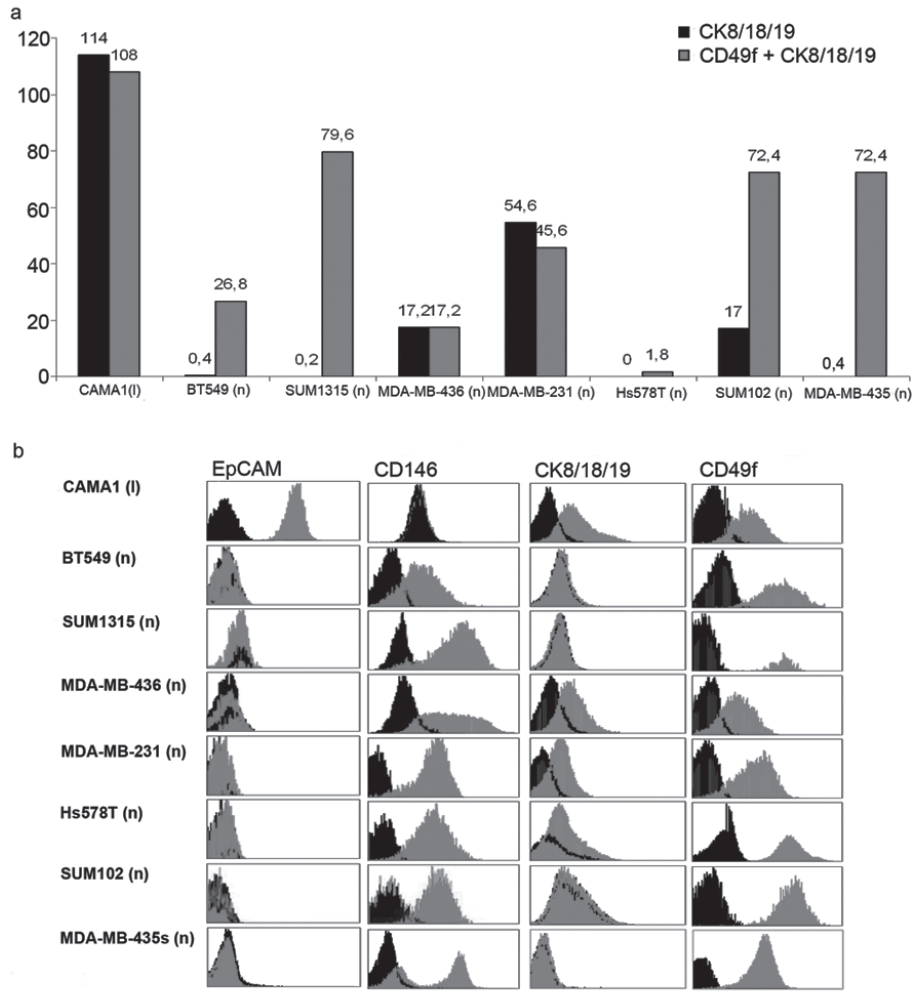

Figure 4

Recovery of luminal (l) (CAMA1, used here as a positive control) and normal-like (n) cell lines with CK8/18/19 (black bar) and CD49f (grey bar) after combined CD146 and EpCAM enrichment (**a**). EpCAM, CD146 and CD49f membrane expression and panCK cytoplasmatic expression in luminal (l) and normal-like (n) cell lines (**b**). Black histograms show unstained cells, grey histograms show expression of EpCAM (first panel), CD146 (second panel), CK8/18/19 (third panel) and CD49f (fourth panel) stained cells of luminal (l) and normal-like (n) cell lines. X-axis; fluorescence intensity; Y-axis; number of counted cells

Supplementary data:

https://docs.google.com/open?id=0B9Etqm_r7T2mNnN5STlLajcyZFU

Chapter 5

mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients

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ABSTRACT

Purpose

Molecular characterization of circulating tumor cells (CTC) holds great promise. Unfortunately, routinely isolated CTC fractions currently still contain contaminating leukocytes, which makes CTC-specific molecular characterization extremely challenging. In this study, we determined mRNA and microRNA (miRNA) expression of potentially CTC-specific genes that are considered to be clinically relevant in breast cancer.

Experimental Design

CTCs were isolated with the epithelial cell adhesion molecule–based CellSearch® Profile Kit. Selected genes were measured by real-time reverse transcriptase PCR in CTCs of 50 metastatic breast cancer patients collected before starting first-line systemic therapy in blood from 53 healthy blood donors (HBDs) and in primary tumors of 8 of the patients. The molecular profiles were associated with CTC counts and clinical parameters and compared with the profiles generated from the corresponding primary tumors.

Results

We identified 55 mRNAs and 10 miRNAs more abundantly expressed in samples from 32 patients with at least 5 CTCs in 7.5 mL of blood compared with samples from 9 patients without detectable CTCs and HBDs. Clustering analysis resulted in 4 different patient clusters characterized by 5 distinct gene clusters. Twice the number of patients from cluster 2 to 4 had developed both visceral and non-visceral metastases. Comparing transcript levels in CTCs with those measured in corresponding primary tumors showed clinically relevant discrepancies in estrogen receptor and HER2 levels.

Conclusions

Our study shows that molecular profiling of low numbers of CTCs in a high background of leukocytes is feasible and shows promise for further studies on the clinical relevance of molecular characterization of CTCs.

INTRODUCTION

Molecular characterization of primary tumors has already greatly contributed to the personalized treatment of cancer patients. High-throughput techniques have yielded the knowledge of mutations or epigenomic changes in certain genes and prognostic and predictive models on the basis of mRNA and microRNA (miRNA) expression profiles[269-274]. Combined with classical tumor characteristics, these models are increasingly used to guide individualized treatment of patients, thereby aiming to avoid over- or undertreatment. However, most of these prognostic and predictive models have been developed based on primary tumor tissue, whereas metastases, rather than the primary tumor, determine the clinical outcome of cancer patients. It has been shown that metastases, which may develop several years after occurrence of the primary tumor and after prior systemic therapy in the adjuvant or neoadjuvant setting, can differ greatly from primary tumor tissue in terms of genetic characteristics[6,275-280]. It is therefore anticipated that molecular characterization of metastases will improve the currently available prognostic and predictive models. Taking biopsies from metastases in patients, however, is an invasive and often painful procedure, and frequently impossible due to the lack of accessible lesions.

Circulating tumor cells (CTC) are found in the peripheral blood of patients and are shed from either the primary tumor or its metastases. A recently developed technology to quantify the number of CTCs in whole blood (WB) is the CellSearch CTC Test (Veridex™ LLC, Raritan). So far, this is the only test that has been approved by the U.S. Food and Drug Administration (FDA[281]) for the detection and enumeration of CTCs in metastatic prostate[282], colorectal[283], and breast[25] cancer as an independent prognostic factor. After enrichment using magnetic beads coated with anti-epithelial cell adhesion molecule (EpCAM) antibodies, isolated cells are stained with fluorescently labeled monoclonal antibodies specific for epithelial cells (CK8/18/19), leukocytes (CD45), and their nuclei with a nuclear staining dye [4', 6 diamidino 2 phenylindole (DAPI)], and subsequently enumerated by a semi-automated fluorescence microscope.

In addition to enumeration, CTCs can also be isolated for molecular characterization. This may enable insight into the molecular biology of metastasis, the association of their molecular profiles with treatment outcomes, and reveal the presence of potential drugable targets. However, although EpCAM-based enrichment eliminates a large proportion of leukocytes (approximately 4-log depletion), there are still considerable quantities of contaminating leukocytes (DAPI+/CD45+) present after this enrichment[9]. This contamination, together with the low frequency of CTCs, forms a challenge when aiming to characterize CTCs by very sensitive molecular methods such as PCR.

Despite these challenges, we have recently shown the feasibility of determining mRNA expression of epithelial-specific genes in CTC-enriched samples[9]. In addition to mRNA, another

class of RNAs that increasingly attracts attention is the group of miRNAs. Each miRNA targets, on average, 200 mRNA transcripts by which miRNAs execute widespread control[284]. As might be expected based on these activities, altered expression of specific miRNA genes has already been shown to contribute to the initiation and progression of cancer[285-287]. Therefore, miRNA-based cancer gene therapy offers the theoretical appeal of targeting multiple gene networks that are controlled by a single aberrantly expressed miRNA[288], making the profiling of miRNAs in cancer even more appealing, especially in the context of CTCs.

Here, we describe the optimization of a method to perform both miRNA and mRNA expression analysis for multiple genes by real-time reverse transcriptase PCR (RT-PCR) on as little as 5 CTCs isolated from 7.5 mL of blood, which is considered the clinically relevant cut-off in patients with metastatic breast cancer[32,237,289], in an environment containing excess quantities of up to $1{,}000^9$ contaminating leukocytes. As shown in this study for patients with metastatic breast cancer, this robust and novel method allows the simultaneous determination of 65 epithelial tumor cell–specific miRNA and mRNA expression levels in CTCs enriched by CellSearch, and the exploration of their clinical relevance on the basis of the identification of 4 different patient clusters with distinct characteristics.

TRANSLATIONAL RELEVANCE

Metastases, which may develop several years after occurrence of the primary tumor and after prior (neo)adjuvant therapy, can differ greatly from primary tumor tissue in terms of genetic characteristics. Taking biopsies from metastases in patients, however, is an invasive procedure and frequently impossible due to the lack of accessible lesions. Circulating tumor cells (CTC) are tumor cells shed from either the primary tumor or its metastases that circulate in the peripheral blood of patients and can thus be regarded as “liquid biopsies” of metastasizing cells. In this study, we show for the first time the feasibility of extensive molecular characterization of CTCs at both the mRNA and microRNA level in a high background of leukocytes and show its applicability in a cohort of 50 metastatic breast cancer patients. It is anticipated that such an extensive molecular characterization of CTCs will improve the currently available prognostic and predictive models on the basis of primary tissue.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Erasmus MC and local Institutional Review Boards (METC 2006-248), and all donors and patients gave their written informed consent.

Breast tumor tissues and blood samples

From 61 patients with metastatic breast cancer, 2 x 7.5 mL blood samples were prospectively taken for CTC enumeration and isolation (for details see next) prior to initiation of systemic therapy for metastatic disease. From these 61 samples, 11 (18%) were excluded because of insufficient RNA quality and/or quantity (for details see next), rendering a total number of 50 patients eligible for further analysis. Metastatic breast cancer patients had been included at the start of systemic therapy between February 2008 and December 2009 in 4 hospitals (9 patients in the Erasmus Medical Center, Rotterdam, The Netherlands, 10 in the Ikazia Hospital, Rotterdam, The Netherlands, and 10 in the Maasstad Hospital, Rotterdam, The Netherlands, and 21 patients in the Oncology Center GZA St-Augustinus, Antwerpen, Belgium). For 8 of 32 patients with at least 5 CTCs, primary tumor tissue containing at least 50% invasive epithelial tumor cells was available for RNA isolation [5 fresh frozen (FF) and 3 formalin-fixed paraffin-embedded (FFPE)]. These 8 specimens were used for comparison of transcript levels between CTCs and corresponding primary tumors. Detailed clinicopathological information for these 50 patients and the 8 matching primary tissues is given in **Table 1** and in **Supplementary Table 1** after dichotomization of patients at the breast cancer clinically relevant level of 5 CTCs[32,237,289]. Fifty-three healthy blood donor (HBD) blood samples were drawn form laboratory volunteers and blood donors of the Sanquin Blood Bank South-west Region.

Enumeration of CTCs

Prior to the administration of first-line systemic therapy, 7.5 mL of blood from HBDs and metastatic breast cancer patients was drawn in CellSave tubes (Veridex). For CTC enumeration, samples were processed on the CellTracks AutoPrep System (Veridex) by using the CellSearch Epithelial Cell Kit (Veridex) and CTC counts were determined on the CellTracks Analyzer (Veridex) according to the manufacturer's instructions and as described previously[64,250].

miRNA and mRNA isolation from CTCs, FF, and FFPE

For gene expression studies, in parallel with the enumeration studies, 7.5 mL blood of the same healthy donors and patients was drawn in EDTA tubes and enriched for CTCs on the CellTracks AutoPrep System using the CellSearch Profile Kit (Veridex). The cells in the enriched CTC fractions were lysed by adding 250 µL of Qiagen AllPrep DNA/RNA Micro Kit Lysis Buffer (RLT+ lysis buffer) (Qiagen BV, Venlo, The Netherlands) and stored immediately at -80°C until RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions. In brief, by using a gradient of ethanol (Absolute Ethyl Alcohol (EtOH) Merck, Darmstadt, Germany) the larger RNAs (>200 nt) were first captured in a RNeasy Mini spin column in the presence of 35% EtOH and eluted separately from the small RNA

molecules (≤200 nt) present in the flow through. These >200 nt aliquots were treated with DNAse I according the manufacturer's instruction. Next, the ≤200 nt molecules present in the flow through were captured in a new RNeasy Mini spin column in the presence of a final concentration of 60% EtOH and thus eluted separately from the >200 nt molecules. Using this approach consisting of two sequential filtrations with different ethanol concentrations, a 12 µL RNA fraction highly enriched in RNA species ≤200 nt and a 14 µL RNA fraction enriched in RNA species >200 nt could be obtained from the same sample.

Total RNA was isolated from FF tissue with RNA-Bee as described before[290] and from FFPE tissue with the column-based High Pure RNA Paraffin Kit (Roche Applied Science, Almere, The Netherlands) according to the manufacturer's instructions.

Stem-loop cDNA synthesis, pre-amplification, and real-time PCR (quantitative RT-PCR)

The generation of pre-amplified cDNA from total RNA from the FF and FFPE tissues and the >200 nucleotide (nt) RNA fractions and subsequent TaqMan-based quantitative RT-PCR (qRT-PCR) analysis, and the validation procedures to ensure homogeneous amplification, were performed as described before[9].

To analyze miRNAs, a multiplex stem-loop cDNA approach was used essentially as described before[286]. In brief, up to 50 different RT primers (250 nmol/L each) were pooled, concentrated for 60 minutes in a speed vacuum centrifuge at 50°C, and resuspended in nuclease-free ddH2O (double distilled water) to a final concentration of 50 nmol/L each. The use of a specific primer with a hairpin structure during cDNA synthesis and mature miRNA-specific detection probes precluded the detection of precursor miRNAs. A total of 25 to 50 ng of total RNA sample aliquots were reverse-transcribed in a final volume of 20 mL with a final concentration of 12.5 nmol/L for each RT primer using the TaqMan miRNA for reverse transcription kit [Applied Biosystems (ABI)] according to the manufacturer's instructions and as described before[286].

For the miRNA quantification in the CTC samples, 3 mL ≤ 200 nt RNA aliquots were reverse-transcribed in a final volume of 7.5 mL with a final concentration of 12.5 nmol/L for each RT primer (ABI), 0.65 mmol/L of each dNTP (ABI), 3 mmol/L magnesium chloride (Invitrogen), 0.3 U/mL RNase inhibitor (ABI), 15 U/mL RevertAidH Minus enzyme (Fermentas), and 1x RT buffer (Fermentas). Cycling conditions were according to the "Megaplex RT reaction for TaqMan miRNA array" protocol from ABI, i.e., 40 cycles of 16°C for 2 minutes, 42°C for 1 minute, and 50°C for 1 second, followed by a final incubation at 85°C for 5 minutes and a cooldown to 4°C. Prior to PCR, half of the resulting multiplex cDNA was linearly pre-amplified in 15 cycles according to the manufacturer's instructions (TaqMan PreAmp from ABI) and as described previously for our multiplex gene expression studies[290]. Before performing real-time PCRs for each of the miRNAs separately, RT samples were diluted in nuclease-free ddH2O and analyzed

by real time.

PCR was performed in a 20-mL reaction volume in an Mx3000P RealTime PCR System (Stratagene) using the individual TaqMan miRNA primer and probe assays in combination with TaqMan Universal PCR Master Mix No AmpErase UNG (ABI) with cycling conditions according to the manufacturer's instructions.

To verify that the multiplex RT approach did not affect the quantification of specific miRNAs, all miRNA data were validated in a uniplex RT reaction. A pool consisting of RNA of different human breast tissues was included in each cDNA synthesis and pre-amplification run, and the resulting data were used to normalize for variation between experiments. In addition, all cDNA synthesis runs incorporated a minus RT reaction, which proved to be negative for all assays in this study. PCR efficiency, linearity, and the upper and lower detection limits of each of the individual miRNA assays were validated with a standard curve prepared of RNA from a pool of breast tumors. Negative controls included samples without RT and samples in which total RNA and cDNA was replaced with ddH2O. Quantitative values were obtained from the threshold cycle (Ct) at which the increase in TaqMan probe fluorescent signal associated with an exponential increase of PCR products reached the fixed threshold value of 0.08, which was in all cases at least 10-fold higher than the background signal.

First selection of potentially CTC-specific mRNA and miRNA transcripts

The specifics of the used TaqMan assays are given in **Supplementary Table 2a** for the miRNAs and **Supplementary Table 2b** for the mRNAs. For the identification phase of potentially CTC-specific miRNA transcripts, the TaqMan Human MicroRNA Assay Set (Sanger miRBase v10; ABI), consisting of 446 unique assays to quantify 436 miRNAs and 10 controls (small nucleolar RNAs; SNORs/RNUs), was used to screen a pool of 150 primary breast cancer RNAs. Of these 446 miRNAs, 253 were expressed in these breast cancer samples and approximately 200 had an expression level of more than 10% of the expression of the reference miRNA set (see next). Next, these levels were compared with those measured in a pool of 6 CellSearch-enriched preparations from HBDs for potentially differentially expressed miRNAs. These prescreen analyses selected 39 miRNAs with both notable expression in breast tumors and at least a 10-fold higher expression in breast tumors relative to CellSearch enriched HBDs. Four additional miRNAs were included for other reasons, i.e., *hsa-miR-452* to compare with *hsa-miR-452#* and *hsa-miR-379* because of the observed difference between estrogen receptor (ER)-positive and ER-negative samples in the prescreen, *RNU6B* as being a potential reference miRNA, and *hsa-miR-210*, which has shown clinically relevance in breast cancer ([286,291]; **Supplementary Table 2a**).

For the mRNA transcripts, clinically relevant and potentially CTC-specific genes were selected

in silico on the basis of literature data and their reported low expression in white blood cells and higher expression in breast tumor tissues, according to the SAGE Genie Database of the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). These prescreen analyses were performed as described in detail before[9] and resulted in 90 mRNA transcripts, including 3 reference genes and 2 reference leukocyte markers that could be measured reliably by qRT-PCR and which were potentially higher expressed in breast tumor cells relative to leukocytes (**Supplementary Table 2b**).

Reference genes, data normalization, and quality control

Unless stated otherwise, levels of *HMBS*, *HPRT1*, and *GUSB* were used to control sample loading and >200 nt RNA quality, as described previously[290]. Bone marrow stromal cell antigen 1 (*BST1*) and protein tyrosine phosphatise receptor type C (*PTPRC* coding for CD45) were the control genes for leukocyte background and keratin 19 (*KRT19*) was the control gene for CTC quantification[290].

Although appropriate reference molecules for miRNAs are still unknown for clinical breast cancer cells with a background of leukocytes, previous studies have shown that normalization on mean or median expression of all miRNAs measured in a sample can adequately reduce technical variation[292]. Therefore, miRNA data of each individual sample were normalized on the median level of all miRNAs measured in that particular sample.

After verification of equal PCR efficiency for all assays, the relative expression levels were quantified by using the delta Ct method, which is the difference between the median Ct of the appropriate control genes and the Ct of the target gene. Only samples that were at the median Ct of all miRNAs and the median Ct of *HMBS*, *HPRT1*, and *GUSB* able to generate a signal within an arbitrarily chosen cut-off set at 26 Ct were considered of sufficient quality and quantity to enter the study. By the use of this threshold, 11 of our initial 61 patient CTC samples (18%) were excluded from further analysis.

Finally, all transcript data of the 50 CTC samples, 53 HBD controls, and 8 primary tumors were normalized to the Ct of the appropriate reference set, after which, for each individual assay, the median Ct measured in CellSearch-enriched HBDs (n = 31 for the mRNAs and n = 8 for the miRNAs) was used as a cut-off Ct for the CTC samples. All genes with Ct values exceeding this cut-off Ct were considered to be undetectable.

Statistical analysis

Statistical analysis was done by SPSS 15.0 and Datan Framework GenEx Pro package version 5.2.5.20 software for real-time PCR expression profiling. Grubbs' test was used to define outlier data points (1.1%) that were replaced with the median value of all samples for the

gene in question. The strengths of the associations between continuous variables were tested with the nonparametric Spearman rank correlation test (rs). Gene expression levels in the various fractions were compared with the nonparametric Wilcoxon's test to test the null hypothesis and the Mann–Whitney U test to identify genes with significantly different expression levels between groups. A false discovery rate (FDR) control of 10% was applied to correct for multiple testing[293]. Cluster analysis (http://rana.lbl.gov/eisen/[294]) was used to cluster the samples on the basis of the gene expression values and TreeView (http://rana.lbl.gov/eisen[294]) was used to visualize the results. DAVID (Database for Annotation, Visualization, and Integrated Discovery, david.abcc.ncifcrf.gov[295-296]) was used to functionally annotate genes and identify the over-represented functions, with *P* values corrected for multiple testing via the Benjamini-Hochberg's procedure. All human genes were used to compare frequencies of functions. Unless stated otherwise, all statistical tests were 2-sided with $P < 0.05$ considered as statistically significant.

RESULTS

Quality control measures taken to ensure reliable measurement of CTC-specific gene transcripts

The first purpose of this study was to establish a sensitive method to perform both mRNA and miRNA expression analysis of transcripts specific for CTCs, in samples often containing only a few CTCs in an environment of excess quantities of contaminating leukocytes.

To select the gene transcripts, we used the approach described in detail in the Materials and Methods section, resulting in 43 putative breast CTC–specific miRNAs, 85 putative breast CTC–specific mRNAs, and 5 control mRNAs. Our first challenge was to find a method that would enable us to measure both mRNAs and miRNAs in RNA isolated from as little as 5 CTCs (approximately 50 pg total RNA), which is considered the clinically relevant cut point in patients with metastatic breast cancer[32,237,289] in a reliable and quantitative manner. In this respect, as already described and tested for the mRNA assays[9], any individual miRNA expression assay showing as a non-homogeneously amplified outlier in our tests should be treated with caution because the data may not be truly representative for the original sample. Therefore, our assay had to have a high sensitivity combined with a minimum number of non-homogeneously amplified miRNA and mRNA assays. To achieve this, we combined the already sensitive multiplex stem-loop cDNA approach with the TaqMan-based linear pre-amplification method, both from ABI. To validate the sensitivity and linear and homogeneous nature of this combined technique, we performed comparative tests between serially diluted non-amplified and multiplexed pre-amplified cDNA from total RNA of pooled primary breast

tumors, as described before[9]. The homogeneity of amplification was set at a cut-off of 2 Ct, i.e., for an assay to be considered homogeneously amplified, the number of cycles that were required after pre-amplification should be within a 2 Ct range of the number of cycles that were required for the non-amplified material. After adjusting for the median 15.5 Ct gain due to the pre-amplification procedure, data of 11 miRNA assays were outside this range (**Table 2**, lower). After testing the 43 miRNAs in a multiplex cDNA PCR reaction in our patient cohort of 50 CTC samples, data of 2 additional miRNAs (*hsa-miR-10b* and *RNU43*) had to be discarded because they generated very poor amplification curves. Finally, the PCR efficiency of 2 of the remaining 30 assays was outside our set range of 75% to 125% (*hsa-miR-135b*, 135%, and *hsa-miR-452#*, 73%) and these miRNAs were therefore also excluded from our final analyses (**Table 2**, column 6). A summary of the results of these quality control experiments, which left us with 28 potentially breast CTC–specific miRNAs that could be measured reliably after our multiplexed cDNA followed by the pre-amplification procedure, is listed in **Table 2**.

Finally, when implementing an assay into clinical diagnostics, it is important that data can be compared in-between qRT-PCR sessions. For our mRNA measurements, we have previously shown that the data are reproducible using the pre-amplification procedure from ABI9. To certify that the miRNA data generated with these assays and the multiplex pre-amplification procedure were also reproducible between different qRT-PCR sessions, a control RNA sample consisting of 300 pg total RNA of a pool of breast tumors was included in each session. The relative expressions (average delta Ct ± 95% CI) of the 28 miRNAs measured in this control sample in 28 independently performed multiplexed pre-amplified qRT-PCR sessions (**Figure 1**) with a median coefficient of variation (CV) at the absolute Ct level of 6%, ranging from 3% for *hsa-miR-200a#* to 15% for *hsa-miR-184*, illustrate the robustness of our method.

mRNAs and miRNAs differentially expressed between CTC preparations and leukocytes

The miRNA analyses showed that of the 446 miRNAs investigated, 28 miRNA transcripts could be measured reliably and linearly in a multiplex pre-amplification reaction with an anticipated more than 10-fold (median 160-fold) higher expression in CTCs relative to blood-derived leukocytes (**Table 2** and **Supplementary Table 2a**). Of these 28 small RNAs, only 1 miRNA (*hsa-miR-183*) was higher expressed in the 32 samples that contained at least 5 CTCs than the 9 samples without detectable CTCs after the CellSearch procedure. At an FDR of 10%, 9 additional miRNA transcripts were more abundantly expressed in the preparations that contained at least 5 CTCs relative to WB preparations of HBDs prior to (n = 14) or after (n = 8) CellSearch enrichment (**Table 3a**).

For the mRNA transcripts, we used the approach described in detail before[9]. Of the thus in silico selected 85 putatively CTC-specific and/or for breast cancer clinically relevant genes

(**Supplementary Table 2b**), 55 were at an FDR of 10% significantly higher expressed in the 32 samples of patients with at least 5 CTCs than 31 CellSearch-enriched HBD samples. A gene expression call rate of 55 of 85 (65%) is within the limits of what can be expected for a profiling study[297]. In addition to these 55 mRNA transcripts, another 6 mRNA transcripts were more abundantly present in the 32 samples that contained at least 5 CTCs relative to the 14 WB samples from HBDs prior to CellSearch enrichment. Of the 55 mRNA transcripts, 14 were also more abundantly expressed in the 32 samples with at least 5 CTCs relative to the 9 enriched metastatic breast cancer blood samples without detectable CTCs. Finally, only 6 genes, including the 2 leukocyte control genes *PTPRC* (CD45) and *BST1*, were found to be significantly higher expressed in the 31 CellSearch-enriched HBD samples than the 32 patient samples with at least 5 CTCs (**Table 3b**).

Unsupervised hierarchical clustering to identify clusters of patients according to gene expression patterns

Next, unsupervised 2-dimensional average linkage hierarchical cluster analysis[294] was done to compare the gene expression profiles of our 50 patients. For this, we used the 65 genes (55 mRNA and 10 miRNA transcripts) that were at a 10% FDR more abundantly expressed in CellSearch-enriched fractions of the 32 patients with at least 5 CTCs (**Table 3** and **Figure 2**). This analysis resulted in a clustering of 4 groups of patients with a clear discrimination between **patient cluster 1** and **patient clusters 2 to 4**. The median number of counted CTCs for cluster 1 was 1 (range: 0 – 173) CTC; for cluster 2, 14 (0 – 138) CTCs; for cluster 3, 41 (0 – 2, 262) CTCs; and for cluster 4, 74 (0 – 886) CTCs (**Figure 2**).

About the gene clustering, 5 gene clusters with a correlation more than 0.2 could be identified. In the largest 18-gene cluster (**gene cluster 1**), "signaling" was the most significant common category for 12 genes (*MUCL1, FGFR4, FGFR3, ERBB4, CXCL14, PLOD2, PIP, TFF3, FKBP10, IGFBP2, TIMP3,* and *PLAU*) as identified by DAVID[295-296] analysis (3.9-fold enriched, P = 0.0014). In addition to these signaling genes, this gene cluster contains some potentially interesting drug targets such as *ERBB4, FGFR3,* and *FGFR4*.

The second-gene cluster (**gene cluster 2**, correlation 0.40) is characterized by luminal genes, such as *CCND1*[226], *ESR1, KRT18*[226], and *MUC1*, of which *MUC1* has previously been used by others for the detection of CTCs in breast cancer[80,109,298-300]. At an enrichment of 8.0-fold, Benjamini *P* = 0.008, "mutagenesis site," i.e., genes with mutational hot spots, was the most significant category identified by DAVID for 6 genes (*MUC1, CCND1, KRT18, ESR1, CEP55*, and *FEN1*) in this 7-gene cluster.

One distinct gene cluster (**gene cluster 3**, correlation 0.35) was responsible for the association with the absence of CTCs, i.e., patient cluster 1. This 14-gene cluster holds in addition to the

previously identified CTC-specific genes *KRT19, AGR2, S100A16,* and *KRT7,* and as could be expected *TACSTD1*, the gene encoding EpCAM, the antigen that was used to enrich for CTCs, also the miRNAs *hsa-miR- 452* and *hsa-miR-34a*.

Notably, the miRNA-cluster (**gene cluster 4**, correlation 0.20) containing *hsa-miR-183, hsa-miR-184, hsa-miR-379,* and *hsa-miR-424* shows an expression pattern that seems to be inversely related to the "mutagenesis" gene cluster 2—which includes *ESR1-*, the gene that encodes for the ER. This suggests that these miRNAs might be negatively regulated by ER or, *vice versa*, that these miRNAs negatively regulate ER.

Although no specific category was identified by DAVID as significantly enriched in the last cluster (**gene cluster 5**, correlation 0.20), this cluster seems to be dominated by genes associated with cell-cycle progression and proliferation such as *DUSP4* (*MKP2*[301]), *KIF11*, *KPNA2*, and *MKI67*. Interestingly, a putative stem cell marker (*ITGA6*[255]) is also included in this last cluster.

To ascertain that the signals we generated were indeed tumor CTC-specific, we also performed a clustering analysis with inclusion of the 14 whole blood HBDs (WB-HBD) from which we had data from both the mRNAs and miRNAs (**Supplementary Figure 1**). These HBDs (marked in green below the cluster) indeed clustered closely together. Also, the patients from patient cluster 1 (**Figure 2**, and marked in red below the cluster diagram in **Supplementary Figure 1**), which were characterized by the lack of expression of epithelial marker genes, remained clustered together, next to the HBD cluster.

To further validate that our identified 65-gene expression profile is able to clearly discriminate between signals derived from leukocytes that remain after CellSearch enrichment and signals derived from epithelial cells, we performed a proof-of-principle spiking experiment. For this, gene expression profiles of cells from 4 different breast cancer cell lines were compared with those of HBD samples of 5 different healthy volunteers and an HBD sample of a healthy volunteer in which the RNAs of the 4 different tumor cell lines were spiked in a final quantity equivalent to approximately 1 CTC (approximately 10 pg) per 1.5 mL blood. As can be appreciated from **Supplementary Figure 2**, a clear distinction can be seen between mixed and unmixed HBD and cell line samples. More importantly, no clear distinction can be seen between the final expression data by using RNA of the cell lines and the cell lines mixed with RNA from HBD.

These data point to a lack of contribution of the leukocytes to the overall gene expression results and confirm that our molecular CTC profile is indeed able to discriminate between signals from leukocytes and epithelial-specific signals from CTCs.

Associations of the CTC molecular profile with primary tumor characteristics

For the association of the molecular profile with primary tumor characteristics, we continued with the 36 patients in patient clusters 2 to 4. These patients displayed a molecular CTC profile

with very distinct patterns from the 14 patients in patient cluster 1, which were characterized by the lack of expression of epithelial marker genes. Detailed clinicopathological information of our patient cohort, subdivided in 2 groups (patient cluster 1 versus clusters 2 to 4) on the basis of our molecular CTC–specific profile, is given in **Table 1**. There were no differences between both groups in terms of nodal status, tumor size, histological tumor type, grade, ER, PR, and HER2 status. The only significant association with clinical information was that the patients of clusters 2 to 4 displayed a 2-fold higher rate of having both visceral and non-visceral metastases, as opposed to only visceral or non-visceral metastasis for the patients of cluster 1.

Almost identical results were obtained when the associations of primary tumor and patient characteristics were studied on the basis of CTC count subdivided in 2 groups (patients with less than 5 CTCs versus patients with at least 5 CTCs; **Supplementary Table 1**).

Associations of gene transcripts measured in CTCs with current drug targets

Although we could not measure *PGR* transcripts reliably in the CTCs due to the relatively high *PGR* levels present in the contaminating leukocytes, we could measure *ESR1* and *ERBB2* mRNA transcript levels, the genes for ER and HER2, respectively, in the CellSearch-enriched CTCs. *ESR1* and *ERBB2* expression levels measured in the 36 patients from clusters 2 to 4 with expression of epithelial marker genes and compared with ER and HER2 status of the primary tumor as assessed by routine pathological immunohistochemical procedures (with additional FISH for the HER2++ cases), respectively, are shown in **Figure 3**.

Comparison of gene profiles measured in the CTCs and corresponding primary tumors of metastatic breast cancer patients

We could retrieve 8 primary tumor tissues (3 x FFPE and 5 x FF) of our cohort of patients with at least 5 CTCs at the time of metastatic disease (median: 174, range 7 – 2,262 CTCs). We measured the 65 genes of our mRNA and miRNA panel in these tissues after adjusting levels measured in FFPE to those measured in FF.

From the unsupervised average linkage correlation clustering (**Figure 4**), it became clear that most CTC samples clustered well with the corresponding primary tumor tissue (T) and that the clustering was not dependent on the origin of the primary tissue (FF or FFPE).

DISCUSSION

In this study, we describe a robust method to simultaneously determine the expression of 65 epithelial tumor cell–specific miRNA and mRNA expression levels in CTCs enriched by

CellSearch. The rationale of our study using the CellSearch technique as a starting point was to develop a simple PCR-based molecular characterization that can be performed on material obtained in a clinical setting. Because the CellSearch method is currently the only FDA-approved semi-automated method to capture CTCs, taking CellSearch-enriched CTCs as a starting point for our method will enable its implementation in clinical studies and broadens its application possibilities. However, although the EpCAM-based enrichment employed by the CellSearch technique eliminates a large proportion of leukocytes (approximately 4-log depletion), there are still considerable quantities of leukocytes present after this enrichment[9]. This remaining leukocyte contamination, together with the low frequency of CTCs, forms a challenge when aiming to characterize CTCs by the expression of multiple genes. Despite these challenges, our data indicate that we have succeeded to measure true epithelial tumor cell–specific genes in CTCs with our CTC-specific 65-gene panel, and managed to avoid generation of a predominant leukocyte–derived signal. First, by only selecting genes highly expressed in breast cancer samples and not, or at a much lower level, in blood from HBDs. Second, by validating the true epithelial-specific expression with clustering analyses, which showed that based on the expression of the 65 genes of our molecular profile, the HBDs and breast cancer patients without detectable CTCs clustered closely together and could be clearly separated from the breast cancer patients with detectable CTC numbers (**Figure 2** and S**upplementary Figure 1**). In addition, after using our 65-gene profile, most CTC samples clustered well with the corresponding primary tumor tissue (**Figure 4**). Finally, as a proof of principle, we showed that profiling with our 65- gene panel before and after spiking RNA of HBDs with RNA from 4 different cell lines in a final quantity equivalent to approximately 1 CTC per 1.5 mL blood clearly separated the mixed and unmixed cells (**Supplementary Figure 2**). These data confirmed that our molecular 65-gene profile is indeed able to discriminate between signals from leukocytes and epithelial-specific signals from CTCs.

On the basis of the expression levels of this 65-gene profile, we could identify 4 different patient clusters characterized by 5 distinct gene clusters (**Figure 2**). One distinct 14- gene cluster (gene cluster 3) was responsible for the association with the absence of CTCs. To further appreciate the strength of our 65-gene profile in relation to CTC count, it should be noted that the CTC counts were derived from 1 of the 2 aliquots of 7.5 mL blood samples that were processed with the CellSearch Epithelial Kit, whereas the other aliquot used for the molecular profiling was processed with the CellSearch Profiling Kit. This inevitably introduced stochastic variation between the tumor cell content in the 2 aliquots, which is more profound in the lower range of CTC counts. Discussion has also started about the actual number of isolated CTCs differing between the enumeration and profiling kit[302]. The given cell counts could therefore only be used as a rough estimate for our molecular profile. Nevertheless, with 14 of 55 mRNAs

(25.4%) and only 1 (*hsa-miR-183*) of 28 miRNAs (3.6%) higher expressed in the 32 samples that contained at least 5 counted CTCs compared with the 9 samples without detectable CTCs after the CellSearch enrichment procedure with the Epithelial Kit, it seems to be easier to discriminate between CTC-specific and leukocyte-derived mRNAs than between CTC-specific and leukocyte-derived miRNAs. Possibly, the detected miRNA transcripts were derived from cell fragments present in the blood of cancer patients without detectable intact CTCs. The fact that we could measure them might be associated with the remarkable stability of miRNA transcripts in blood[303]. Indeed, the detection of an additional 9 of 28 (32.1%) miRNAs that were higher expressed in breast cancer patients without detectable CTCs than in WB preparations of HBDs prior to (n = 14) or after (n = 8) CellSearch enrichment, compared with an additional 6 of 55 (10.9%) for mRNAs, further supports this thought. For these reasons, we felt confident to continue our analyses with those samples that did contain CTCs according to our molecular profile (patient clusters 2 to 4 in **Figure 2**), irrespective of the CTC count in the blood sample that was processed in parallel with the CellSearch Epithelial Kit.

To show the potential clinical utility of measuring these 65 marker genes in CTCs, we had a further look in the data we generated with our molecular profiling on the levels of 2 well-known genes in breast cancer, ER and HER2 (**Figure 3**). For 1 of the patients whose primary tumor was assessed to be ER negative, a clearly positive *ESR1* signal was detected in the CTCs (CTC087 in **Figure 2**) obtained at the time of metastatic disease 7 years after surgical removal of the primary tumor. However, and perhaps even more disturbing, in 11 of 30 patients (37%) whose primary tumor was ER-positive, no detectable *ESR1* transcript levels were measured in the CTCs obtained 1 to 149 months after primary surgery. Thus, although according to the primary tumor characteristics, these patients would have an indication for anti-hormonal treatment, no benefit might be expected from this therapy on the basis of these CTC characteristics. However, due to the limited number of 4 of these 11 patients that were actually treated with anti-hormonal treatment, no conclusion can be drawn yet on the efficacy of hormonal treatment in these patients with *ESR1*-negative CTCs and ER-positive primary tumors. Of note in this respect is that half the patients with relatively high CTC-associated *ESR1* levels expressed relatively low levels of *TFF1* (**Figure 2**). *TFF1* is a gene under the control of ER. Perhaps, assessment of simultaneous *TFF1* expression in CTCs might be able to identify a subset of patients with ER-positive CTCs with functionally active ER, who are more likely to respond to hormonal treatment[304].

Similarly, the CTCs of at least 4 patients with HER2-negative primary tumors showed to be positive at the time of metastatic disease, whereas in 2 patients with an HER2-positive primary tumor, no detectable *ERBB2* mRNA could be measured in their CTCs. For those 4 patients with *ERBB2*-positive CTCs, anti-HER2 therapy is not indicated on the basis of primary tumor

characteristics, whereas this treatment could nonetheless be beneficial based on their CTC characteristics.

No clinically relevant cut point has yet been established for ER and HER2 measured by qRT-PCR in CTCs. Nevertheless, such discrepancies between the levels of ER and HER2 measured in the primary tumor and metastases and CTCs have been described before at both the mRNA and the protein level[80,302,305-308], indicating that the findings with our multigene measuring technique may indeed be relevant, not only for ER and HER2 but also for the other markers included in our panel.

After clustering CTCs and primary tumors based on the expression of all 65 genes, the only obvious discrepancy we observed between the CTCs and the corresponding primary tumors of 8 different patients concerned patient 2. With 2,262 CTCs, this was the patient with the highest number of CTCs, and thus with an expected negligible effect of the presence of contaminating leukocytes in the expression analysis. The primary tumor of this patient was originally assessed as lobular, low-grade, pT2, ER-positive, PR-positive, and HER2-negative. Such a lobular tumor, with scattered epithelial cell clusters, and associated contaminating RNA from many stromal cells[309], may have contributed to this poor correlation with the expression profile of the high number of CTCs.

Although the high degree of homology in the gene expression profiles of CTCs and corresponding primary tumors was reassuring, discrepancies in expression of individual genes, such as for *ESR1* in patients 5, 6, and 8 (**Figure 4**), were detected. Another example in this respect is patient 8, from whom the CTCs expressed much higher levels of markers associated with cell-cycle progression and proliferation such as *DTL*, *KIF11*, *KPNA2*, *KIF11*, and *MKI67* than the primary tumor (**Figure 4**). Such differences between the primary tumor and CTCs isolated at the time of metastatic disease might prove clinically relevant and thus deserve further research.

In summary, by excluding genes with a relatively higher expression in leukocytes, our CTC-specific 65-gene set, consisting of 55 mRNAs and 10 miRNAs, is able to generate a huge amount of highly relevant CTC-specific data, even in the presence of a leukocyte background signal derived of leukocytes cocaptured with CTCs when using the CellSearch procedure.

Although assessed in a relatively small series, we found discrepancies in several important factors such as ER, HER2, and other genes between primary tumor tissue and CTCs. This is not surprising given the time elapsing between primary tumor resection and CTC collection, which occurred at the diagnosis of metastatic disease, and the fact that several patients received prior adjuvant systemic therapy. The discrepancies in molecular characteristics between primary tumor tissue and CTCs clearly stress the importance of further studies on molecular characterization of CTCs.

Table 1

Patients and their clinico-pathological characteristics

Characteristic	No. of patients	%[a]	Primary tissue	Molecular profile			CellSearch		
			Patient cluster 2-4*	Patient cluster 1	Patient cluster 2-4	P^b	<5 CTCs	≥5 CTCs	P^b
All patients	**50**	**100%**	**8**	**14**	**36**		**18**	**32**	
Time between primary surgery and CTC sampling									
≤5 years	23	46%	5	6	17	*1.00*	6	17	*0.48*
>5 years	15	30%	1	4	11		6	9	
Unknown or primary not removed	12	24%	2	4	8		6	6	
Age at CTC sampling									
≤50 years	10	20%	0	4	6	*0.44*	6	4	*0.14*
>50 years	40	80%	8	10	30		12	28	
Menopausal status									
pre	16	32%	0	6	10	*0.51*	9	7	*0.12*
post	30	60%	7	8	22		9	21	
Grade (Bloom-Richardson)									
I, well-differentiated	10	20%	3	3	7	*0.73*	5	5	*0.43*
II, moderately differentiated	17	34%	2	5	12		6	11	
III, poorly differentiated	16	32%	3	3	13		4	12	
Pathological tumor size									
pT1, <2 cm	10	20%	1	3	7	*0.36*	6	4	***0.04***
pT2-4, ≥2 cm	31	62%	7	5	26		6	25	
Lymph nodes involved									
no	12	24%	2	4	8	*1.00*	7	5	*0.16*
yes	29	58%	5	9	20		9	20	
ER status[c]									
negative	12	24%	3	6	6	*0.07*	7	5	*0.09*
positive	38	76%	5	8	30		11	27	
PgR status[c]									
negative	24	48%	5	9	15	*0.20*	11	13	*0.24*

positive	22	44%	3	4	18		7	15	
HER2/neu status[c]									
negative	29	58%	8	7	22	*1.00*	9	20	*1.00*
positive	12	24%	0	3	9		4	8	
Histological type									
lobular	11	22%	3	3	8	*1.00*	4	7	*1.00*
ductal	35	70%	5	9	26		11	24	
Adjuvant chemotherapy									
no	34	68%	5	11	23	*0.50*	14	20	*0.35*
yes	16	32%	3	3	13		4	12	
Adjuvant hormonal therapy									
no	31	62%	7	11	20	*0.20*	14	17	*0.13*
yes	19	38%	1	3	16		4	15	
Any adjuvant therapy									
no	27	54%	5	5	22	*0.13*	7	20	*0.14*
yes	23	46%	3	9	14		11	12	
Site of metastasis									
visceral	12	24%	1	6	6	***0.05***	8	3	***0.02***
non-visceral	7	14%	0	3	4		2	5	
both	31	62%	7	5	26		8	23	

[a]Percentages <100% can be attributed to missing cases, [b]*P* for 2-tailed Fisher's exact test, [c]As retrieved from pathology reports

Table 2

Quality control qRT-PCR miRNA before and after multiplexed pre-amplification

miRNA assay specific for epithelial breast tumor cells	**Serially diluted breast tumor pool 1** median Ct in uniplex cDNA reaction **without** pre-amplification	**Serially diluted breast tumor pool 2** median Ct in multiplex cDNA reaction **with** pre-amplification	**Ct gain due to multiplexed pre-amplification** relative to unamplified uniplex cDNA reaction	**PCR efficiency in uniplex cDNA reaction *without* pre-amplification**	**PCR efficiency in multiplex cDNA reaction *with* pre-amplification**	**Reason for exclusion**
hsa-miR-100	28.67	11.71	16.96	102%	98%	
hsa-miR-10a	31.03	13.86	17.20	98%	108%	
hsa-miR-125b	28.33	12.65	15.68	95%	100%	
hsa-miR-135a	34.89	17.60	17.29	115%	77%	
hsa-miR-141	29.07	12.36	16.71	97%	105%	
hsa-miR-183	32.99	17.57	15.42	92%	120%	
hsa-miR-184	35.66	20.21	15.45	88%	123%	
hsa-miR-187	31.77	17.00	14.77	105%	93%	
hsa-miR-193b	34.05	19.14	14.94	99%	107%	
hsa-miR-200a#	32.73	18.09	14.64	96%	109%	
hsa-miR-200b	27.96	12.65	15.31	97%	89%	
hsa-miR-200c	24.32	10.32	14.00	96%	75%	
hsa-miR-205	29.98	14.15	15.83	94%	93%	
hsa-miR-210	30.15	13.18	16.97	86%	95%	
hsa-miR-214	28.73	12.93	15.80	101%	79%	
hsa-miR-31	29.87	12.40	17.47	101%	76%	
hsa-miR-34a	33.45	17.96	15.49	104%	103%	

hsa-miR-375	30.47	13.24	17.23	90%	75%	
hsa-miR-379	35.42	19.97	15.45	98%	103%	
hsa-miR-424	35.76	19.52	16.24	112%	73%	
hsa-miR-452	35.85	20.67	15.18	108%	94%	
hsa-miR-455	31.00	16.35	14.65	100%	97%	
hsa-miR-497	33.98	17.20	16.78	98%	79%	
hsa-miR-565	30.54	13.98	16.56	98%	123%	
hsa-miR-615	32.47	17.04	15.43	100%	110%	
hsa-miR-9#	33.00	18.59	14.41	104%	117%	
hsa-miR-95	34.47	19.41	15.06	82%	116%	
RNU19	31.78	15.81	15.97	108%	88%	
RNU43	31.02	13.63	17.39	83%	117%	poor amplification curves
hsa-miR-10b	31.71	16.79	14.92	98%	103%	poor amplification curves
hsa-miR-135b	28.86	14.16	14.70	99%	132%	poor PCR efficiency
hsa-miR-452#	31.92	17.98	13.94	105%	73%	poor PCR efficiency
hsa-miR-149	29.82	10.35	19.47	111%	72%	not homogenously amplified
hsa-let-7°	33.61	14.30	19.31	99%	84%	not homogenously amplified
hsa-let-7i	33.14	14.77	18.37	88%	116%	not homogenously amplified
hsa-miR-196a	30.33	17.06	13.27	97%	81%	not homogenously amplified
hsa-miR-199a#	28.18	19.21	8.97	99%	123%	not homogenously amplified
hsa-miR-200a	29.62	17.18	12.44	103%	88%	not homogenously amplified
hsa-miR-203	30.13	11.79	18.34	97%	91%	not homogenously amplified
hsa-miR-429	34.65	25.65	9.00	98%	111%	not homogenously amplified
hsa-miR-511	32.01	18.77	13.24	76%	94%	not homogenously amplified
hsa-miR-99a	34.94	13.13	21.81	89%	104%	not homogenously amplified
snoR-12 (RNU6B)	33.91	16.20	17.71	93%	94%	not homogenously amplified

NOTE (Table 2): RNA of a pool of primary tumors of patients who had not received neoadjuvant systemic therapy was diluted to 100, 25, and 6.25 ng (pool 1) and to 300, 75, and 18 pg (pool 2), respectively. Forty-three serially diluted aliquots of the 3 dilutions of pool 1 were used in 3 x 43 individual RT reactions and after a 20-fold dilution, measured at 25, 6.25, and 1.56 ng in 43 individual miRNA-specific PCRs. For pool 2, only 1 sample of each dilution was used to measure all 43 miRNAs in a multiplex cDNA reaction, resulting, after an additional pre-amplification and dilution step, in a final input of 6, 1.25, and 0.38 pg for each individual miRNA-specific PCR reaction. For all miRNAs, a minus RT sample at the second dilution was included that proved to be negative for all assays.

The median reduction in the number of PCR cycles that were required to generate a signal after pre-amplification was 15.5. The homogeneity of amplification was therefore set at 15.5 ± 2 Ct. All assays that gained less than 13.5 or more than 17.5 Ct after this multiplexed pre-amplification procedure were considered as not homogenously amplified. PCR efficiencies were calculated from the data, thus generated with the 3 serially diluted samples with PCR efficiency between 75% and 125% considered acceptable.

Table 3a

Evaluation of miRNA transcript levels in HBDs and CTC samples

	≥5 CTCs (n = 32)		HBD_CS (n = 8)				HBD_WB (n = 14)				0 CTCs (n = 9)			
	Normalized on the median level of all miRNAs		Normalized on the median level of all miRNAs		Ratio ≥5 CTCs vs. HBD_CS	P-value ≥5 CTCs vs. HBD_CS	Normalized on the median level of all miRNAs		Ratio ≥5 CTCs vs. HBD_WB	P-value ≥5 CTCs vs. HBD_WB	Normalized on the median level of all miRNAs		Ratio ≥5 CTCs vs. 0 CTCs	P-value ≥5 CTCs vs. 0 CTCs
Gene symbol	Av.	SEM	Av.	SEM			Av.	SEM			Av.	SEM		
hsa-miR-379	0.25	0.17	0.05	0.00	**4.77**	**9.40E-05***	0.05	0.00	**4.77**	**1.30E-06***	0.30	0.28	0.84	7.30E-01
hsa-miR-210	10.57	4.69	1.30	2.78	**8.12**	**1.40E-04***	1.32	0.73	**7.99**	**4.30E-06***	11.10	7.77	0.95	8.10E-01
hsa-miR-424	1.87	2.32	0.62	0.00	**3.05**	**1.60E-04***	0.62	0.00	**3.05**	**3.10E-06***	2.96	2.69	0.63	1.50E-01
hsa-miR-452	0.04	0.02	0.01	0.00	**4.72**	**2.80E-03***	0..01	0.00	**3.06**	**3.00E-03***	0.02	0.01	1.80	2.80E-01
hsa-miR-184	0.04	0.02	0.01	0.00	**6.16**	**3.30E-03***	0.01	0.00	**5.76**	**2.60E-04***	0.03	0.01	1.59	4.10E-01
hsa-miR-497	1.43	3.76	0.50	0.19	**2.86**	**4.80E-03***	0.45	0.03	**3.15**	**9.00E-05***	1.13	9.21	1.26	4.90E-01
hsa-miR-183	0.33	0.34	0.09	0.02	**3.49**	**6.50E-03***	0.10	0.00	**3.30**	**2.70E-03***	0.13	0.06	2.55	3.30E-02
hsa-miR-565	13.74	5.66	5.61	2.08	**2.45**	**2.20E-02***	4.41	0.12	**3.12**	**1.80E-04***	23.23	10.81	0.59	4.40E-01
*RNU19**	2.11	2.02	1.30	0.28	**1.62**	**2.60E-02***	1.27	0.00	**1.66**	**1.60E-03***	4.24	2.86	0.50	5.30E-02
hsa-miR-34a	8.85	2.14	6.52	0.00	**1.36**	**8.20E-02***	6.52	0.00	**1.36**	**3.10E-02***	10.85	3.48	0.82	3.40E-01
hsa-miR-125b	2.96	0.46	3.12	0.44	0.95	2.20E-01	3.12	0.15	**0.95**	**2.90E-02***	2.83	0.00	1.04	7.90E-01
hsa-miR-214	1.00	149.28	0.96	0.26	1.04	3.70E-01	1.14	0.82	**0.88**	**1.60E-02***	0.96	0.00	1.04	9.00E-01
hsa-miR-95	0.01	0.00	0.01	0.00	0.96	4.30E-01	0.01	0.00	**0.58**	**3.00E-02***	0.01	0.00	0.79	9.40E-01
hsa-miR-31	3.12	0.22	4.34	0.87	**0.72**	**2.00E-03***	3.69	0.20	**0.85**	**1.30E-02***	3.07	0.00	1.01	9.00E-01
hsa-miR-205	1.11	0.00	1.17	0.36	**0.95**	**7.20E-03***	1.20	0.26	**0.93**	**1.60E-02***	1.11	0.00	1.00	9.00E-01
hsa-miR-200c	7.79	1.83	10.60	2.58	**0.74**	**2.20E-02***	12.27	1.20	**0.64**	**2.40E-02***	8.24	1.06	0.95	2.60E-01
hsa-miR-200b	1.78	1.32	1.81	0.60	0.98	8.20E-02	2.17	0.33	**0.82**	**3.10E-02***	1.81	1.14	0.98	8.60E-01

hsa-miR-455	0.15	0.02	0.17	0.02	0.85	9.40E-02	0.18	0.02	**0.80**	**3.60E-02***	0.14	0.00	1.07	6.80E-01
hsa-miR-187	0.05	0.01	0.05	0.00	1.13	1.60E-01	0.036	0.00	0.88	2.80E-01	0.05	0.01	0.93	8.40E-01
hsa-miR-193b	1.24	3.99	1.03	3.50	1.21	1.80E-01	1.04	1.60	1.19	9.00E-01	1.41	3.20	0.88	5.50E-01
hsa-miR-141	2.62	1.22	2.57	0.30	1.02	2.20E-01	2.78	0.16	0.94	5.60E-02	2.47	0.32	1.06	9.90E-01
hsa-miR-100	3.11	0.84	3.30	0.59	0.94	2.30E-01	2.97	0.06	1.04	1.70E-01	2.81	0.00	1.10	3.30E-01
*hsa-miR-200a**	2.05	1.01	1.73	0.00	1.19	2.30E-01	1.98	0.27	1.04	3.00E-01	1.76	0.17	1.17	2.30E-01
hsa-miR-375	1.66	3.52	1.32	1.62	1.25	2.90E-01	1.25	0.46	1.33	4.20E-01	1.31	2.40	1.26	6.00E-01
hsa-miR-615	0.12	0.05	0.09	0.01	1.29	4.90E-01	0.09	0.00	1.31	6.50E-01	0.12	0.07	0.99	4.40E-01
hsa-miR-10a	0.28	0.21	0.16	0.01	1.73	5.30E-01	0.20	0.02	0.41	4.80E-01	0.44	0.39	0.65	3.90E-02
hsa-miR-135a	0.01	0.00	0.01	0.00	0.92	7.20E-01	0.01	0.00	0.84	8.50E-01	0.01	0.00	1.00	5.20E-01
*hsa-miR-9**	0.03	0.01	0.03	0.00	1.14	8.50E-01	0.04	0.00	0.93	8.30E-01	0.04	0.01	0.93	7.60E-01

NOTE: The expression levels of 28 miRNAs (Table 3a) and 87 mRNAs (Table 3b) were measured in RNA isolated with the CellSearch Profiling Kit, as described in the Materials and Methods section. To identify putative CTC-specific genes, transcript levels were compared between various groups with and without CTCs as established by the CellSearch Epithelial Kit in a separate 7.5mL blood sample. The average (av.) expression±SEM is given by the number of samples indicated at the top in the table. The 2-tailed Mann–Whitney U test was used to identify genes with a significantly different expression level between groups. To compensate for multiple testing, an FDR of 10% was applied on these statistics. *; significant at a 10% FDR (2-tailed $P < 0.05$), CS; enriched by EpCAM-based CellSearch.

Table 3b

Evaluation of miRNA transcript levels in HBDs and CTC samples

	≥5 CTCs (n = 32)		HBD_CS (n = 8)				HBD_WB (n = 14)				0 CTCs (n = 9)			
	Reference gene set normalized ($x10^3$)		Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_CS	P-value ≥5 CTCs vs. HBD_CS	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_WB	P-value ≥5 CTCs vs. HBD_WB	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. 0 CTCs	P-value ≥5 CTCs vs. 0 CTCs
Gene symbol	Av.	SEM	Av.	SEM			Av.	SEM			Av.	SEM		
KRT19	12.01	10.03	0.01	0.00	**1113.54**	**1.0E-08***	0.03	0.00	**428.90**	**9.9E-07***	0.04	0.01	**318.06**	**1.9E-04***
S100A16	3.90	2.70	0.01	0.00	**293.30**	**1.0E-08***	0.03	0.00	**122.38**	**3.4E-05***	0.03	0.01	**125.09**	**3.5E-04***
AGR2	0.52	0.31	0.00	0.00	**135.71**	**3.3E-07***	0.01	0.00	**98.37**	**3.0E-04***	0.01	0.00	**91.98**	**1.9E-03***
IGFBP5	0.26	0.11	0.00	0.00	**66.98**	**1.0E-07***	0.01	0.00	**25.89**	**1.3E-03***	0.01	0.00	**42.10**	**2.4E-03***
KRT7	12.17	8.39	0.51	0.00	**24.08**	**1.0E-08***	0.84	0.02	**14.49**	**4.9E-04***	0.80	0.14	**15.19**	**2.5E-03***
CLDN3	4.67	2.88	0.18	0.01	**54.44**	**1.2E-07***	0.24	0.00	**19.23**	**7.3E-04***	0.29	0.05	**16.12**	**2.9E-03***
FOXA1	0.58	0.31	0.00	0.00	**152.45**	**1.0E-08***	0.01	0.00	**67.05**	**2.0E-04***	0.01	0.00	**63.80**	**2.9E-03***
LAD1	0.13	0.05	0.00	0.00	**33.58**	**1.0E-07***	0.01	0.00	**12.13**	**8.3E-03***	0.01	0.00	**15.18**	**5.3E-03***
PKP3	0.11	0.04	0.00	0.00	**29.90**	**9.9E-07***	0.00	0.00	**29.90**	**1.3E-04***	0.01	0.00	**18.29**	**5.3E-03***
TFF3	9.36	7.67	0.27	0.00	**35.26**	**2.9E-03***	0.37	0.01	**24.97**	**6.5E-03***	0.38	0.05	**24.74**	**5.9E-03***
SCGB2A2	0.33	0.21	0.00	0.00	**86.02**	**5.3E-05***	0.00	0.00	**86.02**	**1.6E-03***	0.00	0.00	**86.02**	**7.4E-03***
SPDEF	1.14	0.57	0.05	0.00	**22.88**	**2.9E-06***	0.09	0.00	**13.04**	**1.7E-02***	0.09	0..01	**13.13**	**1.6E-02***
IGFBP2	13.75	8.70	1.31	0.00	**10.52**	**1.0E-08***	2.42	0.09	5.68	5.8E-02	1.88	0.32	**7.33**	**9.4E-03***
FKBP10	0.28	0.10	0.02	0.00	**13.40**	**9.9E-07***	0.04	0.00	**7.11**	**2.3E-02**	0.04	0.01	**7.23**	**1.2E-02***
SEPP1	3.69	1.76	0.27	0.00	**13.52**	**1.0E-08***	0.46	0.01	**8.06**	**9.9E-04***	0.70	0.19	5.23	1.8E-02
SCGB1D2	0.23	0.14	0.00	0.00	**59.47**	**6.7E-04***	0.00	0.00	**59.47**	**7.8E-03***	0.00	0.00	59.47	2.4E-02
CRABP2	7.29	4.83	0.38	0.00	**19.16**	**1.0E-08***	0.58	0.01	**12.58**	**8.9E-03***	1.10	0.27	6.64	5.3E-02
TNRC9	0.02	0.01	0.00	0.00	**6.43**	**1.4E-03***	0.00	0.00	**6.43**	**1.3E-02***	0.01	0.00	4.13	1.1E-01
SBEM/MUCL1	0.52	0.30	0.00	0.00	**119.71**	**6.7E-04***	0.01	0.00	**35.30**	**1.0E-02***	0.12	0.07	4.38	2.3E-01

Table 3b(continued)

Evaluation of miRNA transcript levels in HBDs and CTC samples

	≥5 CTCs (n = 32)		HBD_CS (n = 8)				HBD_WB (n = 14)				0 CTCs (n = 9)			
	Reference gene set normalized ($x10^3$)		Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_CS	P-value ≥5 CTCs vs. HBD_CS	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_WB	P-value ≥5 CTCs vs. HBD_WB	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. 0	P-value ≥5 CTCs vs. 0 CTCs
Gene symbol	Av.	SEM	Av.	SEM			Av.	SEM			Av.	SEM		
PIP	0.07	0.03	0.00	0.00	**17.68**	**3.0E-04***	0.00	0.00	**15.61**	**9.9E-03***	0.03	0.01	2.15	6.1E-01
IL17BR3	0.13	0.02	0.08	0.00	**1.69**	**1.0E-08***	0.15	0.00	0.89	1.5E-01	0.08	0.00	1.68	3.3E-01
DTX3	3.77	0.71	2.65	0.00	**1.42**	**1.0E-08***	4.19	0.13	0.90	1.0E-01	4.58	1.05	0.82	7.5E-01
PLAU	0.45	0.09	0.30	0.01	**1.52**	**1.5E-08***	0.46	0.01	0.98	2.0E-01	0.42	0.06	1.08	9.4E-01
FGFR4	0.20	0.04	0.10	0.00	**2.09**	**1.0E-08***	0.17	0.00	1.17	4.7E-01	0.30	0.07	0.69	8.6E-01
ITGA6	54.40	25.94	31.74	1.07	**1.71**	**2.1E-05***	45.25	2.17	1.20	2.0E-01	36.40	5.60	1.49	7.2E-01
DTL	1.29	0.31	0.55	0.00	**2.34**	**1.0E-08***	1.00	0.04	1.29	5.9E-01	0.81	0.13	**1.60**	3.9E-01
Ki67	0.66	0.17	0.32	0.00	**2.09**	**1.0E-08***	0.43	0.01	1.54	2.1E-01	0.36	0.02	1.84	6.9E-01
PTRF	1.94	0.49	0.83	0.00	**2.35**	**1.0E-08***	1.23	0.03	1.58	6.1E-01	1.02	0.09	1.91	3.4E-01
CEP55	0.79	0.20	0.28	0.00	**2.76**	**1.0E-08***	0.48	0.01	1.64	9.4E-01	0.65	0.15	1.21	4.9E-01
MELK	0.64	0.16	0.21	0.00	**3.23**	**1.3E-04***	0.40	0.01	1.66	5.6E-01	0.51	0.13	1.30	2.5E-01
SMA/ACTA1	0.01	0.00	0.00	0.00	**3.91**	**1.9E-02***	0.01	0.00	1.68	9.0E-01	0.00	0.00	3.38	4.5E-01
FEN1	4.91	1.48	2.12	0.10	**2.31**	**1.0E-08***	2.88	0.07	1.70	9.2E-01	3.75	0.98	1.31	4.7E-01
KIF11	3.67	1.41	1.13	0.00	**3.21**	**6.7E-04***	1.77	0.05	2.07	6.9E-01	2.70	0.85	1.36	3.2E-01
KPNA2	18.19	8.71	4.80	0.03	**3.79**	**3.0E-05***	7.57	0.28	2.40	2.4E-01	9.39	2.81	1.94	1.3E-01
CD24	3.60	1.52	1.07	0.03	**3.36**	**3.8E-02***	1.47	0.04	2.44	9.5E-01	2.09	0.51	1.72	4.5E-01
TM4SF13	13.88	8.34	2.29	0.00	**6.06**	**5.3E-05***	5.00	0.28	2.78	3.8E-01	8.01	4.20	1.73	1.9E-01
DUSP4	4.41	2.01	1.11	0.00	**3.98**	**2.8E-02***	1.56	0.03	2.84	8.8E-01	1.11	0.00	3.99	1.2E-01
ESR1	3.62	1.78	0.71	0.00	**5.06**	**1.4E-03***	1.15	0.03	3.15	6.7E-01	1.84	0.58	1.97	3.3E-01

Table 3b (continued)

Evaluation of miRNA transcript levels in HBDs and CTC samples

	≥5 CTCs (n = 32)		HBD_CS (n = 8)				HBD_WB (n = 14)				0 CTCs (n = 9)			
	Reference gene set normalized ($x10^3$)		Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_CS	P-value ≥5 CTCs vs. HBD_CS	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_WB	P-value ≥5 CTCs vs. HBD_WB	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. 0	P-value ≥5 CTCs vs. 0 CTCs
Gene symbol	Av.	SEM	Av.	SEM			Av.	SEM			Av.	SEM		
IGFBP4	37.09	28.32	7.55	0.26	**4.92**	**1.7E-03***	11.41	0.46	3.25	4.1E-01	20.40	11.52	1.82	3.1E-01
MUC1 (EMA)	6.66	3.49	1.25	0.00	**5.31**	**6.7E-04***	1.92	0.05	3.46	4.7E-01	2.46	0.55	2.71	3.9E-01
CTTN/EMS1	16.48	10.46	3.49	0.21	**4.73**	**4.7E-04***	4.48	0.13	3.68	2.9E-01	5.61	1.24	2.94	1.2E-01
KRT17	0.02	0.00	0.00	0.00	**4.66**	**1.1E-02***	0.00	0.00	3.71	2.0E-01	0.01	0.00	3.20	3.4E-01
PLOD2	4.57	2.32	0.62	0.00	**7.32**	**1.2E-02***	1.19	0.05	3.85	1.3E-01	1.24	0.27	3.68	6.5E-02
FGFR3	0.05	0.01	0.01	0.00	**8.63**	**1.0E-08***	0.01	0.00	4.02	2.7E-01	0.01	0.00	5.69	1.0E-01
CXCL14	0.02	0.01	0.00	0.00	**6.16**	**5.7E-03***	0.00	0.00	4.97	1.6E-01	0.00	0.00	5.00	1.7E-01
TIMP3	5.01	2.58	0.61	0.00	**8.21**	**1.0E-08***	1.00	0.03	5.03	1.6E-01	0.71	0.05	7.02	3.3E-02
TFF1 (pS2)	0.02	0.01	0.00	0.00	**5.81**	**1.9E-02***	0.00	0.00	5.81	6.8E-02	0.00	0.00	5.71	2.4E-01
TACSTD1	2.23	1.04	0.17	0.00	**12.98**	**7.9E-06***	0.38	0.02	5.83	7.7E-02	0.66	0.20	3.37	1.9E-01
EEF1A2	1.32	0.57	0.11	0.00	**12.44**	**2.9E-03***	0.22	0.01	5.98	4.2E-02	0.46	0.18	2.86	8.9E-02
ERBB3	0.40	0.15	0.04	0.00	**11.17**	**7.9E-06***	0.06	0.00	6.30	7.0E-02	0.09	0.02	4.38	6.3E-02
KRT18	31.07	25.42	3.73	0.45	**8.33**	**4.7E-05***	4.85	0.14	6.40	7.7E-02	8.56	3.61	3.63	1.0E-01
ERBB4	0.03	0.01	0.00	0.00	**7.87**	**2.9E-03***	0.00	0.00	7.26	3.9E-02	0.00	0.00	7.87	4.9E-02
S100A7	0.03	0.01	0.00	0.00	**7.68**	**3.4E-02***	0.00	0.00	7.68	9.7E-02	0.00	0.00	6.74	3.1E-01
CCND1	2.35	1.23	0.18	0.00	**13.35**	**2.1E-05***	0.29	0.01	8.09	5.2E-02	0.48	0.13	4.86	1.0E-01
CEA(CAM-5)	0.10	0.05	0.00	0.00	**26.83**	**3.0E-04***	0.01	0.00	14.65	5.0E-02	0.01	0.00	12.35	1.2E-01
BST1	464.20	927.07	878.90	4353.2	**0.53**	**3.5E-04***	291.33	34.2	1.59	9.8E-01	211.85	20.19	2.19	1.6E-01
CD29	869.70	13784.	2268.1	1165.7	**0.38**	**8.7E-05***	360.54	76.7	2.41	3.9E-01	415.78	676.83	2.09	1.5E-01

Table 3b (continued)

Evaluation of miRNA transcript levels in HBDs and CTC samples

	≥5 CTCs (n = 32)		HBD_CS (n = 8)				HBD_WB (n = 14)				0 CTCs (n = 9)			
	Reference gene set normalized ($x10^3$)		Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_CS	P-value ≥5 CTCs vs. HBD_CS	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_WB	P-value ≥5 CTCs vs. HBD_WB	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. 0	P-value ≥5 CTCs vs. 0 CTCs
Gene symbol	Av.	SEM	Av.	SEM			Av.	SEM			Av.	SEM		
CD44	1552	5355.0	7748.5	1165.7	**0.20**	**3.5E-06***	808.44	496.	1.92	4.6E-01	864.87	5661.3	1.80	1.9E-01
NOTCH3	1.48	0.55	1.70	0.21	**0.87**	**9.9E-07***	0.30	0.00	4.91	9.0E-02	0.29	0.02	5.13	8.3E-02
PSMD10	4.02	1.08	6.86	0.84	**0.59**	**2.5E-04***	2.59	0.07	1.55	7.7E-01	2.97	0.64	1.35	3.2E-01
PTPRC (CD45)	1968	4821.1	21728.	4262.5	**0.09**	**2.1E-07***	1438.2	803.	1.37	7.1E-01	1182.8	6086.1	1.66	7.9E-01
IGFBP3	1.34	0.09	1.24	0.00	1.08	8.4E-01	2.26	0.09	**0.59**	**1.5E-02***	1.24	0.00	1.08	9.0E-01
Nm23-H1/NME1	45.6	25.49	10.10	0.00	4.52	6.4E-02	18.06	0.95	2.53	1.4E-01	24.59	12.02	1.86	3.1E-02
CCNB1	1.69	0.60	0.40	0.00	4.22	2.0E-01	0.71	0.02	2.36	4.0E-01	0.93	0.23	1.81	1.8E-01
CCNE1	1.82	0.41	1.17	0.00	1.56	9.0E-02	1.96	0.06	0.93	1.9E-01	2.26	0.49	0.80	9.9E-01
CCNE2	2.41	0.74	0.87	0.00	2.76	9.0E-02	1.65	0.06	1.46	8.1E-01	1.15	0.14	2.11	2.5E-01
CD133	0.00	0.00	0.00	0.00	1.13	6.7E-01	0.01	0.00	0.72	4.2E-01	0.00	0.00	1.13	7.9E-01
CDH1	7.47	4.17	1.27	0.01	5.87	9.9E-01	1.96	0.05	3.80	3.7E-01	3.32	1.27	2.25	2.5E-01
COL1A1	0.01	0.00	0.00	0.00	1.50	4.0E-01	0.00	0.00	1.50	5.1E-01	0.00	0.00	1.48	9.1E-01
COL2A1	0.00	0.00	0.00	0.00	1.03	8.4E-01	0.00	0.00	1.03	8.5E-01	0.00	0.00	1.03	9.0E-01
EGFR	0.01	0.00	0.00	0.00	1.76	2.9E-01	0.00	0.00	1.76	4.1E-01	0.01	0.00	1.08	8.6E-01
ERBB2	5.79	3.05	1.24	0.00	4.68	2.0E-01	2.62	0.11	2.21	9.0E-01	2.09	0.38	2.77	3.8E-01
FGFR2	0.14	0.02	0.09	0.00	1.67	2.0E-01	0.16	0.00	0.93	2.0E-01	0.09	0.00	1.67	4.0E-01
GALGT	0.01	0.00	0.00	0.00	2.23	9.0E-02	0.00	0.00	2.23	1.9E-01	0.01	0.00	1.23	5.8E-01
GATA3	166.	136.63	155.43	72.22	1.07	2.7E-01	139.97	14.5	1.19	3.2E-01	119.49	52.73	1.40	9.5E-01
KRTHB1/KRT81	0.01	0.00	0.00	0.00	1.72	2.9E-01	0.01	0.00	1.07	1.2E-01	0.01	0.00	0.98	8.9E-01

Table 3b (continued)

Evaluation of miRNA transcript levels in HBDs and CTC samples

	≥5 CTCs (n = 32)		HBD_CS (n = 8)				HBD_WB (n = 14)				0 CTCs (n = 9)			
	Reference gene set normalized ($x10^3$)		Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_CS	P-value ≥5 CTCs vs. HBD_CS	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. HBD_WB	P-value ≥5 CTCs vs. HBD_WB	Reference gene set normalized ($x10^3$)		Ratio ≥5 CTCs vs. 0	P-value ≥5 CTCs vs. 0 CTCs
Gene symbol	Av.	SEM	Av.	SEM			Av.	SEM			Av.	SEM		
MAGEA3	0.00	0.00	0.00	0.00	1.26	5.3E-01	0.00	0.00	1.26	6.2E-01	0.00	0.00	1.26	6.8E-01
MET	0.04	0.00	0.03	0.00	1.32	2.9E-01	0.05	0.00	0.81	1.0E-01	0.05	0.01	0.88	9.1E-01
MSMB	0.05	0.01	0.02	0.00	2.31	9.0E-02	0.04	0.00	1.23	3.1E-01	0.03	0.00	1.50	8.6E-01
MYL3	0.00	0.00	0.00	0.00	1.00	9.9E-01	0.00	0.00	1.00	9.9E-01	0.00	0.00	1.00	9.9E-01
PLK1	1.37	0.47	0.39	0.00	3.54	2.0E-01	0.64	0.02	2.13	9.5E-01	0.84	0.19	1.62	4.9E-01
PTPRK	0.60	0.21	0.10	0.00	5.78	9.9E-01	0.19	0.01	3.15	4.2E-01	0.13	0.01	4.64	7.0E-02
SELE	0.01	0.00	0.00	0.00	1.92	2.9E-01	0.00	0.00	1.92	4.1E-01	0.00	0.00	1.92	4.9E-01
STK6/AURKA	0.01	0.00	0.00	0.00	1.40	1.4E-01	0.01	0.00	0.90	3.5E-01	0.01	0.00	1.04	6.7E-01
TOP2a	5.17	1.99	1.80	0.00	2.88	6.7E-01	2.93	0.10	1.76	9.0E-01	3.36	0.74	1.54	4.1E-01
TWIST	0.02	0.00	0.01	0.00	1.35	2.9E-01	0.02	0.00	0.79	1.0E-01	0.01	0.00	1.30	8.1E-01
VEGFR2	0.01	0.00	0.00	0.00	2.11	1.4E-01	0.01	0.00	1.13	6.3E-01	0.01	0.00	1.31	6.5E-01

NOTE: The expression levels of 28 miRNAs (Table 3a) and 87 mRNAs (Table 3b) were measured in RNA isolated with the CellSearch Profiling Kit, as described in the Materials and Methods section. To identify putative CTC-specific genes, transcript levels were compared between various groups with and without CTCs as established by the CellSearch Epithelial Kit in a separate 7.5mL blood sample. The average (av.) expression±SEM is given by the number of samples indicated at the top in the table. The 2-tailed Mann–Whitney U test was used to identify genes with a significantly different expression level between groups. To compensate for multiple testing, an FDR of 10% was applied on these statistics. *; significant at a 10% FDR (2-tailed $P < 0.05$), CS; enriched by EpCAM-based CellSearch.

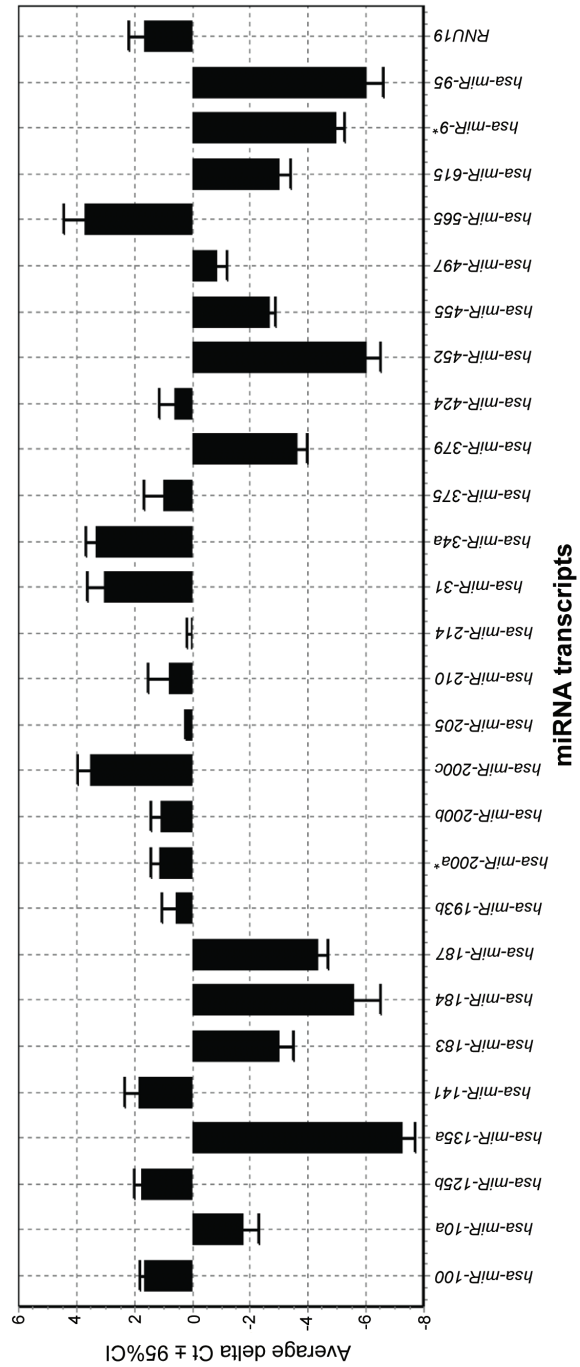

Figure 1

Reproducibility of the measurement of 28 miRNAs in a multiplex cDNA pre-amplified reaction. Bars depict miRNA expression of 28 individual miRNA assays relative to the median level of all miRNA assays (average delta Ct ± 95% CI) as measured in a control sample at 300 pg total RNA input in 28 independently performed multiplexed pre-amplified qRT-PCR sessions.

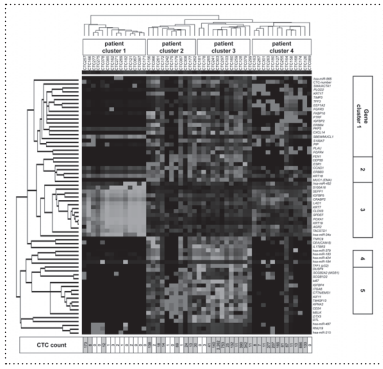

Figure 2: see section 'Color figures'

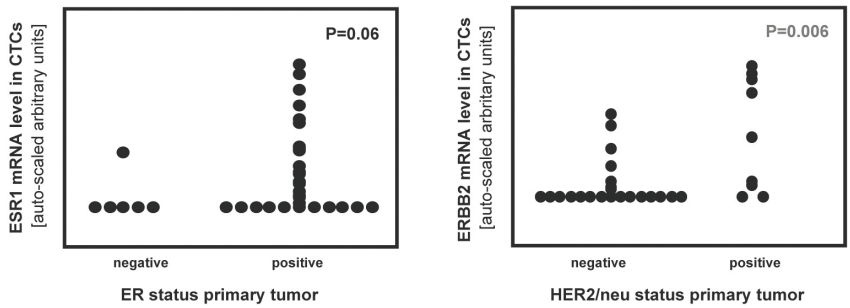

Figure 3

Association between gene expression in CTCs of metastatic breast cancer patients and expression of their corresponding protein in the primary tumor. Gene transcript levels were analyzed with real-time RT-PCR and normalized as described in the Materials and Methods section from CellSearch-enriched fractions of 36 breast cancer patients with molecularly identifiable CTCs. Gene expression levels were compared with expression of their corresponding protein in the primary tumor using the nonparametric Mann–Whitney U test to identify genes with significantly different expression levels between groups.

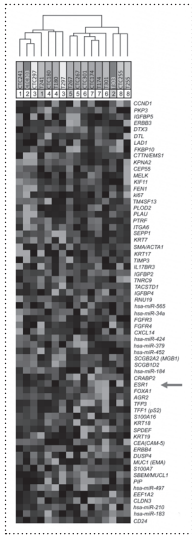

Figure 4: see section 'Color figures'

Supplementary data:

https://docs.google.com/open?id=0B9Etqm_r7T2mNnN5STlLajcyZFU

Chapter 6

Gene expression profiles in circulating tumor cells predict prognosis in metastatic breast cancer patients

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Manuscript in preparation

ABSTRACT

Purpose

A circulating tumor cell (CTC) count is an established prognostic factor in metastatic breast cancer. Besides enumeration, CTC characterization promises to further improve outcome prediction and treatment guidance. After having previously shown the feasibility of measuring the expression of a panel of 96 clinically relevant genes in CTCs in a leukocyte background, we determined the prognostic value of CTC gene expression profiling in metastatic breast cancer.

Patients and methods

CTCs were isolated and enumerated from blood of 130 metastatic breast cancer patients who were about to start first-line systemic, endocrine or chemotherapeutic, therapy. Of these, 103 were evaluable for mRNA gene expression levels as measured by quantitative RT-PCR in relation to time to treatment switch (TTS). Separate prognostic CTC gene profiles were generated by leave-one-out cross validation for all patients and for patients with ≥5 CTCs per 7.5 mL blood, and cut-offs were chosen to ensure optimal prediction of patients in need of an early therapy switch.

Results

In the total cohort, of whom 56% received chemotherapeutic and 44% endocrine therapy, baseline CTC count (≥5 versus <5 CTCs/7.5mL blood) predicted for TTS (Hazard Raio (HR) 2.92 [95% Confidence Interval Cl) 1.71 - 4.95] *P* <0.0001). A 16-gene CTC profile for all patients and a separate 9-gene CTC profile applicable for patients with ≥5 CTCs were generated, which identified those patients with TTS or death within 9 months versus those with a more favorable outcome. Test performance for both profiles was favorable; the 16-gene profile had 90% sensitivity, 38% specificity, 50% positive predictive value (PPV) and 85% negative predictive value (NPV), and the 9-gene profile performed slightly better with 92% sensitivity, 52% specificity, 66% PPV and 87% NPV. In multivariate Cox regression analysis the 16-gene profile was only factor independently associated with TTS (HR 3.15 [95%Cl 1.35 - 7.33] *P* 0.008)

Conclusion

Two CTC profiles were discovered, which both provide prognostic value on top of a CTC count in metastatic breast cancer patients. This study further underscores the potential of molecular characterization of CTC.

INTRODUCTION

In an effort to improve the individualization of metastatic breast cancer treatment, many prognostic and predictive factors have been identified in primary tumors, including mRNA and microRNA (miRNA) expression profiles[137,286,310-312]. However, at the time metastatic disease becomes apparent, the characteristics of metastatic lesions can greatly differ from those of the primary tumor. It has been hypothesized that only specific subclones within the primary tumor have the ability to metastasize, contributing to heterogeneity between primary and metastatic tissue[7]. In addition, genomic instability, a key feature of malignancy, further increases discrepancies between primary tumors and metastatic lesions over time and under pressure of systemic treatment[6]. Heterogeneity between primary tumor and metastasis has been described for a number of clinically highly relevant factors such as ER[47,49], HER2[47-48] and *KRAS*[5,313]. When trying to establish prognostic and predictive factors for metastatic disease, such discrepancies between primary tumor and metastases can be crucial. Consequently, characterization of metastatic tissue rather than that of the primary tumor may lead to better prognostic and predictive models. Unfortunately, metastatic tissue is hard to obtain, which has limited the discovery of predictive and prognostic factors in metastases. Circulating tumor cells (CTCs), which are thought to represent metastatic tissue, can be repeatedly isolated from blood, and present an attractive alternative[314].

A CTC count is an established prognostic factor in metastatic breast cancer[25-27], and a rise or decline in CTC count above or below the clinical cut-off value of 5 CTCs per 7.5 mL blood after the first cycle of systemic therapy is an early predictor of therapy response[25]. Additionally, CTC characterization by immunocytochemistry[43-44], FISH[315], mutation analysis[316] and quantitative reverse transcriptase PCR (qRT-PCR)[38-39] holds great promise as a tool to improve treatment tailoring, but remains challenging. CTCs are extremely rare cells that, even after the sensitive CellSearch® (Veridex™ LLC, Raritan, NJ) EpCAM-based enrichment, need to be identified and characterized among up to a thousand of remaining leukocytes[9]. With respect to CTC characterization by qRT-PCR, one method to overcome this problem of contaminating leukocytes is to focus only on genes that are not, or at a much lower level, expressed in leukocytes. Using these stringent selection methods combined with sensitive pre-amplification, we were able to reliably quantify a CTC-specific gene panel in blood of metastatic breast cancer patients[38].

In this study, we explore the clinical relevance of CTC characterization by assessing the prognostic value of CTC gene expression profiles in metastatic breast cancer patients.

METHODS

Patients

We conducted a prospective trial at six participating hospitals in the Netherlands and Belgium. Inclusion criteria were metastatic breast cancer and start of first-line endocrine or chemotherapeutic treatment; prior adjuvant therapy was permitted. Prior to administration of the first cycle of treatment, two 7.5 mL blood samples were drawn for CTC enumeration and gene expression profiling (for details see next). After 2 – 5 weeks of therapy, two additional 7.5 mL blood samples were taken for CTC enumeration and gene expression profiling. This study was approved by the Erasmus MC and local Institutional Review Boards (METC 2006-248), and all patients gave their written informed consent.

CTC enumeration

For CTC enumeration, 7.5 mL blood drawn in CellSave tubes (Veridex) was maintained at room temperature and processed within 96 hours after collection. Samples were processed on the CellTracks AutoPrep System (Veridex) using the CellSearch Epithelial Cell Kit and CTC counts were determined on the CellTracks Analyzer according to the manufacturer's instructions[8,64,250].

RNA isolation from CTCs, qRT-PCR and quantification of gene transcripts

For gene expression studies, in parallel with the enumeration studies, 7.5 mL of blood was drawn in EDTA tubes and enriched for CTCs on the CellTracks AutoPrep System using the CellSearch Profile Kit (Veridex) within 24 hours after collection. RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen, Venlo, Netherlands), and cDNA synthesis, pre-amplification, PCR and normalization procedures to quantify gene expression levels were performed as described in detail before[38].

Statistical analysis

Primary endpoint was time to treatment switch (TTS), defined as the time elapsed between start of first-line treatment and start of second-line treatment or death, whichever came first. Patients who were alive and had not started second-line treatment were censored at last follow-up date.

Overall survival (OS) was defined as the time elapsed between start of first-line treatment and date of death. All living patients were censored at last follow-up date. Hazard ratios (HR) for TTS and OS were estimated by univariate and multivariate analysis, for the latter of which all variables with $P < 0.05$ in univariate analysis were used.

After quality control and normalization procedures[38] of gene expression data, to establish a prognostic gene score, patients were divided into a poor prognosis and a good prognosis group.

Patients with a therapy switch or death <9 months were classified as the poor prognosis group, and patients alive and with or without a therapy switch ≥9 months after start of treatment were classified as the good prognosis group. The 9-month cut-off was chosen based on the median PFS in first-line metastatic breast cancer patients in the literature[317-318]. In these patients, a leave-one-out cross validation was conducted using the Compound Covariate Predictor (CCP) within Biometric Research Branch ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) starting with the previously established 55 CTC-specific mRNAs[38]. A panel of 16 genes was identified, of which the combined score was used to select a cut-point at which 90% of poor prognosis patients were correctly predicted. The score was calculated by summing the product of the expression and weight, obtained with the CCP, of individual genes. A separate gene profile was constructed in patients with ≥5 CTCs based on all 96 mRNAs in our gene expression panel[9,38] using the same methodology.

All described *P*-values are two-sided. Survival curves were compared by log-rank testing.

RESULTS

Patient characteristics

Between February 2008 and April 2011, 130 metastatic breast cancer patients were included. CTC counts at baseline and follow-up were available for all 130 and for 82 patients, respectively. The possible prognostic value of CTC-gene expression was established in those patients for whom RNA of sufficient quantity and quality [QQ] was isolated and who had a minimal follow-up of 9 months or an event (treatment switch or death) within 9 months. Sixteen of the 130 patients were excluded because of insufficient mRNA QQ and an additional 11 were excluded because less than 9 months had passed since inclusion and no events had occurred yet, leaving us with 103 suitable patients to establish prognostic CTC gene expression profiles (**Figure 1**). Characteristics of all 130 patients and of the 103 patients eligible for generation of the CTC profiles are depicted in **Supplementary Table 1**. Patient characteristics were comparable, with the exception of the number of patients that had received adjuvant endocrine treatment, which was relatively less frequent in the 103 QQ mRNA patients than in the total cohort of 130 patients (Fisher's exact, *P* 0.016).

CTC count predicts TTS

For this study, we chose TTS as the primary endpoint in order to reflect the heterogeneity in daily clinical decision-making in metastatic breast cancer patients. TTS is a reliable reflection of the benefit a patient derives from a certain therapeutic regimen, as it captures the time gained by administering that treatment. However, in contrast to progression-free survival (PFS)[25-26],

TTS has not previously been correlated with CTC count. Therefore, we first verified whether CTC numbers at baseline were associated with TTS in our entire cohort of 130 metastatic breast cancer patients. Indeed, the 63 patients with ≥5 CTCs at baseline had a shorter TTS than the 67 patients with <5 CTCs (median TTS 10.3 vs. 20 months, HR 2.92 [95%CI 1.71 – 4.95] *P* <0.0001) (**Supplementary Figure 1a**). After 2 - 5 weeks of therapy, CTCs were again enumerated in 82 patients. Patients with ≥5 CTCs at follow-up had a shorter TTS (HR 2.83 [95%CI 1.39 – 5.76] *P* 0.004) than did the patients with <5 CTCs (**Supplementary Figure 1b**). Looking at change in CTC count during therapy, all patients with ≥5 CTCs at follow-up, regardless of CTC count at baseline, had a shorter TTS than patients with persistently low CTC counts (HR 3.83 [95%CI 1.71 – 8.86] *P* 0.001, **Supplementary Figure 1c**). The difference in median TTS of patients with persistently low CTC counts versus those with a decline to <5 CTCs after a high CTC count at baseline did not reach statistical significance (*P* 0.066). CTC count at baseline (HR 2.44 [95%CI 1.27 – 4.69] *P* 0.007), at follow-up (HR 2.77 [95% CI 1.18 – 6.52] *P* 0.019) and CTC count change during therapy (HR 3.26 [95%CI 1.23 – 8.61] *P* 0.017) were also associated with OS. In univariate analysis, besides the aforementioned CTC counts, the presence of visceral metastases and the number of metastases were associated with TTS (**Supplementary Table 2**). In multivariate analysis, only CTC count at baseline remained an independent prognostic factor in the analysis for TTS (HR 2.54 [95%CI 1.45 – 4.46] *P* 0.001, **Supplementary Table 2**).

CTC gene expression

After establishing the prognostic value of CTC count for TTS in our patient cohort, we sought to determine the prognostic value of CTC gene expression. We chose to base our initial analysis on the 55 mRNA genes which were previously determined to be CTC-specific, i.e., significantly more abundantly expressed in patients with ≥5 CTCs than in patients without detectable CTCs and healthy blood donors (HBDs)[38]. While this 55-gene panel is based on its expression in patients with ≥5 CTCs, cell line spiking experiments showed the ability of this panel to detect epithelial signal in as little as 1 tumor cell spiked into 7.5 mL blood[9,38]. For this reason, we included all 103 patients with QQ mRNA data and sufficient follow-up, regardless of accompanying CTC count, for the subsequent analyses.

16-gene CTC profile predicts for TTS

Of the 103 patients, 42 patients were classified as the poor prognosis group (therapy switch or death <9 months) and 61 patients as having a good prognosis (no therapy switch or death <9 months). The 9-month cut-off was chosen based on the median PFS in first-line metastatic breast cancer patients in literature[317-318], and was deemed valid as the median TTS in our cohort was 8.9 months (95%CI 7.3 – 10.2).

In these 103 patients, a predictor was built based on the expression of the 55 CTC-specific genes. In univariate analysis, 9 genes were at a *P* of 0.05 (t-test, **Table 1**) and 16 genes at a *P* of 0.1 differentially expressed between the good and poor prognosis group. A leave-one-out cross validation was performed with these latter 16 genes, and a compound covariate predictor was calculated for each sample, for which the ROC-curve is depicted in **Figure 2b**. At an area-under-the curve (AUC) of 0.69 (95%CI 0.59 – 0.80, *P* 0.0001), the 16-gene CTC profile performed at least comparable to the CTC count, which had an AUC of 0.62 (95%CI 0.51 – 0.73, *P* 0.0145) (**Figure 2a**). Because we are primarily interested in correctly predicting patients in need of an early therapy switch, an actionable test result, we aimed for our 16-gene CTC profile to identify poor prognosis patients with 90% sensitivity. At this cut-off, 76 patients had an unfavorable profile and were predicted to belong to the poor prognosis group, half of whom switched treatments before 9 months and half after, resulting in a positive predictive value (PPV) of 50%. Twenty-seven patients had a favorable profile and were thus predicted to belong to the good prognosis group, of whom 23 indeed experienced no treatment switch, conferring to a negative predictive value (NPV) of 85%. The resulting test characteristics of both the 16-gene CTC profile and count in this population are depicted in **Figure 2c&d**.

The Kaplan-Meier curves for the 16-gene CTC profile, CTC count and for the combination of CTC profile and count are shown in **Figure 3**. **Figure 3a** shows that based on a CTC count of ≥5 cells per 7.5 mL blood, the 103 patients in whom the 16-gene CTC profile was generated are separated into a good and a poor prognosis group (Logrank *P* <0.001). In **Figure 3b**, an early and clear distinction into a poor and good prognosis group is seen before the 9 month time point when separating patients according to the 16-gene CTC profile (Logrank *P* <0.001). The added value of the profile appears to lie mainly in its ability to further classify patients with <5 CTCs (**Figure 3c&d**, Logrank for trend *P* <0.001), while in contrast, this profile does not identify groups with different prognosis among patients with ≥5 CTCs.

In univariate analysis, the 16-gene CTC profile was significantly associated with TTS (HR 4.57 [95%CI 2.20 – 9.50] *P* <0.0001, **Table 2**), as were number of metastases (HR 1.39 [95%CI 1.13 – 1.72] *P* 0.002), presence of visceral metastases (HR 1.84 [95%CI 1.05 – 3.23] *P* 0.035) and CTC count at baseline (HR 3.0 [95%CI 1.73 – 5.19] *P* <0.001). In multivariate analysis that included all these prognostic factors, only the 16-gene CTC-profile was an independent predictor of TTS (HR 3.15 [95%CI 1.35 – 7.33] *P* 0.008, **Table 2**).

CTC biology in patients with ≥5 CTCs

As mentioned previously, the 55 mRNAs used for the generation of our 16-gene CTC profile were selected based on their differential expression between patients with ≥5 CTCs versus patients without detectable CTCs and HBDs[38]. These 55 mRNAs are thus by definition highly

expressed in patients with ≥5 CTCs, and are likely to predominantly reflect the presence of an epithelial signal in blood. A further discrimination among patients with ≥5 CTCs might therefore not be expected from the 16-gene CTC profile. Indeed, based on the 16-gene CTC profile generated in all patients irrespective of CTC count, no further distinction in terms of prognosis could be made among patients with ≥5 CTCs (**Figure 3d**). To provide additional information in these patients, we proceeded to perform an exploratory analysis generating a CTC profile aimed at further characterizing CTCs and possibly predicting prognosis in only the 50 patients with ≥5 CTCs, starting with all 96 genes, including 3 reference genes, that can be reliably measured in CellSearch enriched CTC samples[9,38]. This 96-gene panel does not as such discriminate between patients who have ≥5 CTCs and those who have none, and thus had to be disregarded in the analysis of all patients to obtain sufficient specificity to allow identification of circulating tumor load in samples containing <5 CTCs. However, these genes can be reliably measured in samples containing ≥5 CTCs[9] and include potentially prognostic and drug target genes. By focussing only on patients with ≥5 CTCs, we expected a prognostic profile to be less driven by the presence or absence of circulating tumor load, and more by the biology of CTCs. Thus, this time starting with the 96 mRNA genes, the genes that were at a *P* of 0.1 in univariate analysis differentially expressed between the good and poor prognosis group were used to establish a predictor by leave-one-out cross validation. Using a predefined cut-off of 90% sensitivity to identify poor prognosis patients, a 9-gene CTC profile was identified, including among others *ESR1* and *MET*, the genes encoding for estrogen receptor and met oncogene, respectively (**Table 1**). This 9-gene profile identified patients with ≥5 CTCs who were very likely to experience early treatment switch (AUC 0.89 [95%CI 0.80 – 0.98] *P* <0.0001) **(Figure 4a**) with test characteristics and performance at least comparable to the 16-gene CTC profile (**Figure 4b&c**). The Kaplan-Meier curves for the 9-gene CTC profile (**Figure 4d**), also plotted against the CTC count (**Figure 4e**), show that the 9-gene CTC profile identifies two groups with different prognosis among patients with ≥5 CTCs. The good prognosis group experiences rapid relapses after the 9 month time-point, which translates into an inability to predict TTS in univariate Cox regression analysis (HR 1.36 [95%CI 0.66 – 2.78] *P* 0.40 **Table 2**) in the 50 patients with ≥5 CTCs.

DISCUSSION

CTCs provide a unique opportunity to characterize metastatic tumor cells and assess prognostic and predictive markers repeatedly during the course of disease, and increase insight into mechanisms involved in drug resistance. We have previously shown that measurement of a CTC-specific panel of 55 mRNAs in CTCs is feasible despite their low numbers and their

presence in a leukocyte background[38].

In the current study, a 16-gene CTC profile could distinguish patients with poor prognosis (defined as treatment switch or death <9 months after start of treatment) from patients with good prognosis. The profile was designed based on a cut-off with 90% sensitivity to ensure a high NPV, as we are most concerned with identifying poor prognosis patients. With a NPV of 85%, the percentage of patients wrongfully predicted to have a good prognosis is limited to 15%. Of the patients with an unfavorable CTC profile, half indeed experience early treatment switch and would benefit from earlier and more frequent response evaluation in an attempt to minimize prolonged administration of ineffective and toxic therapy. In patients with <5 CTCs, who would be classified as good prognosis according to their CTC count, the 16-gene CTC profile distinguished a truly good from an intermediate prognosis group, providing additional information on top of a CTC count. Because this 16-gene profile is heavily influenced by the presence of epithelial markers such as various cytokeratins and *TACSTD1* (the gene encoding for EpCAM), it probably identifies patients with CTCs or CTC fragments that do not meet the CellSearch criteria in terms of morphology or marker expression[64,319], and could thus identify patients with false-negative CTC counts. It is therefore our hypothesis that in these patients with <5 CTCs as determined by CellSearch CTC count, our 16-gene CTC profile better reflects the actual circulating tumor load than the CTC count does.

However, in patients with ≥5 CTCs, the 16-gene CTC profile did not provide prognostic information, which could be expected as the 55 mRNAs were selected based on their high expression in patients with ≥5 CTCs versus patients without detectable CTCs and HBDs[9]. As a consequence, this 55 mRNA panel comprises predominantly genes associated with epithelial cell load, rather than genes associated with aggressive tumor cell behaviour. To be able to provide prognostic information in patients with ≥5 CTCs, we generated a second CTC profile solely for these ≥5 CTC patients based on all 96 genes that can be reliably quantified in CTCs[9,38]. We expected the profile to be less driven by epithelial gene expression and more by biologic factors associated with tumor aggressiveness. The resulting 9-gene CTC profile could indeed separate patients with ≥5 CTCs according to prognosis, and identified a patient group swiftly progressing under 1st line systemic treatment. Among the 9 genes are epithelial genes such as *KRT17*, but also drug targets such as *MET*[320] and *ESR1. MCAM* (CD146)[224], *CCNE2*[321] and *COL1A1*[139] have previously been associated with poor prognosis, while CD146 also allows for CTC detection in EpCAM-negative breast cancer[250]. These latter genes might thus reflect the malignant potential of CTCs, and allow for the further classification of CTCs. While the 96-gene panel is informative in patients with ≥5 CTCs, we decided to limit ourselves to the 55 CTC-specific mRNAs in the total patient group. We anticipated that the other genes in the 96-gene panel that are not CTC-specific would not be informative in patients without circulating tumor

load, and would strongly dilute the power to detect CTC-derived gene expression correlated with prognosis in patients with <5 CTCs.

This study again shows that while a CTC count is a strong prognostic factor, there are probably more tumor cells of cell fragments that can be detected in the circulation, with each distinct phenotypes and possibly varying clinical value. Breast cancer heterogeneity, which is for instance reflected by its five intrinsic subtypes[220], is likely to lead to differing clinical value of CTCs among breast cancer subtypes. Recently, it was shown that CellSearch CTCs are not prognostic in patients with HER2-positive primary tumors treated with anti-HER2 therapy, in strong contrast to its prognostic value in the overall study population[322]. In our own work, we have shown that breast cancer of the normal-like subtype, which has features of epithelial-to-mesenchymal transition (EMT), lacks EpCAM[64] and cytokeratin expression[319], the markers by which CTCs are commonly defined. Together, these studies show that caution is needed when applying CTC count as a prognosticator to all breast cancer subtypes. Molecular characterization of CTCs allows for the assessment of multiple markers and could lead to detection of a more all-encompassing circulating tumor load.

In conclusion, we show that gene expression signatures of CTCs correlate with prognosis, additional to –but also irrespective of– CTC count. Validation in an independent patient cohort is warranted to confirm whether these profiles can be used as a patient stratification tool at the time of metastatic disease. The ability to measure clinically relevant genes in CTCs underlines the potential of CTC characterization as a tool to improve individualized cancer treatment.

Table 1

Genes associated with prognosis, and those making up the 16-gene and 9-gene CTC panel

Significantly differentially expressed genes between *103* good and poor prognosis patients

Genes	*P*-value
CXCL14	0.003
KRT7	0.007
PKP3	0.021
PTRF	0.021
KRT19	0.031
TIMP3	0.039
LAD1	0.040
S100A16	0.043
FKBP10	0.048

Genes selected from 55 CTC-specific mRNAs through leave-one-out cross validation in *103* patients

Genes	
TACSTD1	*FKBP10*
KRT7	*LAD1*
KRT17	*PIP*
KRT18	*PKP3*
KRT19	*PTRF*
CLDN3	*S100A16*
CXCL14	*S100A7*
ERBB3	*TIMP3*

Genes selected from 96 mRNAs through leave-one-out cross validation in *50 patients with ≥5 CTCs*

Genes	
KRT17	*MET*
CXCL14	*LOXL2*
ESR1	*SNAPC2*
COL1A1	*MCAM*
CCNE2	

Top panel; the nine genes that were at a *P* of 0.05 differentially expressed between 103 good and poor prognosis patients in univariate analysis. Poor prognosis was defined as a switch to 2nd line systemic treatment or death within 9 months after start of 1st line treatment.

Middle panel; the 16 genes that were at a *P* of 0.1 differentially expressed between 103 good and poor prognosis patients in univariate analysis, and thus make up the prognostic CTC gene profile that was generated by leave-one-out cross validation starting with the 55 CTC-specific genes.

Bottom panel; the 9 genes that were at a *P* of 0.1 differentially expressed between good and poor prognosis patients among the 50 patients with a CTC count of 5 or more, and thus make up the prognostic CTC gene profile for patients with ≥5 CTCs that was generated by leave-one-out cross validation starting with all 96 genes that can be reliably measured in CTCs.

Table 2

Prognostic value of established factors and the 16-gene and 9-gene CTC profile

	Univariate analysis			Multivariate analysis		
	TTS			TTS		
Variable	HR	95% CI	*P*-value	HR	95% CI	*P*-value
< 5 vs. ≥5 CTCs at baseline	3.00	1.73 – 5.19	**<0.0001**	1.56	0.82 – 2.99	0.178
Presence of visceral metastases	1.84	1.05 – 3.23	**0.035**	1.40	0.72 – 2.71	0.316
Number of metastases	1.39	1.13 – 1.72	**0.002**	1.06	0.81 – 1.39	0.679
CTC 16-gene profile	4.57	2.20 – 9.50	**<0.0001**	3.15	1.35 – 7.33	**0.008**
CTC 9-gene profile	1.36	0.66 – 2.78	0.40			

Univariate and multivariate analysis are depicted of established prognostic factors, as well as of our 16-gene CTC profile in 103 patients with QQ mRNA data and sufficient follow-up and 9-gene CTC profile in 50 patients with QQ mRNA data, sufficient follow-up and ≥5 CTCs. HR; hazard ratio, TTS; time to treatment switch, QQ; of sufficient quantity and quality.

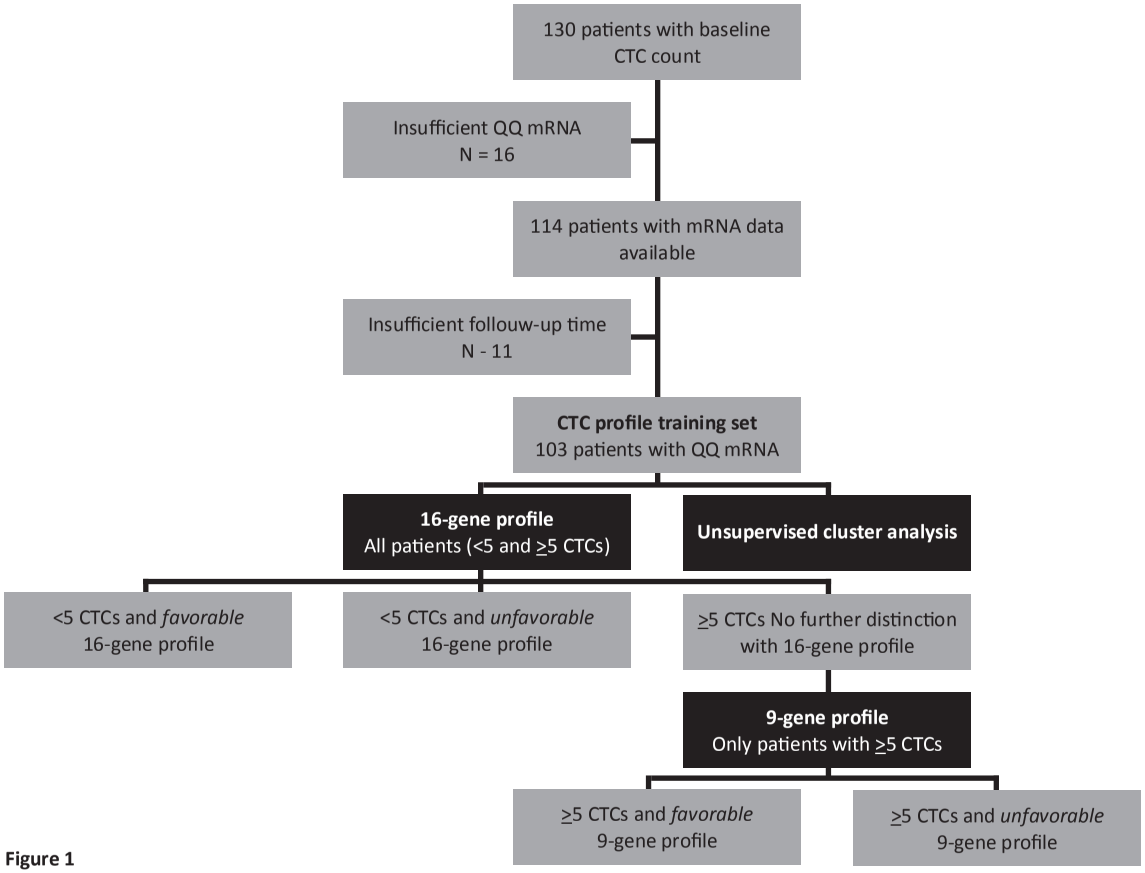
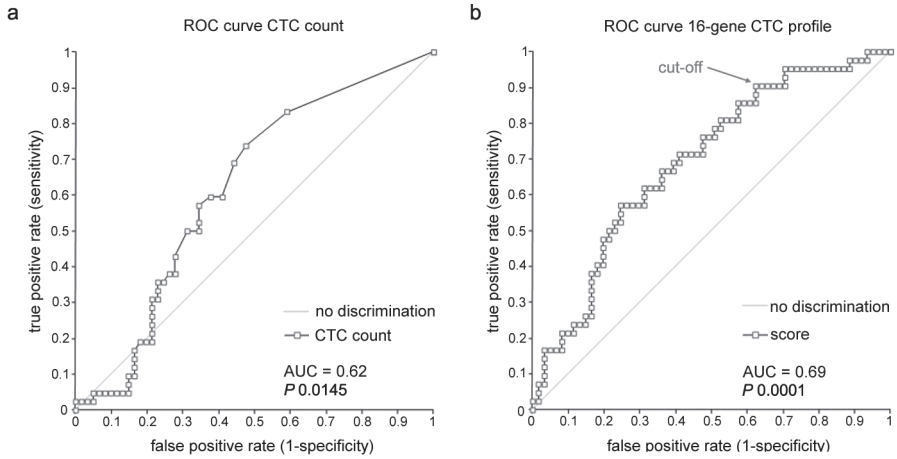


Figure 1

Flowchart depicting the numbers of patients included and excluded from the study, and the reasons for exclusion. All patients with QQ mRNA data and sufficient follow-up, regardless of CTC count, were analyzed with the 16-gene CTC profile. Patients with ≥5 CTCs, in whom the 16-gene CTC profile does not provide prognostic information additional to a CTC count, were subsequently analyzed with the 9-gene CTC-profile. QQ; of sufficient quantity and quality



c

Test performance of CTC count and 16-gene CTC profile

CTC count		TTS <9 months			
		no	yes	total	
CTC count	<5	36	17	53	Pearson's X^2 3.42
	≥5	25	25	50	*P* 0.0643
	total	61	42	103	

16-gene CTC profile		TTS <9 months			
		no	yes	total	
CTC profile	fav	23	4	27	Pearson's X^2 10.21
	unfav	38	38	76	*P* 0.0014
	total	61	42	103	

d

Test characteristics of CTC count and 16-gene CTC profile

	CTC count	16-gene CTC profile
Sensitivity	60%	90%
Specificity	59%	38%
PPV	50%	50%
NPV	68%	85%

Figure 2

ROC-curves (**panel a & b**), test performance (**panel c**) and test characteristics (**panel d**) of CTC count and 16-gene CTC profile in 103 patients. AUC; area under the curve, PPV; positive predictive value, NPV; negative predictive value

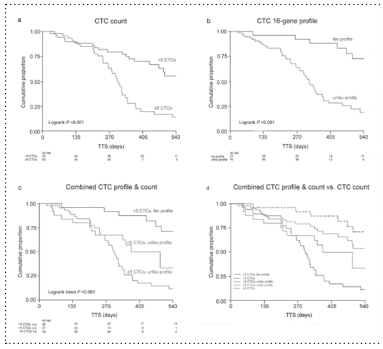


Figure 3: see section 'Color figures'

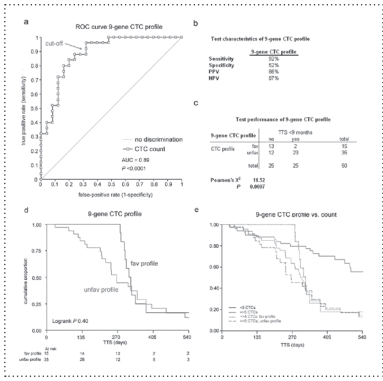


Figure 4: see section 'Color figures'

Supplementary data:

https://docs.google.com/open?id=0B9Etqm_r7T2mNnN5STlLajcyZFU

Chapter 7

mRNA and microRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients

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ABSTRACT

Background & aims

Circulating tumor cell (CTC) counts have prognostic value in metastatic colorectal cancer (CRC), but CTCs can also be isolated for subsequent characterization. CTCs are thus a very promising tool for the repeated and non-invasive evaluation of drug targets and predictive and prognostic factors. We describe the identification of CTC-specific mRNAs and miRNAs in colorectal CTCs.

Methods

For this study, we included 30 healthy donors (HDs) and 161 CRC patients prior to liver metastasis resection. CTCs were enumerated in and isolated from 2 x 30 mL patient blood using the CellSearch Epithelial Cell Kit (Veridex LLC) and Profile Kit, respectively; 30 mL HD blood was subjected to the same isolation procedure. RNA was isolated from the enriched CTC and HD fractions, in which 41 miRNAs and 95 mRNAs were measured by quantitative reverse transcriptase PCR.

Results

miRNA and RNA of sufficient quality and quantity was available for 146 and 98 patients with pathology-confirmed liver metastasis of colorectal origin, respectively. Thirteen CTC-specific miRNAs and 34 CTC-specific mRNAs were identified, of which the transcripts were more abundantly expressed in patients with ≥3 CTCs as compared to HDs (Mann-Whitney U-test $P<0.05$). Cluster analysis distinguished patient clusters associated with epithelial gene expression, among others. Among patients without detectable CTCs, a subgroup was identified of which CTC gene expression suggested the presence of circulating tumor load.

Conclusions

In this study, we show that extensive characterization of colorectal CTCs is feasible and is informative in patients with and without detectable CTCs, greatly increasing the amount of information that can be obtained from colorectal CTCs.

INTRODUCTION

Colorectal cancer (CRC) is a highly heterogeneous disease, in its presentation as well as its prognosis. The liver is a predominant site of metastases; approximately 25% of CRC patients present with synchronous hepatic metastases, and ultimately more than 50% of patients initially presenting with non-metastatic CRC will develop liver metastasis in the course of their disease[323]. When the metastases are confined to the liver and are deemed resectable, patients are increasingly undergoing partial liver resection aiming for curation[324-325]. Nevertheless, up to half of patients undergoing such major abdominal surgery will develop disease relapse in the liver or at other distant sites within one year[326-331]. Despite these disappointing figures, recent data suggest that a selected patient group undergoing this surgical approach achieves long-term survival[332].

While surgical resection might improve outcome for a selected group of patients, the factors on which to base selection for this procedure have not been elucidated, prompting the need for new prognostic factors to identify this specific subgroup. Candidate factors are mRNA and microRNA (miRNA) expression profiles, which have been shown to be prognostic in primary colorectal cancer[333-335]. However, at the time of metastatic disease, clonal selection and genomic instability can lead to discrepancies between primary tumor and metastases, which can be augmented by the passing of time and administration of systemic therapy[6]. Heterogeneity between primary tumor and metastasis has been described for clinically highly relevant predictive factors such as *KRAS*[5,313], and when new prognostic and predictive factors are sought after, such discrepancies between primary tumor and metastases can be crucial. In this regard, better predictive and prognostic models could be established when metastatic tissue, rather than the primary tumor, is used to generate such models on. Unfortunately, metastatic tissue is often hard to obtain for diagnostic purposes, and only through invasive procedures. An alternative approach is the characterization of circulating tumor cells (CTCs) which can be repeatedly isolated from blood[314].

A CTC count has recently been identified as a powerful prognostic marker in metastatic colorectal[29], breast[25] and prostate cancer[31,336], and their rise or decline after the first cycle of chemotherapy predicts therapy response[25,29,31]. Additionally, CTC characterization for drug target expression[43-44,315], mutations[316] and gene expression by quantitative reverse transcriptase PCR (qRT-PCR)[38-39] could greatly improve treatment decision making, but some challenges remain. CTCs are extremely low-frequent in the circulation and, even after CellSearch EpCAM-based enrichment, need to be characterized among up to a thousand remaining leukocytes[9]. To enable CTC characterization by real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR), we have circumvented this problem of contaminating leukocytes by focussing solely on genes that are not, or at a much lower level, expressed by leukocytes.

Using stringent selection methods combined with a sensitive but robust pre-amplification, we were able to reliably quantify a CTC-specific gene panel in blood of metastatic breast cancer patients[38] and have recently established its prognostic value in 103 patients (manuscript in preparation).

In the current study, a large panel of mRNAs and miRNAs is quantified in the CTCs of metastatic CRC patients prior to partial liver resection. From this panel, we identified CTC-specific mRNAs and miRNAs, and explored their clinical relevance both in patients with and without detectable CTCs.

METHODS

Blood samples

From 161 patients with metastatic colorectal cancer, 2 x 30 mL blood samples were taken for CTC enumeration and characterization (for details see next) by way of venipuncture before liver metastasis resection and prior to tumor manipulation. This study was approved by the Leiden University Medical Center and Erasmus University Medical Center Institutional Review Boards (METC P05.182) and all patients were included in the Erasmus University Medical Center, Rotterdam, Netherlands after written informed consent was obtained. Additionally, 30 mL blood samples were drawn from 30 healthy volunteers (age 21 – 58) to evaluate gene expression in healthy donors (HDs).

Enumeration and isolation of CTCs

Two samples of 30 mL blood from the 161 metastatic CRC patients prior to liver metastasis resection were drawn in CellSave™ tubes (Veridex LLC, Raritan, NJ) for enumeration or EDTA tubes for isolation. Prior to CTC enumeration and isolation, a density gradient-based enrichment step was applied as described before[337-338]. Briefly, 30 mL blood was pooled and centrifuged for 10 minutes at 800xg. After removal of plasma, 15 mL CTC buffer was added and mixed, and the total volume carefully placed onto 6 mL of Lymphoprep (Axis-Shield, Dundee, Scotland), a density-gradient medium. After centrifuging at 400xg for 10 minutes, the top buffer layer was discarded. Then, 7.5 mL of suspension including the buffy coat was aspirated with a reversed 10 mL pipette, allowing optimal isolation of the mononuclear cell layer, and pipetted into a regular CellTracks™ tube (Veridex). For CTC enumeration, samples were processed on the CellTracks™ AutoPrep System (Veridex) using the CellSearch™ Epithelial Cell Kit (Veridex) within 96 hours after collection and CTC counts were determined on the CellTracks™ Analyzer (Veridex) according to the manufacturer's instructions and as described before[9,250].

mRNA and miRNA isolation from CTCs, qRT-PCR and quality control

For gene expression studies, in parallel with the enumeration studies, 30 mL of blood from patients and HDs was drawn in EDTA tubes, subjected to Ficoll enrichment as described above and enriched for CTCs on the CellTracks™ AutoPrep System using the CellSearch™ Profile Kit (Veridex) within 24 hours after collection. RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen, Valencia, CA), and cDNA synthesis, pre-amplification, PCR and normalization procedures to quantify gene expression levels were performed as described in detail before[38]. The measures that were taken to ensure the linear and homogeneous nature of pre-amplification, adequate PCR efficiency and reproducibility of each assay have also been described in detail before[38].

Statistical analysis

Stata and Analyse-it were used for statistical analysis and generation of box-plots. The strength of the associations between continuous variables was tested with the non-parametric Spearman rank correlation test. Differences in the median expression levels in various groups were tested with the non-parametric Mann-Whitney U test, and differences in baseline patient and tumor characteristics by the Fisher's exact test. CTC-specific profiles were identified by Class Comparison in Biometric Research Branch ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html), using a permutation *P*-value cut-off of <0.05 (two-sample t-test). Hierarchical cluster analysis was performed using Cluster and Treeview[294] and a custom Perl script to visualize the gene expression values. DAVID (Database for Annotation, Visualization, and Integrated Discovery[295-296]) was used to functionally annotate genes and identify the over-represented functions, with *P*-values corrected for multiple testing via the Benjamini-Hochberg's procedure. Unless stated otherwise, all statistical tests are 2-sided with $P<0.05$ considered statistically significant.

RESULTS

Patient characteristics

Among 161 included patients, four did not have a pathology-confirmed metastasis of colorectal origin (lesions were either benign or from a different primary origin) and 11 had miRNA of insufficient quality and quantity (QQ), leaving 146 patients evaluable for miRNA gene expression. mRNA from 43 of the 146 patients had already been used for mutation analysis (manuscript in preparation), and five did not pass QQ control, leaving us with 98 patients with QQ mRNA. Detailed clinicopathological information for all 146 patients is available in **Table 1** and **Supplementary Table 1a**, and the subset of 98 patients with QQ mRNA data is described in

Supplementary Table 1a. Most patients (60%) presented with synchronous metastatic disease. For the remaining 58 metachronous patients, median time between primary tumor resection and metastasis was 23 months (range 0 – 161). In 25% of patients, the primary tumor was still in situ at the time of blood draw before liver resection, either as part of a liver-first approach, i.e., liver metastasis resection followed by primary tumor resection in a second surgery[324-325], or the primary tumor was resected simultaneously with the liver surgery. Twenty percent of patients had been given neoadjuvant and/or adjuvant chemotherapy prior to or after primary tumor resection, while 57% had received induction chemotherapy before liver resection. Sixteen patients had extrahepatic metastatic disease at the time of liver resection, which were mostly peritoneal lesions discovered during liver surgery. Among all patients, median CTC count was 1 (range 0 – 35) per 30 mL blood and 40 (27%) patients had ≥3 CTCs, the clinically relevant prognostic cut-off level in metastatic CRC patients[29]. The number of patients below and above the cut-off of 3 CTCs was not equally distributed among males and females; both in all 146 patients and in the 98 patients with QQ mRNA, relatively more female than male patients had CTC counts above 3 cells per 30 mL blood (Fisher's exact, *P*=0.001 and *P*=0.004 respectively, **Supplementary Table 1a**). In the total patient cohort, but not in the smaller QQ mRNA cohort, proportionally more patients for whom ≥ 6 months had passed since resection of the primary tumor had 3 or more CTCs than those who underwent metastasis resection at the same time or within 6 months after primary tumor resection (*P*=0.05).

Circulating tumor load in patients without detectable CTCs

Ninety-five mRNAs (**Supplementary Table 2a**) and 41 miRNAs (**Supplementary Table 2b**) were selected to be putatively CTC-specific, i.e., were described *in silico* to be relatively low expressed in leukocytes compared to colorectal cancer (SAGE Genie Database of the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/SAGE/AnatomicViewer), and could be reliably and linearly measured based on the above mentioned quality control measures. For each assay, a differential expression of at least 5 Ct compared to the median of HDs was set as a first cut-off to eliminate false-positive gene expression signals due to leukocyte contamination, leading to the exclusion of genes *H19* and *MMP3*. From the remaining genes, we set out to select colorectal cancer-specific genes, i.e., genes of which the measured expression levels are cancer-related and derived from CTCs, by comparing enriched blood from patients without CTCs with CTC-containing blood fractions. First, we assessed whether patients without detectable CTCs could be grouped with HDs to form the non-CTC group. For this analysis, we performed cluster analysis of mRNA expression data of all 33 patients without detectable CTCs and 30 HDs. As can be seen in **Figure 1**, a subgroup of patients without detectable CTCs was clearly distinct from HDs and other patients without CTCs. These patients had high expression

of epithelial genes such as *KRT19* and *KRT20*, but also of the genes *IGFBP5*, *AGR2*, *S100A16* and *LAD1*, which were previously established to identify epithelial tumor load in breast cancer patients[38]. According to DAVID Functional Annotation Clustering analysis[295-296], "signaling" (15 of 29 genes, 3.1-fold enriched, Benjamini *P*=0.0006) and growth factor binding (7 of 29 genes, 39.3-fold enriched, Benjamini *P*=0.0006) were among the most significant common categories in this gene cluster. By the high expression of these genes, a HD-unlike group was identified, while the remaining HD-alike patients lacked expression of these genes and clustered together with HDs. To further characterize these subgroups, we used a supervised analysis to identify genes that were differentially expressed between 11 HD-alike and 22 HD-unlike patients, which are depicted in **Table 2**. Among the 51 differentially expressed mRNAs were the above mentioned epithelial genes, but also *REG1A*, a prognostic marker[339], *ERBB3*, a drug target[340], and multiple collagens (*COL4A1*, *COL5A1*, *COL1A2* and *COL1A1*), which have previously been associated with prognosis in stage III colorectal cancer[341].

CTC-markers in HDs and patients with low CTC counts

Thus, we had identified a group of patients in whose blood no CTCs were detected with CellSearch CTC enumeration, but whose CTC-enriched blood strongly suggested the presence of circulating tumor content. A possible explanation for the inability to enumerate CTCs in these patients could be an insufficient expression of one of the epithelial markers needed for CTCs to be enumerated according to the CellSearch criteria (the cytokeratins *KRT8*, *KRT18* and *KRT19*) in the presence of sufficient EpCAM expression to enable their capture for subsequent gene expression profiling. To explore this, we focused on the expression of the epithelial markers *EPCAM* (previously named *TACSTD1*) and *KRT8*, *KRT18* and *KRT19* in the RNA fractions isolated from blood of HDs and patients without detectable CTCs after CellSearch enrichment. As expected, both *EPCAM* and *KRT* expression was low in HDs (**Figure 2**). For *EPCAM*, which should be expressed to enable both CTC isolation and enumeration by the anti-EpCAM based CellSearch CTC detection method, no clear difference was noted between HD-alike and HD-unlike patients (**Figure 2a**).

KRT expression is not necessary for the isolation of CTCs through anti-EpCAM capture, but only for their enumeration. *KRT8*, *KRT18* and *KRT19* were higher expressed in HD-unlike than in HD-alike patients without detectable CTCs (Mann Whitney U test, *P*=0.0003, *P*=0.03 and *P*<0.0001, respectively, **Figure 2c-d**).

CTC-specific mRNAs and miRNAs

Based on the presence of a subgroup of patients with a HD-unlike gene expression profile and expression of epithelial-specific genes, we deemed the patient group with 0 CTCs as a whole not suited to combine with the HD group to identify CTC-specific genes. To identify CTC-specific genes we therefore compared the 30 HDs with patients with ≥3 CTCs, i.e., 24 patients in the mRNA analysis and 40 patients in the miRNA analysis. We identified a panel of 34 mRNAs and 13 miRNAs of which the transcripts were at a $P<0.05$ higher expressed in the patients with ≥3 CTCs compared with HDs (Class comparison BRB-array tools, **Supplementary Table 3**).

Unsupervised hierarchical clustering of CTC-specific mRNAs

Next, we used the identified 34 CTC-specific mRNA genes to perform unsupervised 2-dimensional average linking hierarchical cluster analysis of the 98 patients with QQ mRNA data (**Figure 3**). This cluster analysis revealed two main clusters, 1 and 2. Cluster 2 could be further divided into two patient clusters, which, contrarily to patient cluster 1, were both characterized by a relatively high expression of epithelial genes such as *KRT19* and *KRT20*, but were distinguished by two gene clusters. Patient **cluster 2a** was characterized by the expression of genes such as *FABP1*, *CDX1* and *CDH17*. *FABP1* is higher expressed in good-prognosis primary tumors and metastases[342] and a marker of differentiation[343] but has also previously been described as a useful CTC detection marker[39]. *CDX1* has been described as a tumor suppressor gene[344] reducing proliferation through Cyclin D1[345], and is frequently rearranged in relation to rearrangements at the APC locus[346]. *CDH17* mediates cell-cell adhesion in intestinal epithelial cells[347], is specific to cancers of the digestive system[348] and has previously been associated with poor prognosis[349] (genes are marked by the blue rectangle). No specific common category was identified to be significantly enriched in this gene cluster by DAVID functional gene annotation analysis. Patient **cluster 2b** largely lacked expression of these genes, but did express *IGFBP5* and *AGR2*, which were both previously determined to be epithelial-specific in our breast cancer studies[38], while *AGR2* is also upregulated in microsatellite instability-high (MSI-H) tumors[350-351]; and *REG1A*, a gene correlated with poor prognosis[339] and microsatellite instability[352] (red rectangle). DAVID analysis identified "secreted" as the most significant category for seven genes (*PRSS8*, *RARRES2*, *COL4A1*, *LAD1*, *AGR2*, *IGFBP3*, *IGFBP5*) in this 15-gene cluster (5.3-fold enrichment, Benjamini $P=0.04$).

Unsupervised hierarchical clustering of CTC-specific miRNAs

Unsupervised cluster analysis of the 146 patients with QQ miRNA data based on the 13 CTC-specific miRNAs resulted in discrimination into two main groups (**Figure 4**). No relation with CTC count could be established among the clusters, nor were there clear miRNA clusters by which the patients were characterized. **Cluster 2** did have high expression of *hsa-miR-452* and

hsa-miR-187, but no common function of these miRNAs could be identified.

Association with clinicopathologic characteristics

When the patients in the two respective mRNA clusters (1 and 2 a&b) were compared for their baseline clinicopathologic characteristics, no differences between the clusters reached statistical significance (**Supplementary Table 1a**). Contrarily, more patients in miRNA cluster 2 had received induction chemotherapy prior to liver metastasis resection and thus prior to blood draw for CTC gene expression profiling (Fisher's exact, *P*=0.017, **Supplementary Table 1b**).

DISCUSSION

CTCs offer an exciting new opportunity to assess prognostic and predictive markers repeatedly during the course of cancer. CTCs are presumed to represent actual metastatic tumor load, and may thus provide more accurate information to guide treatment decisions than the primary tumor. After having previously shown the feasibility of measuring a CTC-specific gene panel in the CTCs of metastatic breast cancer patients[38], we show here that, this time using genes clinically relevant in CRC, the same method can be successfully applied to CTCs in CRC.

While several studies have described the expression levels of up to 10 different genes in the blood of CRC patients[22,202,353-355], no work has been published on the broad-scale molecular characterization of CTCs of CRC patients after CellSearch enrichment. This EpCAM-based CellSearch enrichment has the significant advantage of being semi-automated and approved by the FDA for use as a prognostic marker, but does not result in a pure CTC population. To still enable reliable measurement of clinically relevant genes, we selected, *in silico*, genes that are not or at a very low level expressed by leukocytes. Among these, we identified CTC-specific genes by comparing HDs and patients with ≥3 CTCs. We first established whether we could group patients without detectable CTCs with the HDs. However, when comparing patients without detectable CTCs with HDs, a cluster of patients had a gene expression profile strongly differing from that of HDs. These HD-unlike patients were characterized by the expression of known epithelial markers such as cytokeratins and *EPCAM*, but also of four other genes previously determined to be epithelial-specific (*S100A16*, *AGR2*, *IGFBP5* and *LAD1*[38]). Two of these, *S100A16* and *AGR2*, have also been described by others to be expressed in CTCs[39]. Together, the gene expression pattern of these patients strongly suggests the presence of circulating tumor load, CTCs or cell fragments, which are not being detected or recognized as such in the blood drawn in parallel for CTC enumeration. A few factors could cause this discrepancy between CTC count and CTC molecular profile. Literature suggests that colorectal cancer cells

can lack CK8, 18 or 19 expression[356], the markers by which a CTC is defined in CellSearch. In breast cancer as well, we have described a lack of cytokeratin expression for certain breast cancer subtypes[319], reflecting the epithelial-to-mesenchymal (EMT)-like phenotype of these cells. A lack of CK8/18/19 expression would result in the enrichment of CTCs based on their EpCAM-expression for both subsequent CTC isolation and characterization, without these cells being counted as CTCs. Indeed, HD-unlike patients had lower expression of all cytokeratins needed for CTC enumeration compared to HD-alike patients, while this difference was much smaller for *EPCAM*. Adding to the discrepancies between CTC counts and gene expression could also be an imperfect correlation of mRNA and protein expression and the well-known issue of stochastic variation[357]. Moreover, the strict morphological criteria that have to be met for cells to be counted as CTCs excludes counting of small CTCs or CTC fragments[61], which nonetheless do confer a mRNA signal. Whatever the cause, measurement of CTC-specific gene expression seems to be able to detect circulating tumor load among patients without detectable CTCs, and may possibly identify a poor prognosis group among patients with a "false-negative" CTC count that would otherwise confer to a good prognosis.

By comparing HDs and patients with ≥3 CTCs, we identified 47 CTC-specific genes, of which 34 were mRNAs and 13 miRNAs. Based on the unsupervised cluster analysis with the 34 CTC-specific mRNAs, we could distinguish three patient clusters, one of them characterized by the absence of epithelial gene expression. We did not see a clear relation with CTC-count, possibly caused by the aforementioned issues resulting in discrepancies between CTC enumeration and gene expression. With regard to the clustering based on miRNA expression data, distinguishing patient clusters was more challenging and like for the mRNA data, no relation with CTC count was seen. These results underline the complexity of miRNA data interpretation due to their regulatory role, which, combined with lack of epithelial association[250], could lead to a decidedly different expression pattern than that of mRNAs.

Our colorectal CTC-specific 47-gene panel enables the large-scale characterization of CTCs despite their presence among remaining leukocytes. The generation of this colorectal CTC-specific gene panel enables the exploration of CTC characterization as a novel means to further individualize cancer treatment based on better prognostic and predictive factors, and specifically in this study population, identify those patients who will derive benefit from liver metastasis resection.

Table 1

Clinicopathological data

Patient characteristics	No. of patients	CTC count		*P*-value
		<3	≥3	
All patients	146	106 (73%)	40 (27%)	
Sex				
Female	55 (38%)	31 (56%)	24 (44%)	*0.001*
Male	91 (62%)	75 (82%)	16 (18%)	
Age at time of surgery				
<60	44 (30%)	31 (70%)	13 (30%)	
≥60	102 (70%)	75 (74%)	27 (26%)	
Presentation of metastasis				
synchronous	87 (60%)	65 (75%)	22 (25%)	
metachronous	59 (40%)	41 (69%)	18 (31%)	
Primary tumor in situ at time of surgery				
yes	37 (35%)	30 (81%)	7 (19%)	
no	109 (75%)	76 (70%)	33 (30%)	
Elapsed time between primary tumor and metastasis resection*				
<6 months	26 (18%)	23 (88%)	3 (12%)	*0.05*
≥6 months	100 (68%)	69 (69%)	31 (31%)	
Primary tumor characteristics				
Location#				
right hemicolon	29 (20%)	22 (76%)	7 (24%)	
left hemicolon	65 (45%)	48 (74%)	17 (26%)	
rectum	51 (35%)	36 (71%)	15 (29%)	
Dukes classification#				
A	2 (1%)	1 (50%)	1 (50%)	
B	23 (16%)	16 (70%)	7 (30%)	
C	26 (18%)	21 (81%)	5 (19%)	
D	89 (61%)	66 (74%)	23 (26%)	
Neoadjuvant chemotherapy#				
yes	21 (14%)	18 (86%)	3 (14%)	
no	106 (73%)	75 (71%)	31 (29%)	
Adjuvant chemotherapy#				
yes	15 (10%)	12 (80%)	3 (20%)	
no	112 (77%)	81 (72%)	31 (28%)	
Induction chemotherapy before liver resection#				
yes	83 (57%)	59 (72%)	24 (28%)	
no	61 (42%)	45 (74%)	16 (26%)	
Site of metastasis				
liver-only	130 (89%)	96 (74%)	34 (26%)	
liver and other sites	16 (11%)	10 (63%)	6 (37%)	

Clinicopathologic characteristics of all 146 patients with pathology-confirmed colorectal liver metastases and miRNA data available. Only significant *P*-values as obtained by the Fisher's exact test are depicted. The subset of 98 patients for whom mRNA data were available is described in Supplementary Table 1. *numbers do not add up to 100% because patients undergoing primary tumor resection after liver resection, and patients who have not undergone primary tumor resection at all, were left out; # numbers do not add up to 100% because of missing data.

Table 2

Genes differentially expressed between HD-unlike and HD-alike patients

Gene symbol	Parametric *P*-value	*P*-value (FDR 10%)	Permutation *P*-value
REG1A	< 1.00E-07	< 1.00E-07	< 1.00E-07
TSPAN8	< 1.00E-07	< 1.00E-07	< 1.00E-07
COL4A1	1.00E-07	2.74E-06	< 1.00E-07
KRT19	1.00E-07	2.74E-06	< 1.00E-07
S100A16	1.00E-07	2.74E-06	< 1.00E-07
PRSS8	7.00E-07	1.60E-05	< 1.00E-07
CKB	2.00E-06	3.91E-05	1.00E-04
ERBB3	4.30E-06	7.36E-05	< 1.00E-07
AGR2	7.90E-06	1.20E-04	1.00E-04
GPX2	1.43E-05	1.96E-04	< 1.00E-07
COL5A1	3.04E-05	3.79E-04	1.00E-04
IGFBP5	4.03E-05	4.60E-04	< 1.00E-07
TM4SF1	4.69E-05	4.94E-04	< 1.00E-07
ASS1	8.61E-05	8.39E-04	1.00E-04
SOX9	9.19E-05	8.39E-04	< 1.00E-07
COL1A2	1.14E-04	9.78E-04	1.00E-04
COL1A1	2.14E-04	1.72E-03	1.00E-04
KRT8	2.35E-04	1.79E-03	3.00E-04
MCAM	2.78E-04	1.90E-03	2.00E-04
DUOX2	2.78E-04	1.90E-03	1.00E-04
RARRES2	3.44E-04	2.25E-03	3.00E-04
TRIM2	4.51E-04	2.67E-03	4.00E-04
COL3A1	4.67E-04	2.67E-03	4.00E-04
LCN2	1.05E-03	5.54E-03	1.30E-03
IGFBP2	1.56E-03	7.92E-03	1.50E-03
MUC1	2.66E-03	1.30E-02	1.80E-03
LAD1	2.98E-03	1.41E-02	2.60E-03
ACTB	3.50E-03	1.54E-02	2.10E-03
CTTN	3.73E-03	1.55E-02	3.70E-03
SPRY4	5.47E-03	2.14E-02	3.80E-03
CDH5	7.52E-03	2.75E-02	6.80E-03
CCND1	7.63E-03	2.75E-02	7.00E-03

KRT20	8.37E-03	2.85E-02	7.80E-03
MT1E	9.17E-03	2.91E-02	9.30E-03
VWF	9.18E-03	2.91E-02	7.50E-03
SLC7A5	9.33E-03	2.91E-02	7.50E-03
LMNA	1.15E-02	3.50E-02	9.40E-03
TGM2	1.37E-02	3.99E-02	1.01E-02
MACC1	1.52E-02	4.33E-02	9.10E-03
IGFBP3	1.69E-02	4.74E-02	1.28E-02
MACROD1	2.02E-02	5.52E-02	1.71E-02
CCNB1	2.11E-02	5.66E-02	1.47E-02
GMDS#	2.23E-02	5.86E-02	1.94E-02
MKI67	2.84E-02	7.07E-02	2.40E-02
LSP1	2.93E-02	7.17E-02	2.90E-02
EPCAM	3.42E-02	8.22E-02	2.60E-02
CDH1	3.57E-02	8.37E-02	2.51E-02
IGFBP4	3.61E-02	8.37E-02	2.60E-02
KRT18	3.88E-02	8.62E-02	2.89E-02
CEACAM5	3.90E-02	8.62E-02	3.58E-02
BST1	4.21E-02	9.15E-02	4.00E-02

Statistical significance, tested with a two-sample t-test and depicted as parametric *P*-value and *P*-value after correction for multiple testing (10% false discovery rate [FDR]) and permutation *P*-value, of the differential expression between HD-unlike and HD-alike patients among patients without detectable CTCs. All genes were higher expressed in HD-unlike patients, with the exception of *GMDS*. #higher expressed in HD-alike patients.

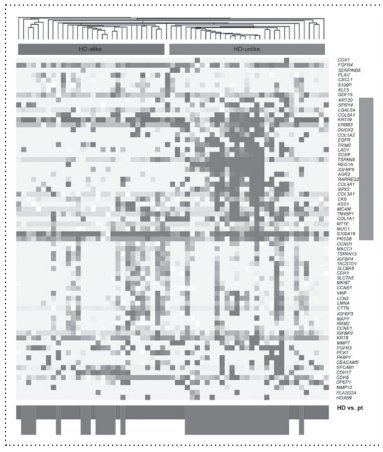

Figure 1: see section 'Color figures'

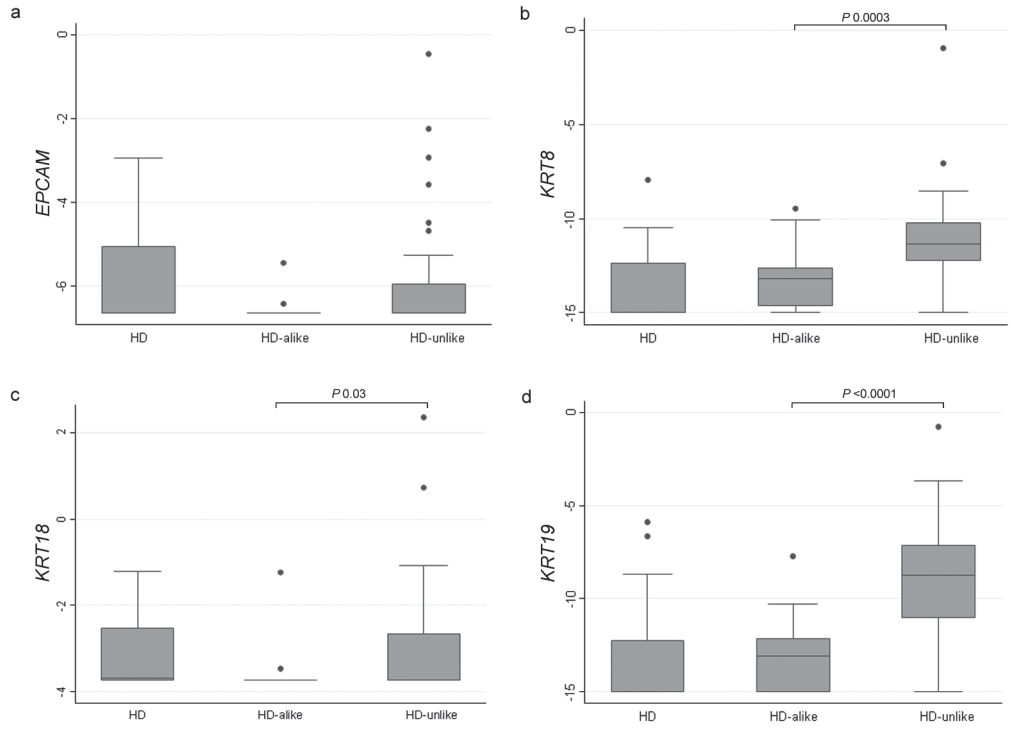

Figure 2: Expression of CTC-markers in HDs, HD-unlike and HD-alike patients with low CTC counts

Expression levels of *EPCAM*, *KRT8*, *KRT18* and *KRT19* are depicted for HDs, HD-alike and HD-unlike patients. Expression levels are depicted as deltaCt to the median of the reference genes (*GUSB*, *HMBS* and *HPRT1*). Box plots represent median, 1st – 3rd quartile (box), 5% – 95% (whiskers) and outlier (dots) expression levels. Level of significance tested with Mann Whitney U test. HD; healthy donor.

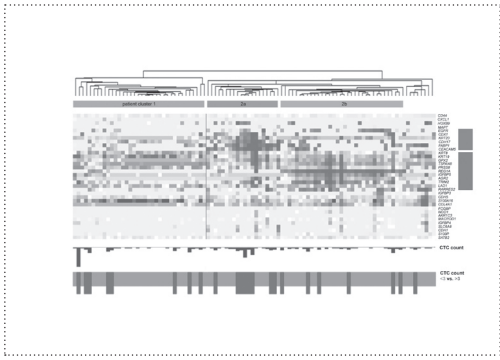

Figure 3: see section 'Color figures'

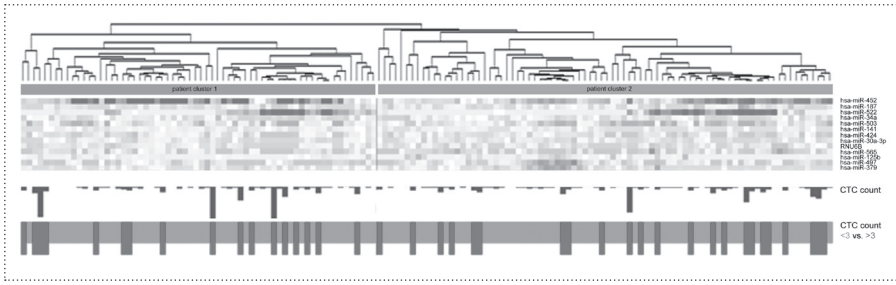

Figure 4: see section 'Color figures'

Supplementary data:

https://docs.google.com/open?id=0B9Etqm_r7T2mNnN5STlLajcyZFU

Chapter 8

KRAS and *BRAF* mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue

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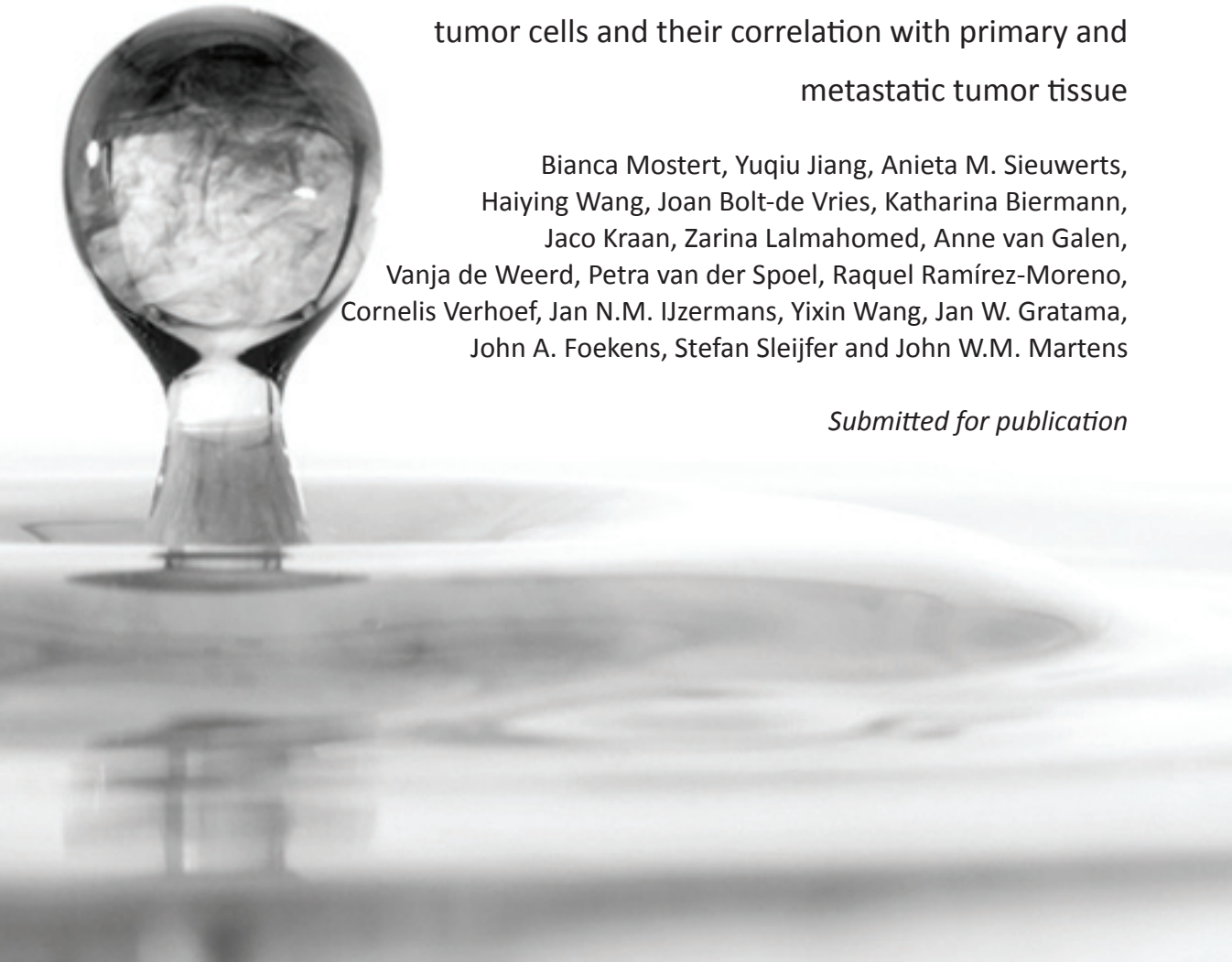


ABSTRACT

Background

Although anti-EGFR therapy has established efficacy in metastatic colorectal cancer, only 10-20% of unselected patients respond. This is partly due to *KRAS* and *BRAF* mutations, which are currently assessed in the primary tumor. To improve patient selection, assessing mutation status in circulating tumor cells (CTCs), which possibly better represent metastases than the primary tumor, could be advantageous. We investigated the feasibility of *KRAS* and *BRAF* mutation detection in colorectal CTCs by comparing three sensitive methods and compared mutation status in matching primary tumor, liver metastasis and CTCs.

Methods

CTCs were isolated from blood drawn from 49 patients before liver resection using CellSearch™. DNA and RNA was isolated from primary tumors, metastases and CTCs. Mutations were assessed by co-amplification at lower denaturation temperature (COLD)-PCR (Transgenomic™), real-time PCR (EntroGen™), and nested Allele-Specific Blocker (ASB-)PCR and confirmed by Sanger sequencing.

Results

In 43 of the 49 patients, tissue RNA and DNA was of sufficient quantity and quality. In these 43 patients, discordance between primary and metastatic tumor was 23% for *KRAS* and 7% for *BRAF* mutations. RNA and DNA from CTCs was available from 42 of the 43 patients, in which ASB-PCR was able to detect the most mutations. Inconclusive results in patients with low CTC counts limited the interpretation of discrepancies between tissue and CTCs.

Conclusion

Determination of *KRAS* and *BRAF* mutations in CTCs is challenging but feasible. Of the tested methods, nested ASB-PCR, enabling detection of *KRAS* and *BRAF* mutations in patients with as little as two CTCs, seems to be superior.

INTRODUCTION

The introduction of new drugs such as monoclonal antibodies directed against EGFR has improved the life expectancy of colorectal cancer patients[358]. Unfortunately, only 10 - 20% of unselected metastatic colorectal cancer patients respond[358-360], which is partly due to activating mutations in genes downstream of the EGF-receptor, such as *KRAS* and *BRAF*.

KRAS mutations are present in 30 - 40% of colorectal cancer patients[361]. Extensive descriptions of the inactivity of anti-EGFR therapy in *KRAS*-mutated patients[4,334,362-365] show that these agents generate a response in 17 - 40% of patients with *KRAS* wild-type (wt) tumors versus 0% of patients with *KRAS* mutated (mt) tumors[4]. Based on these results, the European Medicines Agency has approved the use of monoclonal antibodies against EGFR solely for patients with *KRASwt* tumors[366].

Another potentially predictive mutation for response to EGFR-inhibiting therapy is *BRAF*, which is present in ~10% of colorectal cancer patients[361]. Evidence for the predictive value of *BRAF* mutations is not as abundant as for *KRAS*[367-368], and a mutated *BRAF* is not yet an exclusion criterion for this therapy. However, if the same mechanism applies, which is very likely given the similar important role of *BRAF* and *KRAS* in the EGFR pathway, *BRAF* mutations could be as an important predictive factor as *KRAS*.

Although *KRAS* and *BRAF* mutation status are currently determined in the primary tumor, primary tumor tissue is not always available, is of insufficient quality, or has been obtained years before the diagnosis of metastatic disease. Importantly, mutation status of the primary as well the metastatic lesions can change over time and during the course of therapy[369-370]. In this regard, ideally, mutation status of the patient's actual metastatic tumor load would be assessed right before treatment is started. However, metastatic tissue is often hard to obtain, and usually only through invasive and painful procedures. These drawbacks could potentially be overcome by assessing mutation status of circulating tumor cells (CTCs), which can be present in the peripheral blood of metastatic colorectal cancer patients[371-372].

Here we describe the use of three techniques to assess *KRAS* and *BRAF* mutation status in enriched CTC fractions, consisting of CTCs and >100-fold excess DNA from leukocytes[9]. A fast nested Co-amplification at Lower Denaturation temperature (COLD-)PCR combined with Surveyor®/WAVE® denaturing High-Performance Liquid Chromatography (HPLC) followed by sequencing (Transgenomic®, Omaha, NE), a commercially available real-time PCR kit (EntroGen™, Tarzana, CA), and a nested Allele-Specific PCR with a Blocking reagent (ASB-PCR)[373] were tested and compared for their ability to detect mutations in CTCs of colorectal cancer patients with liver metastases undergoing partial liver resection. In addition, the mutation status in matched primary and metastatic tumor tissue was determined and correlated to each other and to CTC mutation status.

METHODS

Patients and ethics statement

From 63 patients with metastatic colorectal cancer, 2 x 30 mL blood samples were taken for CTC enumeration and characterization by way of venipuncture before liver metastasis resection and prior to tumor manipulation. This study was approved by Leiden University Medical Center and Erasmus University Medical Center Institutional Review Boards (METC P05.182), and all patients were enrolled in Erasmus MC, Rotterdam, Netherlands after written informed consent was obtained.

Cancer cell lines and synthetic DNA

Colorectal cancer cell lines HCT116 and breast cancer cell line SK-BR-3 were obtained from ATCC (Manassas, VA) and cultured under recommended conditions. HCT116 was previously established to harbor a heterozygous G13D, G>A *KRAS* mutation[374], and SK-BR-3 is *BRAF* and *KRAS* wild-type[226]. For cell line experiments, cells were harvested at log phase and counted by Improved Neubauer Hemacytometer (Hausser Scientific, Horsham, PA). A range of synthetic DNA concentrations as supplied in the EntroGen kit (see next) were tested in the EntroGen kit and in the ASB-PCR to assess assay detection limits.

Enumeration and isolation of CTCs

Two samples of 30 mL blood from 49 metastatic colorectal cancer patients about to undergo liver metastasis resection were drawn in CellSave™ tubes (Veridex LLC, Raritan, NJ) for CTC enumeration or EDTA tubes for CTC isolation. Prior to enumeration and isolation, a density gradient-based enrichment step was applied as described before[337-338]. For CTC isolation, samples were then processed on the CellTracks™ AutoPrep System (Veridex LLC) using the CellSearch™ Profile kit (Veridex LLC). For CTC enumeration, samples were processed using the CellSearch™ Epithelial Cell Kit (Veridex LLC) and CTC counts were determined on the CellTracks™ Analyzer (Veridex LLC) according to the manufacturer's instructions.

mRNA and DNA isolation from CellSearch-enriched CTC fractions and tissue

After removal of the supernatant using a MagCellect Magnet (R&D Systems, Minneapolis, USA), the cells in the enriched CTC fractions were lysed by adding 250 µL of Qiagen AllPrep DNA/RNA Micro Kit Lysis Buffer (RLT+ lysis buffer) (Qiagen, Valencia, CA) and stored immediately at -80°C until DNA and RNA isolation was performed with the AllPrep DNA/RNA Micro Kit (Qiagen) according to the manufacturer's instructions and as described before[38]. For analysis of primary and metastatic tumors, total RNA and DNA was isolated from fresh frozen tissue with RNA-Bee (AMSBIO, Abingdon, UK) as described before[290] and from FFPE tissue with the column-

based High Pure RNA Paraffin Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. After DNA and RNA extraction from CellSearch-enriched CTCs and tissues, quality and quantity checks were performed by evaluating the levels of a set of reference genes by real time PCR as described before[38,375]. For tissue RNA, this was preceded by measurements with a Nanodrop Spectrophotometer and agarose gel electrophoresis, and for genomic DNA (gDNA) by measurements with the Quant-iT PicoGreen dsDNA reagent (Life technologies, Carlsbad, CA).

Fast COLD-PCR & Surveyor/WAVE technology

Fast COLD-PCR (Transgenomic, Omaha, NE) exploits the observation that a single-nucleotide mismatch along a double-stranded DNA sequence results in a change of the melting temperature (T_m) for that sequence, so that when PCR denaturation temperature is set to a temperature slightly lower than T_m, DNA amplicons differing by a single nucleotide are selectively denatured and amplified, enriching the sample for mutated alleles[329]. Assay sensitivity was established by the manufacturer to be 0.1% (http://www.transgenomic.com/lib/ps/602136-00.pdf).

For COLD-PCR, 10ng gDNA from CellSearch-enriched CTC fractions, consisting of CTCs and >100-fold excess DNA from leukocytes, was selectively pre-amplified with *KRAS* and *BRAF* primers by two rounds of COLD-PCR as described before[376]. Amplified exons were analyzed by Surveyor digestion using the Transgenomic SURVEYOR kit, in which the surveyor nuclease digests mismatch-containing DNA. After purification of digested DNA using the QIAquick PCR purification kit (Qiagen), samples were analyzed by WAVE technology according to the manufacturer's instructions[376]. For sequencing, amplified exons were fractionated by denaturating HLPC, and fractions of interest were again amplified by PCR. PCR products were purified as above and sequenced using the Applied Biosystems PRISM® 3730 sequencer.

EntroGen KRAS/BRAF kit

EntroGen provides a commercially available real-time PCR kit for *KRAS* and *BRAF* mutation detection, which uses allele-specific primers without employing a pre-amplification step. For the EntroGen assay, 50 ng gDNA from tissues was amplified with gene specific primers and the *KRAS* codons 12, 13 and 61 and *BRAF* V600E mutations were detected using allele-specific probes (EntroGen), as described previously[377]. For CTC samples, a nested PCR was first performed on 10ng gDNA extracted from CellSearch-enriched CTCs, followed by a second PCR with the allele-specific probes as described before[377].

ASB-PCR assay

ASB-PCR is an assay developed to suppress the amplification of primer:template mismatches. Two key features of the assay are a mutant-specific primer that is shortened at its 5'-end to reduce the T_m to approximately 10°C below the annealing temperature of the assay, and the use of a blocking oligonucleotide, which has a sequence complementary to the wild-type sequence but is phosphorylated at the 3'-end to prevent its extension. The combination of these two modifications results in suppression of the amplification of wild-type allele, and the assay has been reported to be capable of detecting mutant alleles with DNA inputs between 2 and 250 pg among a thousand-fold excess of wild-type DNA[373].

ASB-PCR was performed essentially as described before[373]. Briefly, cDNA was synthesized using 5 µL CTC RNA with Invitrogen's cDNA Reverse Transcriptase kit (Life Technologies, Carlsbad, CA). All 10 µL of the resulting cDNA was then used in a nested PCR using 2x Expression Master Mix (TaqMan Gene Expression MasterMix kit, Life Technologies) and 2 µL 0.5 µM Primer Mix in a 20 µL reaction volume. For CTC samples, 2 µL PCR product from the first amplification (above) was used for the second round of amplification; while with DNA isolated from tissues, 510 ng gDNA was used, both in a 20 µL assay with 2 x TaqMan Gene Expression Mastermix and 10x Primer/Probe/Blocker mix (sequences and cycling conditions in **Supplementary Table 1**).

TOPO TA cloning and Sanger sequencing

PCR products from samples that were estimated to have sufficient mutation were cloned with the TOPO® TA Cloning® Kits for Sequencing (Life Technologies) according to the manufacturer's instructions. Positive colonies were amplified individually under the same PCR conditions as the first round of amplification of the nested-PCR. Amplified products were purified using QIAquick PCR Purification kit (Qiagen) and sent to GENEWIZ (South Plainfield, NJ) for Sanger dideoxy terminator sequencing[378].

RESULTS

Patients

DNA and RNA isolated from primary tumor and metastatic tumor tissue was available for analysis from 43 patients. The 20 excluded patients had no primary tumor or metastatic tissue available, either because the primary tumor had not been resected at the time of this analysis or because the liver lesion was benign or not from colorectal cancer origin. CTC count and isolated CTCs were available for 42 of the 43 patients. Median CTC count in all 42 patients was 1 (range 0-37); 16 patients had no detectable CTCs (38%). Most patients had metastatic disease confined to their liver; six patients also had metastases elsewhere, mostly pulmonary.

Twenty-one patients had received induction chemotherapy prior to liver resection, and fifteen patients had undergone neoadjuvant or adjuvant treatment for their primary tumor, leaving 10 patients chemo-naïve at the time of liver resection. Detailed patient characteristics are depicted in **Table 1**.

***KRAS* and *BRAF* mutation status in primary tumors**

Because of the established nature of the test and high sensitivity of 0.1% according to the manufacturer (http://www.transgenomic.com/lib/ps/602136-00.pdf), *KRAS* and *BRAF* mutation analysis was first performed by COLD-PCR in all primary and metastatic tumor tissues. When available, fresh frozen (FF) tissue was used for analysis; in other instances, formalin-fixed, paraffin-embedded (FFPE) tissue was used, resulting in analysis of five FF and 37 FFPE primary tumors. Nine primary tumors (21%) harbored a *KRAS* mutation, which were reproducibly detected in duplicate and confirmed by sequencing (**Table 2**). Mutation frequency ranged from 5 to 100%, and all but one mutations involved codon 12 or 13. One T35I mutation was detected, caused by a C>T substitution, a missense mutation that has previously been described[379]. *BRAF* mutations were detected in three primary tumors (7%), two V600E and one D594G caused by an A>G substitution. No samples harbored both *BRAF* and *KRAS* mutations, in line with other reports[3,5] on their mutual exclusivity.

***KRAS* and *BRAF* mutation status in metastatic tissue**

Determination of mutation status by COLD-PCR was technically feasible in all 31 FF and 12 FFPE metastatic tissue specimens. A *KRAS* mutation was detected in 10 of the 43 patients (23%), all of which involved codon 12 or 13 (**Table 2**). A *BRAF* mutation was present in the metastasis of four of the 43 patients (9%), two of them V600E and two others; D594N and D594G. As with primary tumors, *KRAS* and *BRAF* mutations were never present in the same specimen.

Correlation of primary tumor and metastases mutation status

The same *KRAS* mutation was present in the metastatic tissue of four of the nine patients in whom a *KRAS* mutation was detected in the primary tumor. Of the remaining five patients with a mutated primary tumor, four patients had a *KRASwt* metastasis, and one patient had a different *KRAS* mutation in the metastatic lesion than in the primary tumor (CTC192, **Table 2**). Of 34 patients with a *KRASwt* primary tumor, five (15%) did have a *KRAS* mutation in their metastasis. One of these patients had presented with synchronous metastases but had received induction therapy between primary surgery and metastases resection. The four other patients with discrepancy in *KRAS* mutation status had presented with metachronous metastases, and one had also received induction therapy prior to partial liver resection.

Two of the three patients with a *BRAFmt* primary tumor had the same mutation in their metastases; the third patient's metastasis was *BRAFwt*. Of 40 patients with a *BRAFwt* primary tumor, two (5%) had *BRAFmt* metastases. While one of these patients had presented with synchronous metastases, both of them received induction chemotherapy prior to liver resection.

The only patient in this cohort who had been treated with anti-EGFR monoclonal antibodies before liver surgery had a wild-type primary and metastatic tumor (CTC203, **Table 2**).

COLD-PCR in CTCs

Because of its well-described high sensitivity, *KRAS* mutation detection in CTCs was first attempted by COLD-PCR. The DNA from the enriched CTC fractions of 13 patients, selected to contain both high and low CTC counts, as well as patients with both mutated and wild-type metastases, was analyzed.

A G12D *KRAS* mutation, caused by a substitution of G>A, was detected in one of these samples (CTC208, **Figure 1** and **Table 3**). While no mutation was detected in the primary tumor, this patient's metastasis contained the same mutation as detected in the CTC fraction at an estimated frequency of 80% (**Table 2**). In five other samples, a failed PCR product in the sequencing step meant that no mutation could be detected, and in five other CTC samples, no variant was detected despite a mutated primary or metastatic tumor (**Table 3**).

EntroGen PCR assay in CTCs

Because of the disappointingly few mutations detected in CTCs by COLD-PCR, we tested two other assays with a probable high sensitivity[373,380]. First, the detection limit of EntroGen PCR assay was determined by testing a range of concentrations of synthetic DNA containing a *KRAS* mutation. The assay was able to detect as little as 0.6% *KRASmt* among *KRASwt* DNA (**Table 4**). *KRAS* mutation status was determined by the EntroGen PCR assay in all nine primary tumors with a *KRASmt* according to COLD-PCR, yielding reproducible results (**Supplementary Table 2**). However, when testing gDNA from nine CTC samples for *KRAS* mutation status using the EntroGen kit, no mutations were detected (**Table 3**), even though eight of these patients had a *KRASmt* primary or metastatic tumor tissue according to COLD-PCR.

ASB-PCR in CTCs

Next, CTC mutation detection was attempted by the ASB-PCR approach. Assay sensitivity of the one-run *KRAS* and *BRAF* ASB-PCR assay was determined by testing a range of synthetic *KRASmt* DNA concentrations spiked in wild-type DNA (**Table 5**). As little as 0.2% of mutated among wild-type alleles could be detected, and in the nested ASB-PCR seven different *KRAS*

mutations were tested, which could all be detected in concentrations down to 0.6% mutated among wild-type copies (**Table 6**). Again, confirmatory results were obtained in all *KRASmt* primary tumors and V600E *BRAFmt* primary and metastatic tissues by (**Supplementary Table 2 & 3**). Because our ASB-PCR was designed only for the detection of the most common V600E mutation, two other *BRAF* mutations detected by COLD-PCR could not be validated.

Considering the assay's *in vitro* sensitivity, RNA isolated from the CellSearch-enriched CTCs of all available patients was tested for *KRAS* codon 12 and 13 and *BRAF* V600E mutation status by nested ASB-PCR (**Table 2 & 3**). Using this method, five *KRAS* mutations were detected at a frequency of <0.01 - 8.9%. Accompanying CTC counts ranged from 2 to 37, conferring to CTC input from 0.8 to 15.2 cells, as only 40% of the total sample was used for ASB-PCR. Four of these patients had the same *KRAS* mutation in their metastasis, while one patient (CTC284) had a *KRASwt* primary tumor and metastasis. In patient CTC196, whose primary tumor and metastasis were *BRAFwt*, a *BRAF* V600E mutation was detected. The ASB-PCR assay was designed to detect only the most common V600E *BRAF* mutation, precluding the detection of the D594N and D594G mutations from patients' CTC204 and CTC207 metastases in their CTCs.

Confirmation of CTC mutation status by sequencing

We next sought to confirm the CTC mutations as detected by ASB-PCR through traditional Sanger sequencing. In our hands, Sanger sequencing had a sensitivity of 12.5% in cell line experiments (data not shown). Based on estimates of *KRASmt* frequency in the enriched CTC fractions (**Table 6**), sequencing was potentially only feasible for patients CTC208, CTC245 and CTC284 (estimated mutation frequencies 8.9%, 6.1% and 2.0%, respectively). CTC222 had a too low mutation frequency (<0.01%) for sequencing, and for patient CTC202, no more DNA was available. Sample CTC208 had previously been sequenced following mutation detection with COLD-PCR. The purified PCR products of samples CTC245 and CTC284 were subjected to TOPO TA cloning, and we were able to sequence a G12V, G>A *KRASmt* (2 of 90 colonies) in the CTCs from patient CTC245. In patient CTC284, no *KRASmt* was detected in the 100 selected colonies (**Table 3**).

DISCUSSION

Determining *KRAS* and *BRAF* mutations in CTCs is extremely challenging due to the low number of CTCs, the lack of amplification of the genes of interest, and the presence of up to 1,000 leukocytes despite CTC enrichment[9]. We compared the performance of three mutation assays selected for their high sensitivity. COLD-PCR and ASB-PCR have specifically

been developed to enable detection of low-abundant mutated alleles among wild-type copies through enrichment of mutated alleles and specific blocking steps. Despite these adaptations, *KRAS* and *BRAF* mutation detection in CTCs is pushing the limits of the assays' performance. ASB-PCR proved to be able to detect the most mutations in the CTC-fractions in our hands, as five of six mutations that could be detected by ASB-PCR were not detected with COLD-PCR. Only two of the CTC fractions with a *KRAS* mutation as detected by ASB-PCR were also tested by the EntroGen PCR assay. As no mutations were detected in these two nor in six other CTC fractions of patients with a *KRASmt* primary tumor and/or metastasis, the sensitivity of the EntroGen assay is probably too low. Previous studies on the detection of mutations in the androgen receptor (*AR*)[36] and *EGFR*[381] mutations in CTCs have been more fruitful, quite possibly explained by the amplification of these genes accompanying their mutated status, which results in a larger number of mutated alleles per CTC. Additionally, CTC counts in these studies were generally much higher than in our patient cohort[382].

We were able to detect *KRAS* or *BRAF* mutations in CTCs from six of 43 patients, five of whom had a CTC count above 3 cells in 30 mL blood. In two patients with *KRASmt* metastases and ≥3 CTCs (CTC201 & CTC209), no *KRAS* mutation was detected in the CTCs. Despite optimization of the assays for detection of mutated amplicons amongst abundant wild-type copies, mutant DNA was probably too scarce. The lack of mutant DNA may also be explained by stochastic variation; CTCs can be present in the blood drawn for CTC enumeration, but not in the blood drawn for CTC isolation and mutation assessment[357]. In CTC201, with 28 CTCs, stochastic variation does not explain the inability to detect a *KRAS* mutation. In this and other patients with mutated metastases, CTCs might also be truly wild-type, reflecting tumor heterogeneity[6].

In our 43 colorectal cancer patients, substantial discordance was seen between primary tumors and matched metastases. Five out of 33 initially *KRASwt* patients had a *KRASmt* metastasis; these patients have an indication for anti-EGFR treatment based on primary tumor characteristics, while based on the mutation characteristics of the metastasis, no benefit can be expected. Conversely, four patients whose primary tumor *KRASmt* would exclude them from anti-EGFR treatment had *KRASwt* metastases. While the testing of the *BRAF* oncogene is not yet obligatory before anti-EGFR therapy is started, accumulating data do show its predictive value[367-369]. Two patients with *BRAFmt* metastases would probably not benefit from anti-EGFR treatment, although their *BRAFwt* primary tumor suggests otherwise. In our cohort, a higher discordance in mutation status between primary and metastatic tumors was observed than in earlier studies[5,369-370,383], which could be caused by a number of reasons. First, several other studies used DNA sequencing methods, which are less sensitive than COLD-PCR. Especially given the heterogeneity of tumors and the low percentage of vital tumor cells in fast-growing metastases, which can both result in small mutated cell fractions, sequencing

might underestimate mutation frequency. Also, in accordance with the liver-first approach employed in our clinic[325], almost all patients in our cohort showing discordance between their primary tumor and metastasis had been pre-treated with systemic therapy, which might have led to more discrepancies.

The identification of patients benefitting from targeted treatments is increasingly important[366]. Because of the inherent genomic instability of cancer, possibly augmented by time and treatment, heterogeneity exists between primary tumor and metastases. Predictive factors are therefore probably more informative when assessed on metastases, but these are often not available. Taking CTC mutation status as a surrogate for metastases, *KRAS* and *BRAF* testing in CTCs could spare patients an expensive therapy that would otherwise be both futile and toxic, without the need for invasive biopsies. Our study was initiated to determine if CTC mutation analysis is feasible. At this point we cannot conclude that a CTC *KRAS* and *BRAF* mutation status can be reliably assessed in all patients with a CTC count below 3 cells/30 mL. Especially in patients with a low mutation frequency, the chances of false-negative results are substantial with the currently applied technology. To obtain reliable test results in all patients with CTCs, improvements are necessary. The presence of ~1,000 leukocytes even after CellSearch CTC enrichment complicates subsequent characterization[9,38]. Increasing the purity of the input CTC sample will reduce the number of wild-type alleles, possibly enabling next generation sequencing. Such adaptations should lead to reliable analysis of all patients with one mutated CTC in 30 mL blood, a requirement for this test to be taken into studies investigating the predictive value of CTC mutation status.

To the best of our knowledge, this is the first study to compare *KRAS* and *BRAF* mutation status in matched primary tumors, metastases and CTCs. Mutation assessment on CTCs offers the opportunity to test patients at the time of metastatic disease and to do so repeatedly during the course of treatment.

Table 1

Patient characteristics

Clinicopathological characteristic	No. of patients	%
All patients	43	100%
Age (median, range))	65 (37–84)	
Sex		
Female	15	35%
Male	28	65%
Site of primary tumor		
Right hemicolon	11	26%
Left hemicolon	15	35%
Rectum	17	40%
Tumor stage		
T1	0	0%
T2	6	14%
T3	24	56%
T4	7	16%
pT0	1	2%
Tx	5	12%
Nodal stage*		
N0	21	49%
N1	10	23%
N2	4	9%
Nx	4	9%
Site of metastasis		
Liver only	37	84%
Other sites	6	16%
Dukes' stage*		
A	2	5%
B	10	23%
C	10	23%
D	19	44%
Metastatic lesions*		
Synchronous	18	42%
Metachronous	25	58%
Neo-adjuvant therapy at time of primary tumor*		
Yes	10	23%
No	31	73%
Adjuvant therapy at time of primary tumor*		
Yes	5	12%
No	35	81%
Induction therapy at time of metastasis*		
Yes	21	49%
No	20	47%
Any chemotherapy before partial liver resection*		
Yes	30	70%
No	10	23%
Monoclonal antibodies against EGFR before liver resection		
Yes	1	2%

No	40	93%
Primary tumor in situ at time of CTC blood draw		
Yes	5	12%
No	38	88%
Median time (months) between primary and metastasis resection (range)	18.4 (0 – 51.2)	
CTC count§		
< 3	27	64%
≥ 3	15	36%
Median CTC count (range)§	1 (0 – 37)	

*numbers do not add up to 100% due to missing data; §CTC count per 30 mL blood

Table 2

Comparison of mutation status between matched primary tumor, metastasis and CTCs

	Primary tumor			Metastasis			CTC		
CTC code	type	*KRAS*	*BRAF*	type	*KRAS*	*BRAF*	CTC#	*KRAS*	*BRAF*
CTC189	P	NVD	NVD	F	NVD	NVD	13	NVD	NVD
CTC190	P	NVD	NVD	P	NVD	NVD	3	NVD	NVD
CTC191	P	NVD	NVD	P	NVD	NVD	0	NVD	NVD
CTC192	P	**c.C>T; p.T35I (10%)**	NVD	F	**c.G>T; p.G12V (10%)**	NVD	2	NVD	NVD
CTC194	P	**c.G>T; p.G12V (5%)**	NVD	P	**c.G>T; p.G12V (40%)**	NVD	1	NVD	NVD
CTC195	P	NVD	NVD	P	NVD	NVD	5	NVD	NVD
CTC196	P	NVD	**c.T>A; p.V600E (80%)**	P	NVD	NVD	5	NVD	**c.T>A; p.V600E (0.5%)**
CTC198	P	NVD	NVD	F	NVD	NVD	2	NVD	NVD
CTC200	P	NVD	NVD	F	**c.G>A; p.G13D (30%)**	NVD	0	NVD	NVD
CTC201	P	NVD	NVD	F	**c.G>C; p.G12R (30%)**	NVD	28	NVD	NVD
CTC202	P	NVD	NVD	F	**c.G>A; p.G13D (40%)**	NVD	7	**c.G>A; p.G13D (0.7%)**	NVD
CTC203	P	NVD	NVD	F	NVD	NVD	33	NVD	NVD
CTC204	F	**c.G>A; p.G13D (5%)**	NVD	F	NVD	**c.G>A; p.D594N (20%)#**	1	NVD	NVD
CTC207	P	NVD	**c.A>G; p.D594G (20%)#**	F	NVD	**c.A>G; p.D594G (30%)#**	0	NVD	NVD
CTC208	P	NVD	NVD	F	**c.G>A; p.G12D (80%)**	NVD	17	**c.G>A; p.G12D (8.9%)**	x
CTC209	P	**c.G>T; p.G12V (100%)**	NVD	F	**c.G>T; p.G12V (40%)**	NVD	3	NVD	NVD
CTC210	P	NVD	NVD	F	NVD	NVD	4	NVD	NVD
CTC211	F	NVD	NVD	F	NVD	NVD	1	NVD	NVD
CTC215	F	**c.G>A; p.G13D (10%)**	NVD	P	NVD	NVD	0	NVD	NVD
CTC216	P	NVD	NVD	F	NVD	NVD	2	NVD	NVD
CTC217	P	NVD	NVD	P	NVD	NVD	1	NVD	NVD
CTC218	P	NVD	NVD	F	NVD	NVD	5	NVD	NVD

CTC219	P	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC220	P	NVD	NVD	F	NVD	**c.T>A; p.V600E (10%)**	0	NVD	NVD
CTC221	P	NVD	NVD	F	NVD	NVD	11	NVD	NVD
CTC222	P	NVD	NVD	F	**c.G>T; p.G12V (20%)**	NVD	37	**c.G>T; p.G12V (<0.01%)**	x
CTC225	P	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC226	P	**c.G>A; p.G12D (50%)**	NVD	P	NVD	NVD	0	NVD	NVD
CTC227	P	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC243	P	NVD	NVD	P	NVD	NVD	0	NVD	NVD
CTC244	P	NVD	NVD	P	NVD	NVD	1	NVD	NVD
CTC245	F	**c.G>T; p.G12V (20%)***	NVD	F	**c.G>T; p.G12V (20%)**	NVD	3	**c.G>T; p.G12V (6.1%)**	NVD
CTC246	P	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC247	P	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC248	P	NVD	NVD	F	NVD	NVD	1	NVD	NVD
CTC249	P	NVD	NVD	F	NVD	NVD	3	NVD	NVD
CTC250	F	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC251	P	NVD	NVD	F	NVD	NVD	0	NVD	NVD
CTC252	P	**c.G>A; p.G12D (30%)**	NVD	P	**c.G>A; p.G12D (20%)**	NVD	0	NVD	NVD
CTC253	P	**c.G>T; p.G12V (50%)**	NVD	P	NVD	NVD	0	NVD	NVD
CTC254	P	NVD	NVD	F	NVD	NVD	1	NVD	NVD
CTC284	P	NVD	NVD	F	NVD	NVD	2	**c.G>T; p.G12V**	NVD
x	P	NVD	**insTAC; p.T599_V600insT (20%)**	F	NVD	**c.insTAC; p.T599_V600insT (70%)**	na	x	x

Correlation between *KRAS* and *BRAF* mutation status in the primary tumor, metastasis (as assessed by COLD-PCR) and CTCs (as assessed by ASB-PCR). COLD-PCR results for CTC mutation detection are depicted in Table 3. ASB-PCR data are depicted here to enable optimal judgement of concordance between three tumor compartments. CTC count depicted as number of cells per 30 mL blood. Estimated mutation frequency depicted in parentheses. F; fresh-frozen, P; formalin-fixed paraffin-embedded, NVD; no variant detected, *; no variant detected in ffpe primary tumor specimen, #; mutation only detected by sequencing, x; no sample available.

Table 3
Mutation detection in CTCs by different sensitive techniques

	KRAS	*BRAF*			*KRAS*			*BRAF*
Patient CTC code	mt in tissue	V600E mt in tissue	**CTC count**	**CTC equivalent in ASB-PCR**	nested ASB-PCR	EntroGen	COLD-PCR	nested ASB-PCR
CTC189			13	5,3	NVD	NVD	NVD	NVD
CTC190			3	1,2	NVD	x	x	NVD
CTC191			0	0,0	NVD	x	x	NVD
CTC192	+		2	0,8	NVD	x	x	NVD
CTC194	+		1	0,4	NVD	x	x	NVD
CTC195			5	2,1	NVD	x	x§	NVD
CTC196		+	5	2,1	NVD	x	x§	**c.T>A; p.V600E (0.5%)#**
CTC198			2	0,8	NVD	x	x	NVD
CTC200	+		0	0,0	NVD	NVD	NVD	x
CTC201	+		28	11,5	NVD	x	NVD	NVD
CTC202	+		7	2,9	**c.G>A; p.G13D (0.7%)#**	x	x§	NVD
CTC203			33	13,5	NVD	x	x§	NVD
CTC204	+		1	0,4	NVD	NVD	x	NVD
CTC207			0	0,0	NVD	x	x	NVD
CTC208	+		17	7,0	**c.G>A; p.G12D (8.9%)**	x	**c.G>A; p.G12D**	x
CTC209	+		3	1,2	NVD	NVD	x	NVD
CTC210			4	1,6	NVD	x	x	NVD
CTC211			1	0,4	NVD	x	x	NVD
CTC215	+		0	0,0	NVD	NVD	x	NVD
CTC216			2	0,8	NVD	x	x	NVD
CTC217			1	0,4	NVD	x	x	NVD
CTC218			5	2,1	NVD	x	NVD	NVD
CTC219			0	0,0	NVD	x	x	NVD
CTC220		+	0	0,0	NVD	x	x	NVD
CTC221			11	4,5	NVD	x	NVD	NVD
CTC222	+		37	15,2	**c.G>T; p.G12V (<0.01%)#**	NVD	NVD	x
CTC225			0	0,0	NVD	x	x	NVD

CTC226	+		0	0,0	NVD	NVD	x	NVD
CTC227			0	0,0	NVD	x	x	NVD
CTC243			0	0,0	NVD	x	x	NVD
CTC244			1	0,4	NVD	x	x	NVD
CTC245	+		3	1,2	**c.G>T; p.G12V (6.1%)***	NVD	NVD	NVD
CTC246			0	0,0	NVD	x	x	NVD
CTC247			0	0,0	NVD	x	x	NVD
CTC248			1	0,4	NVD	x	x	NVD
CTC249			3	1,2	NVD	x	x	NVD
CTC250			0	0,0	NVD	x	x	NVD
CTC251			0	0,0	NVD	x	x	NVD
CTC252	+		0	0,0	NVD	NVD	NVD	NVD
CTC253	+		0	0,0	NVD	x	x	NVD
CTC254			1	0,4	NVD	x	x	NVD
CTC284			2	0,8	**c.G>T; p.G12V (2.0%)§**	x	x§	NVD
NA		+	NA	NA	x	x	x	NVD

KRAS and *BRAF* mutation status as detected by COLD-PCR, nested ASB-PCR and sequencing in the CTCs of 42 colorectal cancer patients. mt; mutation, +; *KRAS* or *BRAF* V600E mutation in primary tumor and/or metastasis (for details see Table 2), NVD; no variant detected, x; sample not available, *; confirmed by sequencing. #no sample left for sequencing, §; failed PCR product in sequencing, dHPLC; denaturing high-performance liquid chromatography, ASB-PCR; allele-specific PCR with a blocking reagent. EntroGen data are not depicted in this table because no mutations were detected with this method in the nine tested CTC samples. Estimated mutation frequencies are depicted in parentheses.

Table 4

Detection limit of Entrogen qRT-PCR

	***KRAS* Ct**	
% mutated alleles	**G13D**	**Δ Ct to wt**
20%	26.1	13.9
10%	27.1	12.9
5%	27.3	12.7
2.5%	28.8	11.2
1.25%	30.1	9.9
0.625%	31.4	8.6
0%	NVD*	NA

EntroGen assay sensitivity in mixed synthetic DNA samples. Delta Ct cut-off mutation call criteria were -3.0. Sensitivity of the assay is determined by the minimum percentage of mutated (mt) alleles that could be detected among wild-type (wt) alleles at a delta Ct of - 3.0. Table shows the sensitivity of the *KRAS* EntroGen kit using synthetic DNA. NVD*; no variant detected after 40 Ct, NA; not available.

Table 5

Detection limit of one-run ASB-PCR

	***KRAS* Ct**		***KRAS* Ct**		***KRAS* Ct**		***KRAS* Ct**	
% mt alleles	**12CGT**	**Δ Ct to wt**	**12TGT**	**Δ Ct to wt**	**12AGT**	**Δ Ct to wt**	**12GGT**	**Δ Ct to wt**
20%	29	11	24.8	15.2	26.4	12.1	30.0	10.0
2%	32.5	7.5	28.0	12.0	29.9	8.6	33.4	6.6
0.2%	35.8	4.2	31.7	8.3	33.2	5.3	36.9	3.1
0%	NVD*	NA	NVD*	NA	38.5	NA	NVD*	NA

	***KRAS* Ct**		***KRAS* Ct**		***KRAS* Ct**	
% mt alleles	**12GCT**	**Δ Ct to wt**	**12GAT**	**Δ Ct to wt**	**12GAC**	**Δ Ct to wt**
20%	25.0	15.0	22.7	17.3	22.5	17.5
2%	28.5	11.5	26.1	13.9	25.9	14.1
0.2%	31.7	8.3	29.6	10.4	29.2	10.8
0%	NVD*	NA	NVD*	NA	NVD*	NA

One-run ASB-PCR (allele-specific PCR with a blocking reagent) assay sensitivity in mixed synthetic DNA samples. Delta Ct cut-off mutation call criteria were -3.0. Sensitivity of the assays is determined by the minimum percentage of mutated (mt) alleles that could be detected among wild-type (wt) alleles at a delta CT of -3.0. Table shows the sensitivity of the *KRAS* one-run ASB-PCR using synthetic DNA. NVD*; no variant detected after 40 Ct, NA; not available

Table 6

Detection limit of nested ASB-PCR

	KRAS Ct		*KRAS* Ct	
%mt alleles	**12GGT**	**Δ Ct to wt**	**13GAC**	**Δ Ct to wt**
5%	26.7	8.3	26.4	7.0
2.5%	27.6	7.4	27.6	5.8
1.25%	28.2	6.8	28.4	5.0
0.6125%	29.4	5.6	28.6	4.8
0%	35.0	NA	33.4	NA

Nested ASB-PCR (allele-specific PCR with a blocking reagent) assay sensitivity in mixed synthetic DNA samples. Delta Ct cut-off mutation call criteria were -3.0. Sensitivity of the assays is determined by the minimum percentage of mutated (mt) alleles that could be detected among wild-type (wt) alleles at a delta Ct of -3.0. Table shows the sensitivity of the nested ASB-PCR for *KRAS*mt detection sensitivity, including the reproducibility of the detection of a G12V and a G13D mutation using synthetic DNA. NA; not available

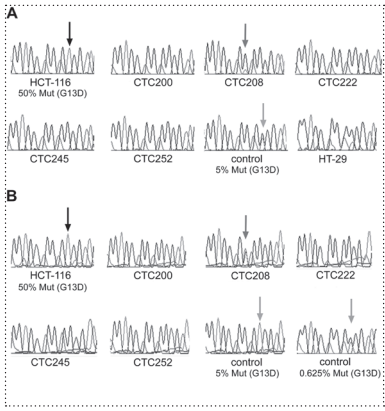
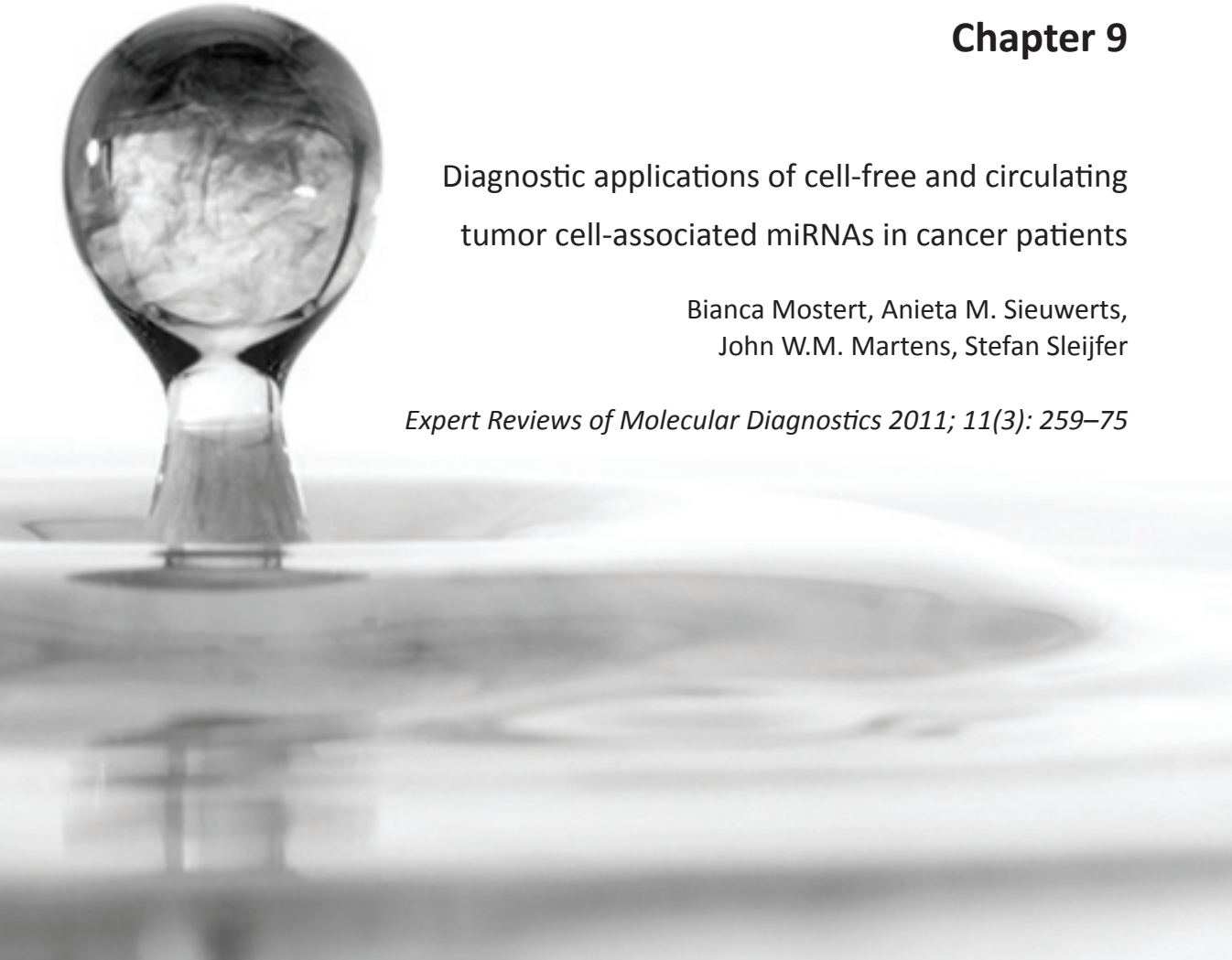


Figure 1: see section ' Color figures'

Supplementary data:

https://docs.google.com/open?id=0B9Etqm_r7T2mNnN5STlLajcyZFU



Chapter 9

Diagnostic applications of cell-free and circulating tumor cell-associated miRNAs in cancer patients

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SUMMARY

Recently, miRNA-expression profiling in primary tumors has yielded promising results. However, establishing miRNA expression in the circulation probably has advantages over determination in primary tumor tissue, further augmenting the potential applications of miRNA determination in oncology. Circulating tumor cells (CTCs) have rapidly developed as important prognostic and therapy-monitoring biomarkers in metastatic breast, colorectal and prostate cancer when enumerated, and their isolation enables subsequent analysis using various molecular applications, including miRNA-expression analysis. In addition to CTC-associated miRNAs, free circulating miRNAs have been identified in whole blood, plasma and serum. Determination of miRNAs in peripheral blood, either cell-free or CTC-associated, is expected to become important in oncology, especially when linked to and interpreted together with epithelial CTCs. In this article, we will discuss miRNA-expression profiling in primary tumors, depict the potential applications of measuring miRNA in the circulation and review the literature on cell-free circulating miRNAs, as well as offering some methodological and technical considerations on the measurement of circulating miRNAs.

INTRODUCTION

The implementation of assays enabling the detection of circulating tumor cells (CTCs) has sparked an additional boost of interest in blood-derived biomarkers for cancer patients. Numerous assays for CTC enumeration have been described lately and for one, the CellSearch® Epithelial Cell test (Veridex™ LCC, Raritan, NJ), FDA-approval has been acquired for use as a prognostic factor when measured in patients with metastatic breast[25-26], colorectal[29] and prostate cancer[31]. Additionally to its application as a prognosticator prior to treatment start, enumeration of CTCs may also guide treatment decisions, as a rise or decline in the number of CTCs after the first cycle of chemotherapy predicts for therapy response earlier than conventional radiographic evaluation does[237].

Probably even more interesting than mere counting, CTCs can be isolated from the blood of cancer patients for further analysis. In the case of metastatic disease, analysis of metastatic tissue can be very informative. Often, a considerable amount of time has passed since the occurrence and resection of the primary tumor and in many cases systemic adjuvant treatment has been administered. This means that clinically relevant changes can have occurred in the geno- and phenotype of residual cancer cells, and these changes could and probably should affect treatment decisions. Therefore, characterization of metastatic tissue, rather than that of the primary lesion, may show a better association with outcome in cancer patients.

Clinicians are however understandably reluctant to perform invasive and complicated procedures to obtain tissue from patients for whom quality of life is a major concern. The isolation and subsequent characterization of CTCs provide the opportunity to bypass the problems associated with obtaining metastatic tissue, and serve as a 'liquid biopsy'. CTCs have already been characterized for presence of gene amplification[40,306,315] and genetic aberrations[36,381], for expression of proteins[41] and several mRNAs[9,43,244], and recently, also for expression of certain miRNAs[38].

In recent years, miRNAs have been revealed as key regulators of gene expression. Given this crucial role, it is not surprising that miRNA expression in primary tumor tissue associates with outcome in several studies. However, determination of miRNA expression in the peripheral circulation, either CTC-associated or as cell-free circulating molecules, likely has several advantages over determination in primary tumor tissue, thereby further augmenting the potential applications of miRNA determination in oncology.

In this review, we will discuss the measurement of cell-free and CTC-associated miRNAs present in the peripheral circulation, and give examples of the clinical applications of this upcoming research field.

MiRNAs

MiRNAs (miRs) are small single-stranded RNA molecules, measuring 21 - 23 nucleotides in length, which have, since their discovery in 1993[384], been shown to play important roles in regulating gene expression[385]. Until recently, miRNAs were disregarded as degraded RNA fragments or non-translated small RNAs, but the discovery of their aberrant expression in a wide array of pathological events and their involvement in carcinogenesis has made them a hot topic in cancer research[270,386]. One of the big advantages of miRNAs is their stability, and it has been shown that cell-free miRNAs in body fluids are stable under harsh conditions such as high temperatures, extreme pH values, repeated freeze-thaw cycles and long-term storage[63,387-391]. They are well preserved, not only in blood, but also in tissues that have been formalin-fixed and paraffin-embedded years before[392]. This enables the retrospective analysis of large tissue collections, providing researchers with massive amounts of information. The identification of miRNAs has yielded an exciting new array of easy accessible molecular features, which may be employed in diagnostic and therapeutic decision making in cancer patients.

MiRNA biology

In the nucleus, miRNAs are transcribed by RNA polymerase II into large polyadenylated, capped primary miRNA transcripts (pri-miRNAs)[386,393] (**Figure 1, left panel**). These pri-miRNAs are subsequently cleaved by a complex formed by the RNAse II enzyme Drosha and its binding partner DGCR8 (DiGeorge syndrome critical region 8, or Pasha) into precursor miRNAs (pre-miRNAs). These pre-miRNAs are 70 - 90 nucleotides in length and have an imperfect stem loop hairpin structure. They are transported into the cytoplasm by Exportin 5 where the hairpin precursors are cleaved by a complex formed by the RNAse III enzyme Dicer and its binding partner TRBP (HIV-1 transactivating response RNA binding protein); resulting in a small dsRNA duplex that contains both the mature miRNA strand and its complementary strand. The mature miRNA strand is then incorporated into a RNA-induced silencing complex (RISC), which inhibits the function of its target mRNA by mRNA degradation or, most commonly, by translational repression after binding of the RISC to the target mRNA. Additionally, miRNAs can directly or indirectly increase the expression of their target mRNA[386,394]. An example of the multiple functions of *miR-210* in the for cancer important process of hypoxia, which allows cancer cells to adapt to a low oxygen environment, is shown in **Figure 1, right panel**.

MiRNA function

MiRNAs are estimated to regulate up to 30% of all protein-coding genes[395]. They regulate post-transcriptional gene expression in a sequence-specific manner, recognizing their mRNA target with the 5'-end of the mature miRNA strand, which is often referred to as the 'seed-

sequence'[396]. After recognition of the target mRNA, regulation of gene expression can occur through two different mechanisms, depending upon the complementarities of the miRNA sequence with its target mRNA. When perfect base-paring homology exists between the miRNA and the mRNA, the RNA-mediated interference pathway is induced, which leads to cleavage of the mRNA by Argonaute, present in the RISC complex. When imperfect binding to partially complementary sequences in the 3'-untranslated region of target mRNAs occurs, which is more frequent than perfect binding, the target mRNA is regulated by repression of protein translation. Consequently, proteins are regulated by miRNAs without significantly affecting the corresponding mRNA expression levels. Such knowledge underscores the need to combine mRNA and miRNA data to generate improved predictive and prognostic models.

MIRNAS IN PRIMARY CANCER AND THEIR POTENTIAL APPLICATIONS

MiRNAs are thought to play two distinctly different roles in carcinogenesis, functioning both as 'oncomirs' and as tumor suppressors. This hypothesis is supported by the observation that miRNA expression in tumors can be up- or downregulated compared to normal tissue[397]. The miRNA expression profiling of tumors has provided many new insights into states of differentiation and lineages within different tumor types.

As a consequence of the crucial role of miRNAs in tumor biology, there is a broad range of potential applications of miRNA measurement in oncology. Besides being informative of tumor biology, miRNA signatures can also be a diagnostic tool, serve as prognostic factors, predictive factors, potential drug targets and as pharmacodynamic markers. All of these applications are possible in primary tumors and metastases, but the stability of miRNAs also enables their detection in the circulation. In this field, circulating miRNAs can serve as biomarkers that can be measured repeatedly and non-invasively in a wide array of cancer types.

Research to date has however mainly focused on primary tumor tissue. We will, without attempting to give a complete overview, provide examples of miRNAs being used as any of the aforementioned biomarkers, before proceeding with how this knowledge can and has been applied to circulating miRNAs.

MiRNAs to identify cancer tissue origin

MiRNAs can serve to determine the tissue of origin for cancers of unknown primary origin, as has been shown with a classifier based on 48 miRNAs[398-399]. This microarray-based classifier was generated on 205 primary tumors and 131 metastases of 22 different tumor origins. The classifier was validated in an independent test set, in which it reached an overall sensitivity of 72% and a specificity of 99%. This application could be very informative in the still existing problem of metastatic cancer patients in whom no primary tumor can be identified, and for

whom no standard chemotherapy exists.

MiRNA expression profiles to classify cancers

Lu and co-workers were also able to successfully classify poorly differentiated tumors using miRNA expression profiles. Contrarily, messenger RNA profiles were highly inaccurate in classifying tumors when applied to the same samples[397]. Breast cancer is a notoriously heterogeneous disease, but miRNAs can help to identify the subtype origin of tumor cells, as was demonstrated by Sempere and co-workers using an in situ hybridization method to reveal the spatial distribution of miRNA expression in archived formalin-fixed, paraffin-embedded breast tumors[400].

MiRNAs as prognostic factors

Many investigators have focused on identifying miRNAs that can separate patient groups according to prognosis. It would be beyond the scope of this review to discuss all studies that have identified such prognostic miRNAs, and we refer to Ferracin et al[401] for a complete and comprehensive overview.

Predictive miRNAs

Not many data have been generated identifying specific miRNAs that can predict response to systemic therapy. This is not surprising, as determining true predictive value of a miRNA requires studies designed very carefully specifically for that research question.

Ovarian cancer

In ovarian cancer, *miR-214* has been identified as a miRNA involved in resistance to cisplatin, through targeting of *PTEN*[402]. In this study, 4 of the most differentially expressed miRNAs among a total of 515 miRNAs tested in 10 ovarian tumors and 10 normal cell line pools were further validated. *MiR-214* was one of the most frequently upregulated miRNAs in 30 primary ovarian tumors; and the expression of *miR-214* in *miR-214*-negative cell lines led to resistance to cisplatin-induced cell death, and subsequent knockdown of *miR-214* resulted in increased sensitivity to cisplatin-induced cell death[402]. These promising results should be validated in patients treated with cisplatin before *miR-214* can be used as a valid biomarker to predict cisplatin response.

Non-small cell lung cancer

This validation in patient samples was performed in a study looking at the predictive value of *miR-128b* expression on response to gefitinib, an EGFR inhibitor, in non-small cell lung cancer (NSCLC)[403]. *MiR-128b* was chosen based on its regulatory role for EGFR and the fact that loss of chromosome 3p, where *miR-128b* is located, is one of the most frequent and earliest events

in lung carcinogenesis. An inverse relationship between *miR-128b* and EGFR expression was observed in NSCLC cell lines, and while EGFR expression as assessed by immunohistochemistry did not correlate with gefitinib response in 58 NSCLC patients, *EGFR* mutations and loss of *miR-128b* were associated with improved response to gefitinib. In multivariate analysis, only histology, line of treatment and loss of *miR-128b*, and not EGFR expression or mutation, were found to be predictive of response[403].

Hepatocellular carcinoma (HCC)

Ji et al. undertook a carefully designed study in three independent cohorts of a total of 455 HCC patients, and identified *miR-26* to be lower expressed in tumors than in paired noncancerous tissue[404]. Additionally, of the patients who were not treated with interferon, the control arm of the cohorts, those with lower expression of *miR-26* in their tumor had a shorter overall survival. Contrarily, of the patients in the treatment arm of the cohorts who did receive interferon, those with lower *miR-26* expression had an improved survival compared to patients with higher *miR-26* expression. In multivariate analysis too, a significant interaction was observed between *miR-26* expression and response to interferon therapy[404].

Breast cancer

While data have been generated on breast cancer cell lines[405-406] we recently selected 5 candidate predictive miRNAs from 249 miRNAs measured in a small discovery set of breast cancer specimens and analyzed their expression in an independent series of 246 ER-positive primary breast tumors. In multivariate analysis, higher expression of *miR-30c* was associated with benefit from first line tamoxifen monotherapy and longer progression-free survival[407].

MiRNAs as drug targets

Because of their pivotal role in cancer development, progression and treatment, several preclinical findings point at the great potential to use miRNA as drug targets, either by inhibiting overexpressed 'oncomirs' or replacing underexpressed tumor suppressor miRNAs. Inhibition of *miR-21* has been shown to reduce tumor development and metastatic potential in breast cancer cells[408]. Inhibition of *miR-21*, combined with *miR-200b*, also enhanced response to gemcitabine in cholangiocarcinoma cells[409]. In breast cancer cells, reintroducing *miR-205* resulted in improved response to tyrosine kinase inhibitors through *HER3* silencing[410].

Finally, researchers have demonstrated that hepatocellular carcinoma cells have reduced expression of *miR-26*, while this miRNA is highly expressed in normal tissues. Re-expression of *miR-26* caused cells to arrest in G1, probably through repression of *cyclin D2* and *cyclin E2*. When administering *miR-26* to a mouse model using an adeno-associated viral vector, cancer cell proliferation was reduced and apoptosis increased[411].

MEASURING MIRNAS IN THE CIRCULATION

As depicted above, promising results have been obtained in primary tumor material with respect to miRNAs as cancer biomarkers. There are however a number of situations in which it is likely that the value of miRNAs can be further augmented by measuring miRNAs in blood, either as cell-free circulating miRNAs or as CTC-associated miRNAs. Below we will now discuss the potential applications of circulating miRNAs.

Circulating miRNAs can help to more accurately predict patient outcome

Many studies have focused on identifying prognostic miRNAs in primary tumors, and these prognostic factors are now known for a large variety of tumor types. While these prognostic miRNAs do distinct those patients with a favorable outcome from those with an unfavorable outcome, measuring miRNAs in the primary tumor does not take into account two important things; not all cells in the primary tumor have the ability to metastasize, and the subset of spreading tumor cells might differ in genetic make-up. Secondly, at the time of metastatic disease, genetic characteristics of the remaining or relapsing tumor cells can differ from those of the primary tumor, because by the time a patient presents with metastatic disease, years might have gone by since first presentation, and various different anti-tumor treatments can have been administered. Both these factors can cause profound differences in genetic and epigenetic make-up between primary tumor and metastatic tissue. At the time of disseminated disease, optimally, metastatic tissue would be used to determine prognosis; however, acquiring such tissue can often only be done through painful and invasive procedures. Circulating tumor cells can serve as a 'liquid biopsy' representing the patient's tumor load, and thereby provide a unique opportunity to assess prognosis in real time.

Furthermore, the presence of miRNAs that are associated with the process of metastasis or epithelial-to-mesenchymal transition, a process that is thought to be necessary for haematogenous spread of disease to occur, might identify those patients that already have distant micrometastases too small to diagnose otherwise.

Circulating miRNAs to predict response to anti-tumor therapy

When systemic therapy is warranted, either in the adjuvant or metastatic setting, the choice of first line treatment can be crucial for ultimate patient outcome. Depending on tumor type, various patient and tumor characteristics are taken into consideration when deciding on the optimal treatment, but still, for a proportion of patients, ineffective therapy is started. Especially in patients receiving targeted therapy, such as EGFR inhibitors, factors determining their benefit are still being discovered. While most attention has been given to predictive factors such as *KRAS* mutations and EGFR expression, it may be expected that miRNAs will turn out

to play an equally important role, given their pivotal role in cancer progression. As discussed before, circulating miRNAs can be measured repeatedly, which is especially important for their use as a predictive factor. One can imagine wanting to administer a certain systemic therapy as second-line treatment, for which a predictive miRNA has been established. While this miRNA can be measured in the primary tumor, earlier administered systemic therapy can have affected the expression of this miRNA in residual cancer cells. It is, therefore, very conceivable that a treatment adapted to circulating tumor characteristics is more beneficial than a treatment based on primary tumor characteristics.

Whenever a certain treatment has been started based on miRNA expression data, re-assessment can occur each time the patient becomes refractory to the installed treatment. Acquired resistance to systemic anti-tumor treatment is a major problem in cancer treatment, and overcoming that resistance by administering targeted therapy based on changed tumor characteristics might greatly improve patients' prognosis.

Circulating miRNAs as a monitorable drug target

One of the big theoretical advances of targeting miRNAs is the ability to monitor their expression in the circulation. When anti-miRNA treatment is started, miRNA expression levels could be monitored in blood at various time points, and their increase could predict treatment resistance and warrant a switch in therapeutic regimen. Already, it was shown that the administration of intravenous anti-miR-16, anti-miR-122, anti-miR-192 and anti-miR-194 caused a decrease in the levels of the corresponding miRNAs across all organs in mice[412]. In this sense, circulating miRNAs could serve as combined drug targets and pharmacodynamic markers.

CELL-FREE CIRCULATING MIRNAS

In view of the potential advantages of determining miRNA expression in the peripheral circulation over that in primary tumor tissue, several studies have already identified free circulating miRNAs that are expressed in the circulation of cancer patients. Importantly, most of these miRNAs were found to be differentially expressed between cancer patients and healthy donors. These miRNAs were found to be either diagnostic or prognostic, but little study has been done on their potential roles as predictive factors or drug targets. The main findings of the studies on circulating miRNAs in relation to diagnosis and prognosis are reviewed below and summarized according to 12 different primary tumor types in **Table 1**. We have focused on solid tumors for this review, and refer to Fabbri et al[413] for a comprehensive review of the many research advances in the field of miRNAs in hematological malignancies.

Carcinomas of unknown primary

MiRNAs can serve to determine the tissue of origin for cancers of unknown primary origin, as has been shown with a classifier based on 48 miRNAs determined in primary or metastatic tumor tissue[398-399]. Lodes et al. focused on the evaluation of miRNA expression patterns in human serum for five types of human cancer, prostate, colon, ovarian, breast and lung, using a pan-human microRNA, high density microarray, and identified a serum classifier based on 28 circulating miRNAs able to separate cancer cases from normal individuals[414]. In serum of cancer patients, specific miRNA expression patterns for lung cancer and colorectal cancer have been identified[387], providing evidence that miRNAs present in the circulation contain fingerprints for various diseases.

Breast cancer

In 148 breast cancer patients and 44 healthy controls, seven candidate miRNAs were measured in whole blood by RT-PCR without a preceding enrichment step. All miRNAs could be measured in patients and controls alike, but *miR-195* and *let-7a* were expressed higher in breast cancer patients than in controls, with a mean fold change of 19 and 11, respectively. Additionally, the levels of these two miRNAs decreased significantly after curative tumor resection[415].

The first study that reported circulating miRNAs as potential biomarkers of early stage breast cancer with different results for Caucasian American (CA) versus African American (AA) women, concerns the study of Zhao and co-workers. After comparing levels of circulating miRNAs in plasma samples of 20 patients with early stage breast cancer and 20 matched controls, they reported 17 upregulated and 14 downregulated miRNAs in the 10 CA women and 9 upregulated and 9 downregulated miRNAs in the 10 AA women. Furthermore, they were able to link these differentially expressed miRNAs to specific pathways using target prediction algorithms[416].

In a larger study evaluating *miR-21* expression in the serum of 102 breast cancer patients and 20 healthy controls, this miRNA was found to be higher expressed in patients, especially in stage IV breast cancer[417]. Recently, 4 breast cancer associated miRNAs were measured in the serum of 59 localized breast cancer patients after primary tumor surgery, 30 metastasized breast cancer patients and 29 healthy controls. *MiR-10b*, *miR-34a* and *miR-155* discriminated metastasized breast cancer patients from controls, and the latter was higher expressed in localized breast cancer patients than healthy controls but also than metastasized breast cancer patients[418]. Another study measured three miRNAs (*miR-16*, *miR-145* and *miR-155*) in the serum of 13 breast cancer patients and 8 healthy controls, but did not find a difference in expression between these two groups[419].

Non-small cell lung cancer

Hu et al. used serum of NSCLC patients to look for miRNAs that were differentially expressed between 30 patients with longer survival and 30 patients with shorter survival, matched by age, sex and stage. Eleven miRNAs were found to differ more than five-fold between the two groups, and four of those were confirmed by RT-PCR to be associated with survival, also in a larger validation set of 243 NSCLC patients. While these data are very encouraging, the investigators unfortunately measured these miRNAs in only one healthy donor, and their specificity for NSCLC is thus not sufficiently clear[420]. A comparison with more healthy controls was done with a pooled sample of 11 Chinese lung cancer patients, in whom 28 miRNAs were downregulated and 63 miRNAs were upregulated compared to 11 male and 10 female normal controls. Two of the highest expressed miRNAs, *miR-25* and *miR-223*, were validated in an independent set of 152 lung cancer sera and 75 normal sera and also found to be higher expressed in these patients[387].

A different approach was used by Silva et al, who preceded their tests by an EpCAM-based immunomagnetic enrichment step. Out of 365 candidates, no miRNAs were found to be upregulated in 28 patients as compared with 20 controls, but 10 miRNAs were downregulated. Three of these were differentially expressed in the validation step as well, and lower levels of *let-7f* were associated with shorter overall survival, while patients with lower levels of *miR-30e-3p* had shorter disease-free survival, without difference in overall survival[421].

Prostate cancer

In prostate cancer patients, a panel of six candidate miRNAs, selected on their expression in prostate tumors and lack of expression in healthy donor blood, was analyzed in two pools of 25 metastatic prostate cancer patients and 25 healthy controls, respectively. Out of the candidate miRNAs, *miR-141* showed the greatest differential expression between the two pools, and this miRNA was confirmed to be higher expressed in cancer patients on an individual level as well[389].

Brase and co-workers unfortunately did not validate their interesting findings of the upregulation of 5 miRNAs out of a panel of 667 candidate miRNAs in the serum of prostate cancer patients in healthy controls. They did observe that the expression of two of the 5 miRNAs, *miR-375* and *miR-141*, was upregulated in malignant prostate tissue compared to benign prostate tissue, but concerns about the specificity of these miRNAs in serum remain[422].

Using an array method, Moltzahn and co-workers screened the expression level of 384 miRNAs in 12 healthy controls and 36 prostate cancer patients divided into three groups according to a validated risk score. The twelve miRNA candidates that were most differentially expressed between cancer patients and controls were validated by individual qRT-PCR, which confirmed

the differential expression of nine miRNAs. No significant correlation was seen with risk scores or other clinicopathological parameters[52].

Lodes et al. used microarray profiling and found 15 miRNAs to be over-expressed in serum from 6 prostate cancer patients (all stage 3 and 4) relative to expression in 8 normal male controls[414].

Ovarian cancer

In ovarian cancer too, interest has been generated to detect miRNAs in the peripheral blood of cancer patients. Comparing 9 serum samples from ovarian cancer patients to 4 serum samples from healthy donors, 21 differentially expressed miRNAs were identified. Eight could be confirmed in 19 cancer versus 11 normal specimens to be differentially expressed by RT-PCR, of which 5 (*miR-21*, *-29a*, *-92*, *-126* and *-29a*) were upregulated expression in cancer patients, probably making these more suitable for clinical implementation[423].

MiRNA expression was also measured in EpCAM-positive exosomes. Exosomes are microvesicles that are actively released by tumors into the peripheral circulation[424] and it was hypothesized that miRNAs detected in exosomes reflect those present in CTCs. Exosomes were isolated from 50 ovarian cancer patients and 20 controls using an immunomagnetic enrichment method based on anti-EpCAM. Eight miRNAs were found to be differentially expressed between the two groups[63].

Gastric cancer

Analyzing plasma samples of 69 gastric cancer patients taken before surgery and 30 healthy donors, five miRNAs were found to be differentially expressed[425]. Two of these five, *miR-106a* and *miR-17*, were also identified in samples of 90 patients (of which, remarkably, 49 were taken after resection of the primary tumor), to be differentially expressed compared to 27 healthy donors. Both miRNAs were expressed at a lower level after surgery compared to before surgery, but still differed about 10-fold from healthy controls[426].

As part of a larger study looking at liver pathology-specific miRNAs, gastric cancer patients were also evaluated for differential miRNA expression compared to controls. *MiR-885-5p*, which was also found to be upregulated in hepatocellular carcinoma patients (see below), was higher expressed in gastric cancer patients[427] compared to controls.

Hepatocellular carcinoma

MiR-500 was identified as highly expressed during fetal liver development and thus postulated to be involved in proliferation. Indeed, *miR-500* was highly expressed in hepatocellular carcinoma cell lines, but its expression was higher in only 18 of 40 hepatocellular carcinomas

compared to adjacent non-tumorous tissue, and in the serum of 3 of ten HCC patients[428].
Another study looking at HCC identified *miR-885-5p* as a miRNA of interest in this disease, being expressed higher in HCC patients than in healthy controls, liver cirrhosis and chronic hepatitis B patients[427].

Colorectal cancer

At least three studies have looked into the occurrence of selected candidate miRNAs in the plasma of colorectal cancer patients. A large study looked at samples from 120 primary colorectal cancer patients and 37 advanced adenoma patients, both taken before surgery, and compared them to 59 age-matched healthy controls who were confirmed to be without colorectal cancer by extensive diagnostic procedures including colonoscopy and CT scan. Two miRNAs, *miR-29a* and *miR-92a*, were identified from a training set and confirmed in the larger validation set to be upregulated in CRC plasma compared to controls. In adenoma patients too, these miRNAs were expressed higher than in controls, but significantly lower than in true cancer patients. Additionally, these two miRNAs decreased after surgery in another 20 colorectal cancer patients, suggesting that these miRNAs are in fact cancer-specific[429].
Another study also found *miR-92a* to be higher expressed in CRC patients. Five miRNAs were selected based on higher expression in CRC plasma compared to healthy control plasma and higher expression in primary cancerous biopsies compared to adjacent non-cancerous colon tissue. Of these miRNAs, the two that were significantly elevated in 25 CRC patients compared to controls and decreased after tumor resection (*miR-92a* and *miR-17*), were validated in an independent cohort of 90 CRC patients and 50 controls. Additionally, both *miR-92a* and *miR-17* were not expressed higher in patients with gastric cancer or inflammatory bowel disease, confirming their specificity[430].
Pu et al. chose to investigate *miR-221* out of three miRNAs abundantly expressed in CRC, because of the good linearity in spiking samples obtained with this miRNA. In 103 CRC patients, *miR-221* expression was higher than in 37 controls, however with a low specificity of 41% at the optimal cut-off level. *MiR-221* expression did correlate with overall survival and p53 expression[431].

Pancreatic cancer

In pancreatic cancer, two miRNAs, *miR-200a* and *miR-200b*, involved in epithelial mesenchymal transition, were identified to be hypomethylated and overexpressed in primary tumors compared to surrounding normal pancreas tissue. In 45 serum samples obtained from pancreatic cancer patients before surgery, both miRNAs were expressed at a higher level than in samples from 32 healthy controls and 11 chronic pancreatitis patients[432].

Ho et al. looked for pancreatic cancer-specific expression of *miR-210* in the circulation, as this miRNA increases under hypoxic conditions, which are known to correlate with poorer prognosis. *MiR-210* expression was measured in the plasma of a total of 22 locally advanced pancreatic cancer patients and 25 age-matched healthy controls, and confirmed to be 1.7 – 4-fold higher expressed in the patients[433].

Head and neck cancer

Wong et al. examined the expression of a large panel of miRNAs in tongue carcinomas and paired normal tissues, which identified 24 up regulated and 13 down regulated miRNAs. Because of its 59-fold higher expression in tumor tissue, *miR-184* was further validated in an independent dataset and observed to be more abundant in plasma of patients with tongue squamous cell carcinoma than in controls. Additionally, *miR-184* levels dropped after resection of the primary tumor[434].

Esophageal squamous cell carcinoma (SCC)

In esophageal SCC, one large study was recently published in which 25 miRNAs measured in serum were found to be upregulated in a pool of 141 cancer patients compared to controls. Of these 25, 7 miRNAs were confirmed to be differentially expressed by individual qRT-PCR in a separate patient cohort, yielding higher AUCs than carcinoembryonic antigen (CEA)[435].

Rhabdomyosarcoma

Besides carcinomas, research has also been focused on specific miRNAs in rhabdomyosarcoma (RMS). Looking at RMS cell lines and primary tumor tissues, *miR-206* was found to be most abundantly expressed among several muscle-specific miRNAs. *MiR-206* was also the marker with the highest sensitivity and specificity in discriminating 10 RMS-patients from 28 patients with other pediatric tumor and 17 healthy donors, but *miRs-1*, *-133a* and *-133b*, involved in muscle proliferation and differentiation[436], were also higher expressed in RMS patients than in controls or non-RMS patients[437].

CTC-ASSOCIATED MIRNAs

CTC-associated vs. cell-free miRNAs

As depicted above, studies on cell-free circulating miRNAs yield very interesting results and show the measurement of miRNAs in the circulation to be both feasible and clinically relevant. However, it is to be expected that not all miRNAs can actually be measured in the peripheral circulation. Especially in view of the fact that at least 100 different miRNAs already circulate

in the blood of healthy donors[387,416], it is very likely that measuring these miRNAs in whole blood, serum or plasma from cancer patients can yield false-positive results. Several studies have identified circulating miRNAs that are differentially expressed between patients and healthy donors (**Table 1**). Most of these studies have measured miRNAs in the serum, plasma or exosome fractions of blood, instead of using whole blood. Using serum or plasma does for the most part eliminate the leukocyte background present in whole blood, but evidence has been presented that most miRNAs measured in these fractions are not actually derived from circulating epithelial cells[389]. Also, cellular miRNA expression patterns can differ from miRNA patterns released into the blood[438]. These studies raise the concern that cell-free miRNAs present in the circulation may not be a reliable representation of metastatic or primary tumor tissue, and that measuring CTC-associated miRNAs would be preferable. Besides possibly better representing the tumor load, measuring miRNAs in CTCs has the additional benefit of being able to correlate a miRNA signal to a CTC count, which aids in the interpretation of epithelial specificity.

Despite the potential benefits of measuring CTC-associated miRNAs, most work so far has been done on cell-free miRNAs. Data have been generated suggesting that the large majority of miRNAs are present in cell-free form and are not cell-associated[389]. These cell-free miRNAs can enter the circulation roughly through three different potential pathways: (1) passive leakage from apoptotic or necrotic cells, which can occur in tissue damage or chronic inflammation, and has been shown to occur after heart tissue injury[439]; (2) active and selective secretion of microvesicle-free miRNAs, which could be derived from tumor cells or circulating microvesicles; and (3) active and selective secretion of miRNA-containing microvesicles, including microparticles and exosomes. These mechanisms can occur in malignant cells, enabling miRNA from circulating tumor cells or primary or metastatic tumor cells to enter the circulation, but also in non-malignant cells with a short half-life, such as platelets, or upon tissue damage in non-malignant cells.

Another question surrounding cell-free miRNAs is what enables them to remain in the circulation despite the presence of endogenous RNAses. In this regard, the secretion of microvesicles is made more plausible, as the inclusion of miRNA in microvesicles could protect them from degradation[440]. However, more hypotheses have been postulated to explain the stability of miRNAs in the circulation, including modification of circulating miRNAs through processes such as methylation and adenylation[441] or binding of circulating miRNAs to as of yet unknown proteins[442].

Function of cell-free miRNAs

The function of the release of cell-free miRNAs as an active process remains largely unclear.

Recent evidence suggests that the transportation of miRNAs in microvesicles results in regulation of gene expression in the recipient cells[443]. Exosomes in general are thought to play a role in the communication between cells[424], as has been shown in vitro by Skog et al., who showed that glioblastoma-derived microvesicles were incorporated by human brain microvascular endothelial cells[62].

It is an attractive hypothesis that exosomal miRNAs can be selectively transferred to other cells, thus enabling tumor cells to manipulate both their direct and distant environment, possibly leading to increased metastatic potential. These microvesicles containing miRNAs could then theoretically also form an attractive drug target.

Enrichment of CTC-specific miRNAs

When testing whether identified cell-free miRNAs can be measured in CTCs, or identifying new CTC-associated miRNAs, an enrichment step is crucial. Most methods aimed at specifically molecularly characterizing CTCs in whole blood are preceded by such an enrichment step. Many methods are available, including enrichment based on size, density or specific marker expression[219]. These enrichment steps aim to isolate all CTCs from full blood, while getting rid of as many contaminating peripheral blood mononuclear cells (PBMCs) as possible. However, even when applying tumor-specific marker enrichment, hundreds to thousands of leukocytes are still present in the CTC-enriched fraction[9]. Also, the actual number of leukocytes may differ depending on tumor stage[418]. These leukocytes generate a background signal and thus complicate the measurement of CTC-specific miRNA expression, as only epithelial-specific miRNAs that are hardly expressed in leukocytes can be reliably measured. Many efforts are being made to develop a CTC isolation method that provides a purer CTC fraction for downstream analysis, based on for instance micromanipulation techniques[444]. Obtaining a higher purity of the enriched CTC fraction through more specific CTC isolation techniques would eliminate the need to only measure epithelial-specific genes, i.e., genes that are much higher expressed in CTCs than in leukocytes. So far, however, these techniques have not become widespread available and need further validation.

Despite these challenges, measuring CTC-associated has proven to be feasible. In our own work (manuscript submitted), we were able to identify 10 miRNAs more abundantly expressed in patients with >5 CTCs compared to patients without detectable CTCs and healthy donors.

Remaining technical issues concerning the measurement of CTC specific miRNAs

It is to be expected that the development of enrichment methods that provide a purer CTC fraction will simplify the measurement of CTC-associated miRNAs. In the meantime, a number of aspects need to be taken into consideration when measuring these CTC-specific miRNAs.

Because of the low numbers of CTCs in the circulation, frequently less than 5 CTCs in 7.5 mL blood[8], sensitive RNA isolation techniques and unbiased pre-amplification steps are needed. Fortunately, kits are now on the market which enable the isolation of DNA, large RNA fragments (mRNA, >200 bp), small RNA fragments (micro- and non-coding RNA, <200 bp) and proteins in 4 separate aliquots from as little as one cell (**Figure 2**). After this sensitive fractionated RNA isolation, it is crucial that only epithelial-specific miRNAs are measured that are not or very weakly expressed in leukocytes. To estimate the ratio of the tumor cell-specific signal over leukocyte-derived signal, which is unfortunately present even after enrichment procedures, transcript levels of CTCs-specific miRNAs such as those in the *miR-200/141* family[389,445] and leukocyte-specific miRNAs such as *miR-429* can be compared[445]. The suitability of any miRNA combination to estimate epithelial-specific signal does however depends on the epithelial tumor cell type or subtype studied. Furthermore, due to the presence of cell-free EpCAM-positive exosomes in serum[63], it remains to be established which part of the miRNA signal from EpCAM-enriched CTC-fractions is actually derived from CTCs and which part from other EpCAM-enriched cells or cell fragments including exosomes. Another factor complicating miRNA measurement is the lack of an established constitutively expressed set of reference miRNAs that can be used to normalize candidate miRNA expression levels. *MiR-16* has been used as a reference in several studies[390,418], but concerns have been raised due to its inconsistent expression in sera[414]. RNU/SNORs are also frequently used (**Table 1**), but it needs to be realized that these small RNA's are longer than the actual mature miRNAs being studied, making them less suitable for normalization. Furthermore, *RNU6B (U6)* has been reported to be degraded in serum samples[435]. Until consensus has been established on a robust reference miRNA set, normalizing on the mean expression of all expressed and CTC-specific miRNAs is probably the optimal method when multiple miRNA transcripts are measured at the same time[292]. In **Figure 2** we summarize the steps that need to be taken to ensure epithelial tumor cell specific gene expression profiling of CTCs.

CONCLUDING REMARKS

When critically looking at the data generated thus far measuring miRNA expression in the circulation, a few remarks must be made.

Firstly, the methods that are used to identify differentially expressed miRNAs vary greatly; many researchers start off with candidate miRNAs of interest that have previously been associated with a cancer type, while others look at all differentially expressed miRNAs between healthy controls and patients or between tumor tissue and normal adjacent tissue. This latter approach also enables the identification of potential up- or downmodulated pathways associated with

differentially expressed miRNA transcripts. Furthermore, a combination of several higher and lower expressed miRNAs is likely more informative than analysis of the expression of a single marker alone.

Secondly, some studies have unfortunately failed to validate their results in healthy controls, raising concerns about the specificity of potentially interesting miRNAs. Similarly, as with CTC enumeration, and also with regard to gene expression profiling in CTCs, specificity is pivotal when trying to identify tumor-specific signal particularly when contaminating leukocytes are present.

Lastly, any miRNA that is identified in a patient cohort to be differentially expressed or associated with prognosis should be validated in an independent cohort and reach acceptable sensitivity and specificity before it can be implemented into the clinic for routine analysis. While some studies have used validation sets, most have not, hampering the translation from bench to bedside.

Despite these possible flaws in some studies, measuring circulating miRNAs, cell-free or CTC associated, has proven to be feasible, can generate tumor-specific results, and may thus be of clinical relevance if their expression can be robustly measured and is sufficiently correlated with clinical outcome parameters such as overall survival or therapy response. The value of circulating miRNAs is expected to increase rapidly with the development of techniques that are able to isolate a more pure CTC fraction. Such an improvement will enable the measurement of any of the discussed prognostic and predictive miRNAs that have been identified in primary tumor tissue. Even more so than prognostic factors, the oncology field is devoid of reliable and robust predictive factors that adequately guide oncologists in the choice of optimal treatment for their patients. MiRNAs in the circulation may provide a new opportunity in this direction because of their stability and far-stretched effects in cancer biology and disease progression, and research should be aimed towards identifying and validating the predictive potential of these markers.

MiRNAs are a valuable addition to the information that CTCs already provide. In **Figure 3** we have depicted our view of the future role of CTC analysis before and during treatment of cancer patients, providing both prognostic, predictive and drug target information at different time points.

EXPERT COMMENTARY

MiRNAs have become an important research field, and have proven their value as regulators in carcinogenesis and cancer progression in many different cancer types. If these relatively stable small RNAs can be robustly detected by highly sensitive PCR methods routinely available in

most laboratories, they can become a new class of biomarkers. More important for the clinical setting, the prognostic value of certain miRNAs has been established in various cancer types and data are slowly emerging on their predictive value. These clinically relevant miRNAs are subsequently sought for in the circulation of cancer patients to enable their repeated and non-invasive measurement. A few issues have to be accounted for before miRNA expression in the circulation as a tool to predict prognosis or therapy response is ready for the clinic.

Firstly, consensus should be established on which fraction of peripheral blood should be used to measure miRNAs. Current studies have been performed in non-enriched or enriched whole blood, serum and plasma, without clear data being available on the distribution of miRNAs is these different blood compartments. It is conceivable that only a selection of miRNAs is, actively or passively, shed from circulating tumor cells. Also, the use of an enrichment marker for whole blood can greatly influence the amount and type of CTCs that are subsequently characterized.

Secondly, more data should be generated on the occurrence and expression levels of circulating miRNAs in healthy individuals. This can be done either by testing selected panels of miRNAs in a large cohort of gender- and otherwise matched healthy controls in parallel with cancer patients.

Thirdly, the discussion on which constitutively expressed miRNAs to use as a reference gene set is ongoing and it remains to be seen if a cell type-independent panel can be identified. Until that time, it is imperative that each study clearly states their normalization method and their reasons to choose said method.

FIVE-YEAR VIEW

While enumeration of CTCs has already proven its strength, the future of molecular analysis lies in the development of CTC isolation assays that generate higher CTC numbers and thus increased tumor DNA, mRNA and miRNA content, and reduce the number of contaminating leukocytes. We have already shown that the enrichment marker used in one of the most common CTC detection assays, the EpCAM-based CellSearch technique, is not expressed in all breast cancer subtypes[64]. This epithelial cell adhesion molecule (EpCAM) does not detect normal-like breast cancer cells, which can however be detected by adding CD146 in the enrichment step[250]. This is just one example of a probably more widespread problem of marker heterogeneity among cancer subtypes, which causes us to miss a subset of CTCs. The development of a method that either uses a panel of antibodies to detect CTCs, or a method that is independent of marker expression, but instead on for example physical properties of tumor cells, is probably the answer to this problem. Especially the latter option, selecting CTCs

based on unique properties such as membrane stiffness, is a still developing field[446]. Following this selection step by micromanipulation will enable the isolation of single CTCs, which makes them available for downstream applications such as whole genome DNA or transcriptome sequencing, but also allowing culturing of these cells.

In a few years time, anti-miRNA treatments will probably become available, and it is very likely that at least some of these miRNAs will be detectable in the circulation. This could simplify and fasten the testing of these drugs in phase I and II clinical trials, as the level of the target miRNAs can be directly measured in the patients' blood.

KEY ISSUES

- There is an urgent need for additional diagnostic, prognostic and predictive markers in oncology
- These markers should preferably be measurable at any time during the course of the disease
- Circulating tumor cells provide an unique opportunity to diagnose the origin and type of primary tumor, and to assess prognosis, response to therapy and drug targets non-invasively and repeatedly
- Among these markers, miRNAs are especially promising because of their stability and pivotal regulatory role in carcinogenesis
- Many miRNAs have already been identified to be of prognostic value in primary tumors
- Some miRNAs have shown predictive value in cell lines and patients
- Measuring miRNAs in the circulation is feasible and, depending on the choice of miRNA, can be cancer-specific
- A properly sized control cohort of healthy blood donors is a pre-requisite for these kind of studies
- So far, very few circulating tumor cell associated miRNAs have been associated with prognosis or therapy response
- The technical challenge is to discriminate between epithelial tumor cell-specific miRNAs and miRNAs from background leukocytes

Table 1
Circulating cell-free miRNAs

	Tumor type	Sample type	Enrich-ment step	Method	# pts	# HDs	Normalization procedure based on	# candidate miRNAs	Differentially expressed miRNAs		Progn	Ref
1	**Carcinoma of unknown primary**	serum	no	microarray	21	15	one HD	547	28 miRs		no	[414]
2	**Breast cancer**	whole blood	no	qRT-PCR	148	44	*miR-16*	7	*miR-195* *let-7a*		no	[415]
		plasma	no	Illumina microarray	10 CA	10 CA	Quantile normalization algorithm	1.145	17 miRs up	14 miRs down	no	[416]
					10 AA	10 AA			9 miRs up	9 miRs down		
		serum	no	qRT-PCR	102	20	*miR-16*	1	*miR-21*		no	[417]
		serum	no	qRT-PCR	89	29	*miR-16*	4	*miR-10b* *miR-34a*	*miR-155*	no	[418]
3	**NSCLC**	serum	no	Solexa sequencing †	2 x 30‡	none	spiked-in miR	101/109§	3 miRs up§	8 miRs down§		[420]
				qRT-PCR	303	1	one HD	11	*miR-486*¶ *miR-30d*¶	*miR-1** *miR-499**	yes	
		serum	no	Solexa sequencing †	11‡	21‡	total RNA	190	63 miRs up	28 miRs down		[387]
				qRT-PCR	152	75	average of HDs	3	*let-7a* *miR-25*	*miR-223*	no	
		exosomes	EpCAM-based	qRT-PCR array†	28	20	*miR-142-3p* and *miR-30b*	365	0 miRs up	10 miRs down		[421]
				qRT-PCR			*miR-142-3p*	5	*let-7f*	*miR-30e-3p*	yes	

						and *miR-30b*					
								miR-20b		no	
4	**Prostate cancer**	serum	no	qRT-PCR	25	25	spiked-in miRs	6	*miR-141*		no	[389]
		serum	no	qRT-PCR†	21	none	spiked-in miRs	667	69 miRs up††	0 miRs down††		[422]
				qRT-PCR	45	none	spiked-in miRs	5	*miR-9†*	*miR-516a-3p*	no	
					116	none	spiked-in miRs		*miR-141*	*miR-375*	yes	
									miR-200b		no	
		serum	no	qRT-PCR	27	9	median	384	*miR-26b*	*miR-30c*	no	[52]
									miR-24	*miR-874*		
									miR-93	*miR-1274a*		
									miR-106a	*miR-1207-5p*		
									miR-223	*miR-451*		
5	**Ovarian cancer**	serum	no	qRT-PCR array†	9	4	*U44* and *U48*	365	21 miRs			[52]
				qRT-PCR	19	11	*miR-142-3p* and *miR-16*	21	*miR-21*	*miR-99b*	no	
									miR-29a	*miR-126*		
									miR-92	*miR-127*		
									miR-93	*miR-155*		
		exosomes	EpCAM-based	microarray†	50	20	spiked-in miRs	467	*miR-21*	*miR-200c*	no	[63]
									miR-141	*miR-203*		
									miR-200a	*miR-205*		
									miR-200b	*miR-214*		
6	**Gastric cancer**	plasma	no	qRT-PCR	69	30	spiked-in miRs	5	*miR-106a*	*miR-21*	no	[425]
									miR-106b	*let-7a*		
									miR-17-5p			
		whole blood	Ficoll	qRT-PCR	90	27	*U6*	2	*miR-106a*		no	[426]
									miR-17			
		serum	no	qRT-PCR	17	16	*U6*	5	*miR-885-5p*		no	[427]

				array							
7	**HCC**	serum	no	qRT-PCR	10	none	*U6* and total RNA	1	*miR-500*	no	[428]
		serum	no	qRT-PCR array[†]	15[‡]	10[‡]	*U6*	380	26 miRs up		[427]
				qRT-PCR	46	24	*U6*	5	*miR-885-5p*	no	
8	**Colorectal cancer**	plasma	no	qRT-PCR	100	59	*miR-16*	12	*miR-29a*	no	[429]
		plasma	no	qRT-PCR array[†]	25	20	*U6*	95	*miR-17-3p* *miR-92* *miR-95*	*miR-135b* *miR-222*	[430]
				qRT-PCR	90	50	*U6*	5	*miR-17-3p* *miR-92*	no	
9	**Pancreatic cancer**	serum	no	qRT-PCR	45	32	*miR-16*	2	*miR-200a* *miR-200b*	no	[432]
		plasma	no	qRT-PCR	22	25	spiked-in miRs	1	*miR-210*	no	[433]
10	**Head and neck cancer**	plasma	no	qRT-PCR	30	38	*miR-16*	1	*miR-184*	no	[434]
11	**Esophageal SCC**	serum	no	Solexa sequencing[†]	141[‡]	40[‡]	total RNA	na	25 miRs up		[435]
				qRT-PCR	149	100	serum volume	25	*miR-10a* *miR-22* *miR-100* *miR-127-3p*	*miR-133a* *miR-223* *miR-1248b*	no
12	**Rhabdomyosarcoma**	serum	no	qRT-PCR	10	17	*miR-16*	4	*miR-206*	no	[437]

†discovery phase; ‡pooled samples; §differentially expressed between long and short survival groups; ¶higher expressed in short survival group; #higher expressed in long survival group; ††higher expressed in metastatic compared with localized prostate cancer patients; AA: African-American; CA: Caucasian-American; HCC: hepatocellular carcinoma; HD: healthy donor; miR: miRNA; NA: not applicable; NSCLC: non-small cell lung cancer; qRT-PCR: quantitative reverse-transcriptase PCR; RMS: rhabdomyosarcoma; SCC: squamous cell cancer

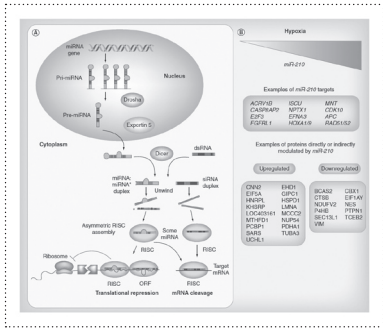


Figure 1: see section 'Color figures'

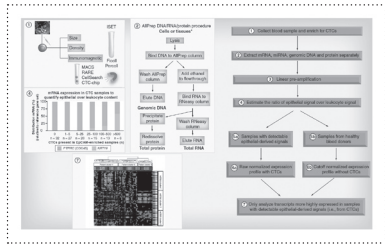


Figure 2: see section 'Color figures'

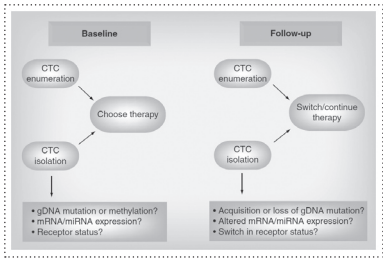
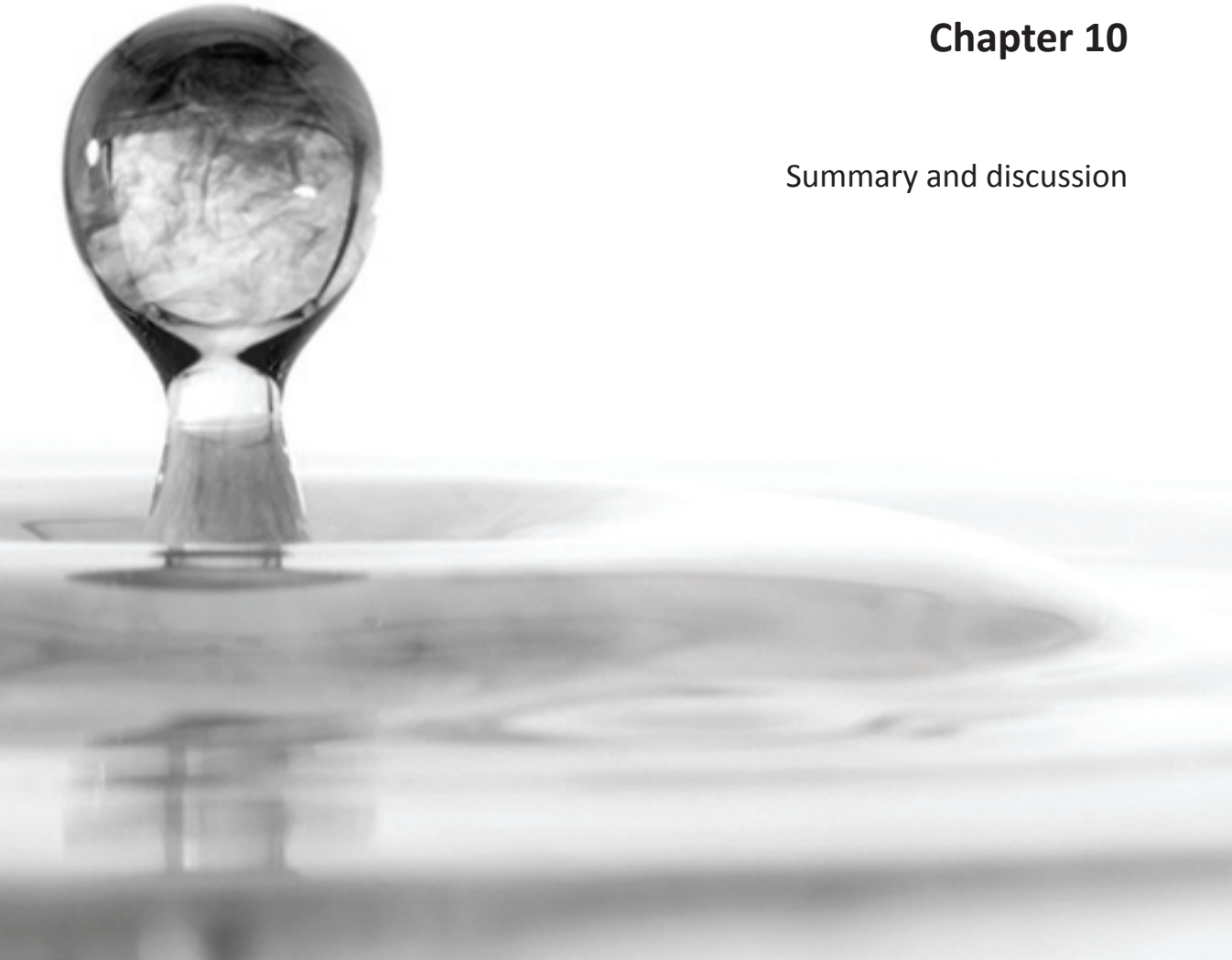


Figure 3: see section 'Color figures'



Chapter 10

Summary and discussion

Over the past years, the field of oncology has seen rapid and profound changes. High-throughput genomic, proteomic and epigenomic analyses have enabled the deeper understanding of crucial signal transduction pathways involved in tumor cell proliferation, metastasis and drug resistance. This surge of new data has, if anything, convinced researchers and physicians that big improvements in the treatment of cancer are probably further away than was envisioned before the omics-era. The complexity of the mechanisms involved in metastasis and drug response has convinced us that patient or tumor response prediction is not a black-or-white matter, which is probably why good predictive and prognostic factors remain an unmet need in cancer treatment.

THE NEED FOR PREDICTIVE AND PROGNOSTIC FACTORS

The implementation of new cancer therapies has improved patient's chances of survival and prolonged quality of life, but at the same time, worldwide cancer burden is increasing. Higher cancer incidence due to environmental and lifestyle factors as well as a higher prevalence due to early detection and improved treatment options result in a dramatic increase in the cost of cancer care worldwide, jeopardizing its affordability in both high- and low-income countries. While this increase in cost is multifactorial[447], and many of the most important drivers are beyond the scope of this thesis, cost-effectiveness can be markedly improved by the proper selection of patients for both clinical research trials and off-study treatment. As a consequence of specific patient and tumor characteristics, only a subset of patients will benefit from cancer treatment at all, and, perhaps more importantly, benefit from specific anti-tumor agents. Better response prediction will improve cancer care affordability but could also aid in ensuring optimal quality of life for cancer patients, as the trade-off between efficacy and toxicity can become more favorable. In this regard, better prognostic factors and predictive factors are urgently needed to more reliably estimate patients' life expectancy and predict their response to specific anti-tumor agents. Especially in the field of targeted treatment, the specific inhibition of a component of a signaling pathway is often followed by the counter-reactive activation of downstream targets of alternative pathways, which enables the tumor to circumvent the inhibitory effect of the targeted agent. Moreover, not all tumors are equally addicted to certain targeted pathways, and inhibition of such a bystander pathway will not result in a significant tumor response. Predictive factors should therefore ideally identify which patients' tumors are dependant upon the targeted pathway and are not inherently or acquired resistant to the drug of choice, and are thus most likely to show a clinically relevant response. Patient selection can determine the success of clinical trials; some of the hallmark cancer therapies of today, such as trastuzumab, had not passed phase II/III trials had it not been for the right selection

of patients[448]. Certain therapies might only work in a few percent of all patients, but deliver significant clinical benefit in that small subgroup; their efficacy will only come to light in a properly designed clinical trial pre-selecting for sensitive patients based on reliable predictive factors. With the conduction of such clinical trials, treatment can be adapted for small patient subgroups or even individual patients, resulting in truly personalized medicine.

TUMOR HETEROGENEITY

Even when selection is based on tumor characteristics, response prediction in patients is often far from perfect. Markers that are expected to correspond to a black-or-white tumor response often fail to show such predictive value in clinical trials. Partly, this could be due to the tissue in which the marker is determined, which is currently almost always the primary tumor. However, in the metastatic disease setting, years have often passed since removal of the primary tumor, leading to heterogeneity between these tissues[7] due to processes such as clonal selection and genomic instability. This heterogeneity between primary and metastatic tissue could explain why patient selection based on primary tumor characteristics does not always result in a response in metastatic lesions, which ultimately determine the faith of most cancer patients.

Heterogeneity between primary tumor and metastases is a compelling argument for the assessment of predictive and prognostic markers on metastatic tumor tissue, and important efforts are ongoing to initiate trials based on in-depth genomic analysis of metastatic lesions[449]. The implementation and physician and patient acceptance of metastatic biopsies can however be limited by their invasive and painful nature, hampering especially the repeated analyses needed to determine drug resistance. To overcome these hurdles, circulating tumor cells (CTCs) present an attractive alternative.

CTCs can be analyzed repeatedly during the course of treatment to re-assess predictive factors as time and drug pressure influence the predominant clones and sensitivity of tumor lesions. Moreover, CTC counts can predict patients' prognosis[25,27,29,31,34,450] and can be used as an early marker of therapy response[451-452]. Together, counting and characterizing CTCs hold great promise as a tool for patient stratification, selection and monitoring, but important steps forward are needed to fulfill this promise. In this thesis, we have described a number of advances in the field of CTCs, both in terms of optimization of their detection and of their large-scale characterization.

ADVANCES IN CTC DETECTION

Many different CTC detection methods have and are continuing to be developed in an effort to obtain an as big and representative fraction of these rare cells as possible, while preserving them for downstream characterization and culturing. Because of the scarcity of CTCs and the overwhelming number of peripheral blood mononuclear cells amongst which they have to be identified, extremely high sensitivity and specificity is needed from these assays. Until now, only CellSearch® (Veridex™ LLC, Raritan, NJ) has obtained FDA approval for use as a prognostic marker in metastatic breast[25-27], colorectal[29], lung[30] and prostate cancer[31,453]. While the papers in this thesis and other work[64] have shown that CellSearch is not the perfect assay, its FDA-clearance does enable its use in the clinic and as a stratification marker in clinical trials. To further improve the amount and quality of information that can be obtained from peripheral blood, numerous advances can and have been made, specifically in the choice of enrichment[454] and selection markers to enable detection of all circulating tumor cells.

Detection of EMT-like CTCs

We have described the value of adding CD146 to anti-EpCAM as an enrichment marker to the CellSearch assay, in order to enable the detection of EpCAM-negative CTCs. These EpCAM-negative cells are predominantly of the normal-like subtype, which has been described to have EMT characteristics[455] and stem-cell features[456], and display an aggressive behavior[10]. CD146 is expressed in the majority of normal-like breast cancer cell lines and thus enables their detection. For CTC characterization purposes, the additional cell populations that are isolated by combined anti-EpCAM/CD146 enrichment do result in a less favorable tumor cell-leukocyte ratio, as a result of which tumor cell transcripts will have to be measured among even more leukocyte transcripts. For this reason, the characterization of CD146-positive CTCs will probably benefit from a single-cell approach[457], in which single cell EpCAM-positive and CD146-positive CTCs from the same patients can be compared and their individual characteristics correlated with disease outcome and patient characteristics.

The counting of CD146-positive CTCs can readily be implemented into clinical trials, and we are currently conducting a prospective multicentre clinical study in primary breast cancer patients treated with neoadjuvant chemotherapy, in whom both EpCAM-positive and CD146-positive CTCs are enumerated. This study will allow us to establish the frequency of CD146-positive CTCs, and especially in primary breast cancer patients, in whom EpCAM-positive CTCs are even less frequent[34], the addition of CD146 might increase CTC detection frequency. Importantly, recent evidence shows that CD146 expression in breast cancer cell line cells is associated with down regulation of epithelial marker expression in favor of mesenchymal marker expression and increased tumor and metastasis formation in mice models[458]. EpCAM-negative, CD146-

positive CTCs could thus represent cells that have undergone EMT and are possibly more malignant in nature, and the dual-enrichment of CD146 and EpCAM positive CTCs may increase the clinical relevance of CTC counting.

While the addition of CD146 as an enrichment marker resulted in an important increase in coverage of breast cancer subtypes, a subgroup of EMT-like breast cancer cells still remained undetected as a consequence of insufficient expression of the cytokeratins 8, 18 and 19, which are the positive selection markers employed in the standard CellSearch assay. Loss of cytokeratin expression also occurs in the context of EMT[11], and these cytokeratin-negative cells could be highly relevant in the process of metastasis. Other studies have shown that CK-negative circulating cells display properties of CTCs, such as *HER2* amplification[454], adding to the notion that these CK-negative cells too are in fact derived from tumors and clinically relevant. CD49f, a stem-cell marker[254] associated with increased tumor aggressiveness[262,264], was broadly expressed across breast cancer subtypes, including cytokeratin-negative cells. We showed that the addition of CD49f to cytokeratin 8/18/19 as CTC selection markers resulted in the improved detection of cytokeratin-negative CTCs.

Together, CD146 and CD49f have improved the ability of the CellSearch assay to detect all breast cancer subtypes, and will hopefully lead to increased clinical value in more breast cancer patients.

Room for improvement

With the difficulty in finding one or a set of markers covering all tumor types, one has to wonder whether CTCs should be detected based on marker expression at all. Assays have also been described isolating CTCs based on morphological or functional characteristics, such as size[19,76,459], cell stiffness[460] or cell membrane capacitance[446]. Besides possibly enabling higher CTC detection rates, these methods do not require cell fixation and permeabilization steps, resulting in viable cells suitable for subsequent cell culturing.

In addition to the choice of method, the preferred timing and place of blood draw for CTC detection also needs further elucidation. CTCs have been speculated to be released into the blood stream in a circadian rhythm[461], but are also heavily influenced by the administration of anti-tumor therapies[462]. When it comes to the preferred place of blood draw, CTCs are most often obtained by venipuncture because of its convenience and minimally invasive nature. However, the size of CTCs in relation to that of capillaries will probably lead to trapping of CTCs in capillaries, and a lower CTC count in the periphery compared to the central venous compartment or arteries has been described[463].

Despite all the unknowns in the field of CTC detection, clinical studies should continue with currently available and validated methods while efforts to improve detection methods are

ongoing. For all of the uncertainty, the value of CellSearch-enumerated CTCs as a prognostic and therapy response marker is well-established, and should not be disregarded while we await identification of the 'perfect' circulating particle.

ADVANCES IN CTC CHARACTERIZATION

CTC-specific genes in colorectal and breast cancer

When going beyond CTC counting and employing their features as prognostic and predictive factors, a number of challenges arise. Probably most crucial is the low amount of tumor-derived nucleic material that can be obtained from a tube of blood. This can in part be overcome by analysis of larger blood volumes[337] or optimization of the sensitivity of the CTC enrichment assay[20,250], but also by employing a pre-amplification step before molecular analysis[9,64]. Additionally, most current CTC isolation methods, such as the CellSearch EpCAM-based immunomagnetic enrichment, result in a cell fraction containing a few CTCs amongst still up to a thousand leukocytes[9], significantly complicating subsequent molecular analysis. We have shown that this problem can at least in part be tackled by strictly selecting genes with no or very low expression in leukocytes and a much higher expression in breast cancer, followed by careful validation of their CTC-specificity.

Through comparison of healthy donors (HDs) with patients with 5 or more CTCs, we were able to identify a large panel of CTC-specific genes. These genes identified distinct patient subgroups based on epithelial, ER-associated and growth factor receptor-associated gene expression. The CTC-specific gene panel also allowed for the comparison of primary tumors and CTCs, which revealed clinically relevant discrepancies for ER and HER2 expression.

Similarly, we selected genes clinically relevant in colorectal cancer and searched for CTC-specific genes by comparing healthy donors with patients with 3 or more CTCs. We did not feel confident to group patients without detectable CTCs together with the healthy donors to make up the no-CTC group, because among patients without detectable CTCs we identified a subgroup with a gene expression profile clearly distinct from that of HDs, suggesting the presence of circulating tumor load. This discrepancy between CTC counts and molecular profile can be the consequence of low CK8/18/19 expression in CTCs[356,464], which can lead to the inability to count CTCs while, based on EpCAM expression, they are enriched for subsequent molecular analysis. Consequently, CTC-specific mRNAs and miRNAs were identified in CRC patients by comparing HDs to patients with 3 or more CTCs, resulting in a panel of 47 genes that clustered patients primarily based on epithelial gene expression.

CTC gene expression profiles to predict treatment benefit

In our clinical breast cancer study, we were able to proceed with the CTC-specific gene expression panel and assess its prognostic value in a large set of 103 metastatic breast cancer patients. By leave-one-out cross validation, a 16-gene CTC profile was discovered that could predict time to treatment switch, and was able to further divide the good-prognosis group as defined by a CTC count <5 in a good and intermediate prognosis group. In patients with 5 or more CTCs, prognosis was almost universally poor, and the 16-gene CTC profile was not capable of identifying patients with an even more dismal prognosis. This lack of value in CTC-high patients is probably the result of the strong influence of the presence or absence of epithelial cell content on the 16-gene profile. Many genes in the 16-gene profile are epithelial markers, allowing for identification of patients who, despite a CTC count below 5, have circulating tumor load in their blood. However, they also assign an unfavorable profile to almost all patients with a high CTC count and thus high epithelial gene expression.

In patients with more than 5 CTCs, we also wished to better predict prognosis, but more importantly, provide information on CTC characteristics in terms of their biology and malignant potential. To his end, we developed a separate prognostic 9-gene profile in patients with 5 or more CTCs, starting with all 96 mRNAs that could be reliably measured in CTCs[9,38]. The presence in the 9-gene profile of *ER*, a well-established predictive[465] and prognostic[466] factor in breast cancer, and *MET*, which encodes for the oncogene met[320], suggests that this 9-gene profile is less driven by circulating tumor load and more by CTC characteristics.

The external validity of these two CTC gene profiles awaits validation in an independent patient cohort, but already CTC characterization has proven to be able to be of additive value to a CTC count.

DNA mutations in colorectal CTCs

Besides CTC gene expression, we have also shown the feasibility of detecting DNA mutations in colorectal CTCs, which is an important advancement in this era of targeted treatments. Many of these targeted treatments can be used solely in the presence of companion diagnostics, which identify patients sensitive to these therapies. Like gene and protein expression, important discrepancies can exist between primary and metastatic tumors in terms of their mutation status[369-370,467-468], and patient selection could possibly improve by selecting therapies based on CTC mutation status. For monoclonal antibodies targeting the EGF-receptor, a potent treatment in a variety of solid tumors[358,469], *KRAS*[4] and, to a lesser extent, *BRAF*[367] mutations are important predictors of response, resulting in approval of their use solely for *KRAS* wild-type patients[366]. In our prospective clinical study in colorectal cancer patients about to undergo partial liver resection for their metastases, we assessed *KRAS* and *BRAF* mutation status in matching primary tumors, metastases and CTCs by three different highly sensitive

assays. Important discrepancies were seen between primary tumors and metastases, but mutation detection in CTCs proved to be challenging due to low CTC counts in this patient population, the known abundance of remaining leukocytes and lack of amplification of the genes of interest. *KRAS* and *BRAF* mutations were detected in CTCs, but false-negative results can currently not be excluded in patients with low CTC counts and low mutation frequencies.

Increasing the clinical use of CTCs

Our difficulties with assessing *KRAS* and *BRAF* mutation status in CTCs, but also the current inability to measure all genes of interest in CTCs as we are limited to truly cancer-specific genes, demand attention. If anything, they are compelling evidence of the fact that the road to more in-depth molecular characterization methods such as whole-genome sequencing would be greatly facilitated by the further development of CTC detection methods that result in a pure CTC population[470].

Nonetheless, CTC counts are an established prognostic factor in metastatic breast[25], colorectal[283], prostate[471] and lung cancer[30], and we and others have started to elucidate the prognostic value of their characteristics. While prognostic factors are important stratification tools in clinical studies, can be informative of tumor biology and guide the administration of adjuvant treatment, they usually do not guide oncologists in the optimal choice of a specific therapeutic agent for an individual patient. For this, predictive factors are needed, and given the poor patient selection for most current cancer therapies[447], this need is urgent. Fortunately, CTC characterization provides ample opportunity to establish better predictive factors, and in the near future we aim to evaluate the predictive value of numerous CTC characteristics, on the protein, mRNA as well as DNA level, in prospective clinical studies. At the same time, we are continuing our efforts to improve the methods by which CTCs are isolated from and counted in the blood. Together, this can lead to the identification and validation of better predictive factors, and maximize the potential of CTC counts and characterization as a means to individualize and improve cancer treatment.

Samenvatting

De levensverwachting van patiënten met kanker is de laatste jaren sterk verbeterd, onder andere door de introductie van nieuwe behandelingen. Echter met de komst van meer effectieve therapieën is ook de noodzaak om de juiste therapie voor de individuele patiënt te selecteren groter geworden. Voor het bepalen van de juiste therapiekeuze zijn betrouwbare prognostische en predictieve factoren onontbeerlijk. De afgelopen jaren zijn veel van dergelijke factoren geïdentificeerd in primair tumormateriaal, maar de uiteindelijke prognose van patiënten met kanker wordt bepaald door het gedrag van metastases en niet zozeer door dat van de primaire tumor. Daarnaast wordt het de laatste jaren steeds duidelijker dat er grote discrepanties kunnen bestaan tussen de kenmerken van de primaire tumor en die van de metastasen. Vaak zijn, alvorens anti-tumor therapie gestart wordt voor gemetastaseerde ziekte, jaren verstreken sinds het chirurgisch verwijderen van de primaire tumor en is tussentijds anti-tumor therapie gegeven. Door genetische instabiliteit, die karakteristiek is voor kankercellen, kunnen zo grote verschillen ontstaan in allerlei kenmerken die van invloed zijn op het gedrag van tumorcellen. Predictieve en prognostische factoren zouden dus waarschijnlijk het beste in metastatisch weefsel bepaald kunnen worden, maar het verkrijgen van weefsel van metastasen is vaak technisch moeizaam en pijnlijk voor de patiënt.

Circulerende tumorcellen

Een veelbelovend alternatief voor de analyse van metastasen is de detectie en karakterisatie van circulerende tumorcellen (CTC's). CTC's zijn cellen afkomstig van primaire tumor of metastasen die zich bevinden in het perifere bloed. CTC's zijn voor het eerst beschreven in de 19^{e} eeuw, maar lange tijd waren er geen goede methodes om CTC's te identificeren. Vooral tijdens het laatste decennium zijn er vele pogingen gedaan om betrouwbare, reproduceerbare technieken te ontwikkelen, wat uiteindelijk geresulteerd heeft in verschillende methoden waarmee CTC's gedetecteerd kunnen worden.

CTC's kunnen op verschillende manieren worden toegepast in de behandeling van kankerpatiënten. Een CTC-aantal kan gebruikt worden als prognostische factor voorafgaand aan therapie, als parameter om de effecten van anti-tumortherapie te meten en voor de vroegtijdige detectie van recidieven.

Naast het tellen, kunnen CTC's ook worden geïsoleerd uit bloed en vervolgens op moleculair niveau verder worden gekarakteriseerd. Mogelijke toepassingen van het karakteriseren van CTC's zijn het identificeren van eiwitten of genen die kunnen helpen bij de keuze van therapie, het detecteren van het ontstaan van resistentie tegen de ingestelde therapie en het vergroten van het inzicht in de tumorbiologie, door bijvoorbeeld eigenschappen van de primaire tumor te vergelijken met die van de CTC's.

HET TELLEN VAN CTC'S

Het tellen en isoleren van CTC's is een grote uitdaging gebleken, vooral doordat er meestal sprake is van slechts één of enkele cellen te midden van miljarden leukocyten. Pas sinds een aantal jaren is een semigeautomatiseerde methode beschikbaar, de CellSearch methode genaamd, die goedgekeurd is voor het bepalen van CTC's als prognostische factor in verschillende tumortypes. Deze methode maakt gebruik van de aanwezigheid van specifieke eiwitten op CTC's. In de eerste stap van de test wordt bloed verrijkt voor cellen die expressie vertonen van EpCAM, een kankerspecifiek antigeen. Deze cellen worden uit het bloed geïsoleerd door gebruik te maken van zogenaamde ferrofluids, kleine ijzerdeeltjes, die beladen zijn met een antistof tegen EpCAM. Deze verrijkte celsuspensie kan worden gebruikt voor verdere moleculaire karakterisatie. Wanneer CTC's geteld worden, volgt een aantal stappen waarin CTC's in de verrijkte celsuspensie worden gedetecteerd op basis van de expressie van cytokeratines, een kernkleuring en de afwezigheid van CD45, een leukocytenmerker. Het met deze methode verkregen aantal CTC's per 7.5 mL bloed is van prognostische waarde gebleken voor patiënten met gemetastaseerd prostaat-, colorectaal, long- en mammacarcinoom. Daarnaast voorspelt het stijgen of dalen van het CTC-aantal tijdens therapie, kort na het starten van de behandeling, de respons op die therapie.

Verbeterde CTC-detectie door additionele merkers

Hoewel het tellen van CTC's van grote waarde is gebleken, is de huidige detectiemethode niet perfect. Bij een substantieel deel van de patiënten worden, zelfs wanneer sprake is van uitgebreid gemetastaseerde ziekte, geen CTC's gedetecteerd. Borstkanker is een heterogene ziekte waarin verschillende subtypes kunnen worden onderscheiden. Eerder hebben we aangetoond dat in mammacarcinoompatiënten deze suboptimale detectie deels te verklaren is door het ontbreken van EpCAM, het membraaneiwit dat essentieel is voor de verrijking van CTC's uit bloed, op de cellen van één van die subtypes mammacarcinoom. Cellijncellen van dit type, het zogenaamde 'normal-like' mammacarcinoom, werden dan ook niet gedetecteerd met de standaard CellSearch methode. In dit proefschrift hebben we laten zien dat het toevoegen van CD146, een eiwit dat wel voorkomt op het 'normal-like' type mammacarcinoom, als merker voor CTC-verrijking de detectie van ook dit mammacarcinoomsubtype mogelijk maakt. Daarnaast hebben we laten zien dat deze CD146-positieve CTC's, al dan niet in combinatie met EpCAM-positieve CTC's, voorkomen in het bloed van patiënten met gemetastaseerd mammacarcinoom.

Het toevoegen van CD146 aan EpCAM als merker voor CTC-verrijking verbeterde de CTC-detectie in mammacarcinoom, maar er waren nog steeds mammacarcinoomcellijnen die niet gedetecteerd konden worden met deze nieuwe benadering. Dit bleek veroorzaakt te worden

door een te lage cytokeratine-expressie op diverse cellijnen, de merkers op basis waarvan CTC's worden geselecteerd na verrijking uit het bloed. Een ander eiwit, CD49f, dat ook beschreven is als merker voor kankerstamcellen, kwam wel op deze cellen tot expressie en de toevoeging ervan aan cytokeratines als selectiemerker leidde tot een betere CTC-detectie.

CTC KARAKTERISATIE

CTC-specifieke genen in mammacarcinoom

Wanneer CTC's geïsoleerd worden uit bloed voor verdere moleculaire karakterisatie, kan de analyse van CTC's dienen als een mogelijk alternatief voor analyse van metastatisch weefsel, dat lang niet altijd te verkrijgen is. In CTC's kunnen vervolgens zowel prognostische als predictieve factoren bepaald worden, bijvoorbeeld door het meten van genexpressie of DNA mutaties. Het karakteriseren van CTC's is daarmee veelbelovend, maar is technisch moeilijk gebleken. Net zoals bij het tellen van CTC's is er sprake van zeer lage aantallen CTC's te midden van een veelvoud aan leukocyten, zelfs na EpCAM-gebaseerde verrijking van bloed. Het is momenteel nog niet goed mogelijk om een suspensie te krijgen die alleen uit CTC's bestaat. Dit maakt het lastig om in die gemengde suspensie van CTC's en leukocyten de expressie van alleen de genen van de CTC's te bepalen. Omdat de meeste genen niet volledig kankerspecifiek zijn, brengt het meten van die genen in een gemengde populatie het risico met zich mee dat de gevonden expressie van een gen grotendeels afkomstig is van de aanwezige leukocyten en niet van de CTC's. In dit proefschrift beschrijven we een methode om toch tumorcelspecifieke genexpressie te meten in een verrijkte celsuspensie. Na een eerste selectie op klinisch relevante genen die niet of slechts zeer laag in leukocyten tot expressie komen, hebben we de expressie van deze genen in verrijkt bloed vergeleken tussen gezonde proefpersonen en patiënten met 5 of meer CTC's. Op deze manier hebben we een set CTC-specifieke genen geïdentificeerd die patiëntengroepen kon onderscheiden op basis van expressie van epitheliale, oestrogeen receptor (ER)-geassocieerde en groeifactorreceptor-geassocieerde genen. Ons CTC-specifieke genenpanel liet in een kleine groep patiënten ook mogelijk klinisch relevante verschillen zien tussen de primaire tumor en CTC's wat betreft de expressie van genen zoals *ER*.

CTC-specifieke genen in colorectaal carcinoom

In analogie aan de studie in mammacarcinoompatiënten zijn we ook in de CTC's van patiënten met gemetastaseerd colorectaal carcinoom op zoek gegaan naar een panel van CTC-specifieke genen met mogelijke prognostische waarde. Na een voorselectie op klinisch relevante genen die niet of nauwelijks tot expressie komen in leukocyten, hebben we deze genen gemeten in patiënten met gemetastaseerd colorectaal carcinoom bij wie bloed voor CTC-telling en -isolatie

was afgenomen vlak voor operatieve verwijdering van hun levermetastasen. Door het verrijkte bloed van gezonde proefpersonen te vergelijken met dat van patiënten met in dit geval 3 of meer CTC's hebben we een panel van 47 CTC-specifieke genen kunnen identificeren. In deze patiënten met colorectaal carcinoom konden we niet de patiënten zonder detecteerbare CTC's groeperen met de gezonde donoren. In een subgroep van patiënten waarin geen CTC's gedetecteerd werden met de CellSearch methode wees de expressie van diverse epitheelspecifieke genen namelijk op de aanwezigheid van tumormateriaal in het bloed. In deze subgroep van patiënten is waarschijnlijk sprake van tumormateriaal dat niet voldoet aan de criteria om in de CellSearch methode als een CTC geteld te worden, ofwel op basis van morfologische kenmerken, dan wel door een te lage expressie van de cytokeratines. Het genexpressieprofiel lijkt dus een gevoeliger methode om ook in deze patiënten zonder detecteerbare CTC's de aanwezigheid van tumormateriaal aan te tonen.

Prognostische waarde van CTC genexpressieprofielen

Na de identificatie van het CTC-specifieke genenpanel in mammacarcinoompatiënten zijn we op zoek gegaan naar de mogelijke prognostische waarde van dit panel. Voor dit doel hebben we de genexpressieprofielen gemeten in 103 patiënten met gemetastaseerd mammacarcinoom bij wie CTC's waren afgenomen voor start van eerstelijns medicamenteuze behandeling in de vorm van hormonale therapie of chemotherapie. We hebben de expressie van dit genenpanel vergeleken tussen een groep van patiënten met een overstap naar een volgende lijn medicamenteuze therapie of die overleden waren binnen 9 maanden na start en een groep van patiënten die geen nieuwe vorm van therapie nodig hadden en niet overleden waren binnen die periode. Hieruit hebben we een 16-genenprofiel geïdentificeerd dat dit verschil in prognose kon voorspellen. Dit 16-genenprofiel had een toegevoegde prognostische waarde aan een CTC-telling, en onderscheidde de patiënten die op basis van een CTC-getal <5 per 7.5 mL bloed een goede prognose zou hebben, in twee groepen met een verschillende prognose. In de groep van patiënten met 5 of meer CTC's kon het 16-genen profiel geen additionele prognostische informatie geven, waarschijnlijk door de grote invloed van de aanwezigheid van epitheliaal signaal op de score van het profiel. In deze groep hebben we om die reden een apart 9-genenprofiel gegenereerd dat wel prognostische waarde had. Dit 9-genenprofiel is minder afhankelijk van de expressie van epitheelspecifieke genen en is dus waarschijnlijk een betere afspiegeling van de biologie en mate van kwaadaardigheid van de aanwezige CTC's.

DNA mutaties in CTC's

Naast het meten van genexpressieprofielen in CTC's hebben we ook de haalbaarheid onderzocht van het meten van DNA mutaties in de CTC's van colorectaal carcinoompatiënten. Een relatief nieuwe optie binnen de behandeling van het colorectaal carcinoom is behandeling met monoklonale antistoffen gericht tegen de EGF-receptor (EGFR). Deze zijn echter alleen werkzaam in patiënten met een tumor zonder een mutatie in het *KRAS* gen. Deze *KRAS* mutaties, die een activerende werking hebben op de EGFR-RAS-RAF cascade, worden gevonden in de tumoren van ongeveer 40% van de patiënten met colorectaal carcinoom. Het is gebleken dat deze patiënten geen baat hebben bij behandeling met een antistof gericht tegen EGFR, reden waarom de behandeling met anti-EGFR therapie is voorbehouden aan patiënten zonder een gemuteerd *KRAS* gen. Ook een mutatie in het *BRAF* gen, die in ongeveer 10% van de patiënten voorkomt, zorgt voor resistentie tegen anti-EGFR therapie. Net als voor genexpressieprofilering geldt ook voor mutatieanalyse dat het bepalen in CTC's waarschijnlijk van toegevoegde waarde is boven het bepalen in de primaire tumor. Het geven of onthouden van anti-EGFR therapie aan patiënten op basis van de mutatiestatus van deze genen in de primaire tumor, terwijl deze mogelijk verschillend is in de metastasen, zou onnodig toxiciteit induceren dan wel een kans op effectieve behandeling ontnemen aan deze patiënten. Gezien het grote klinische belang van mutatiedetectie hebben we mogelijke verschillen in de mutatiestatus van *KRAS* en *BRAF* bestudeerd in de primaire tumoren ten opzichte van de CTC's en metastasen van patiënten die een gedeeltelijke leverresectie hadden ondergaan. Hoewel we klinisch relevante discrepanties vonden tussen de primaire tumoren en de metastasen, was de mutatiestatus van deze twee genen in de CTC's moeilijk te interpreteren gezien de kans op vals-negatieve resultaten in patiënten met lage CTC-aantallen. Desondanks is het gelukt drie verschillende detectietechnieken te vergelijken, en met de meest sensitieve techniek *KRAS* en *BRAF* mutaties te vinden in patiënten met slechts 2 CTC's.

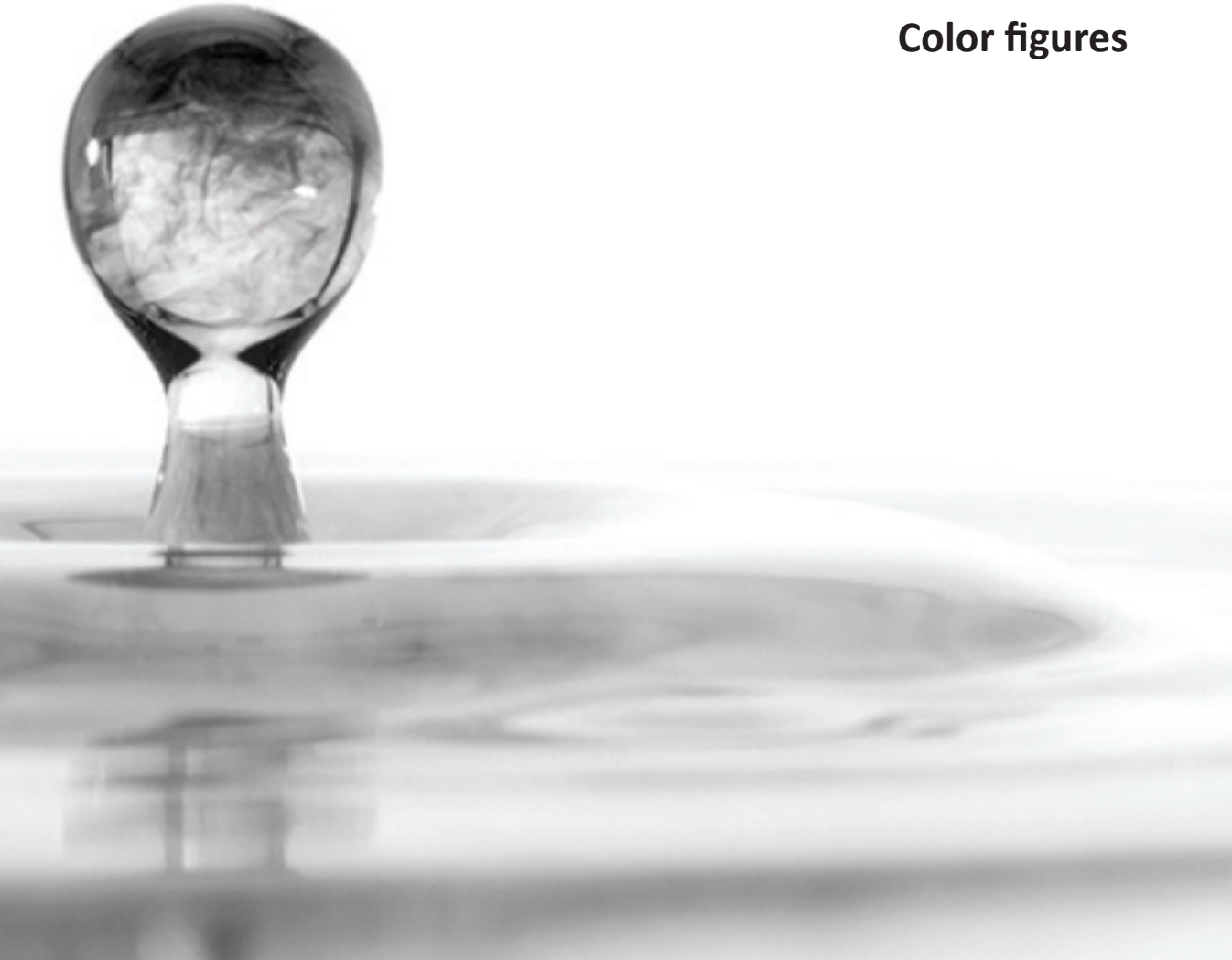
CTC's op weg naar de kliniek

In dit proefschrift worden methodes beschreven die de detectie van CTC's verbeteren door het gebruik van alternatieve verrijkings- en selectiemerkers voor CTC-telling. Daarnaast beschrijven we een uiterst gevoelige methode waarmee een uitgebreid panel klinisch relevante genen gemeten kan worden in de CTC's van mammacarcinoom en colorectaal carcinoompatiënten door ons te richten op kankerspecifieke genen. Het bleek bovendien dat een selectie van die genen van prognostische waarde is in mammacarcinoompatiënten. Deze ontwikkelingen samen maken het mogelijk in meer patiënten meer informatie uit CTC's te winnen, wat de klinische relevantie van CTC's vergroot.

In de komende jaren kan de rol van CTC's als prognostische en predictieve factor groter

worden wanneer het mogelijk wordt een volledig pure populatie CTC's te verkrijgen, zodat we niet langer gehinderd worden door de aanwezigheid van leukocyten in de te analyseren celpopulatie. Daarnaast zullen de moleculaire karakterisatietechnieken gevoeliger worden en meer informatie opleveren, bijvoorbeeld door de mogelijkheid het volledige genoom van een enkele cel te analyseren. Al deze nieuwe toepassingen van CTC's moeten uiteindelijk leiden tot een betere behandeling van kanker en een optimale therapiekeuze voor elke individuele patiënt.

Color figures



Chapter 2

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

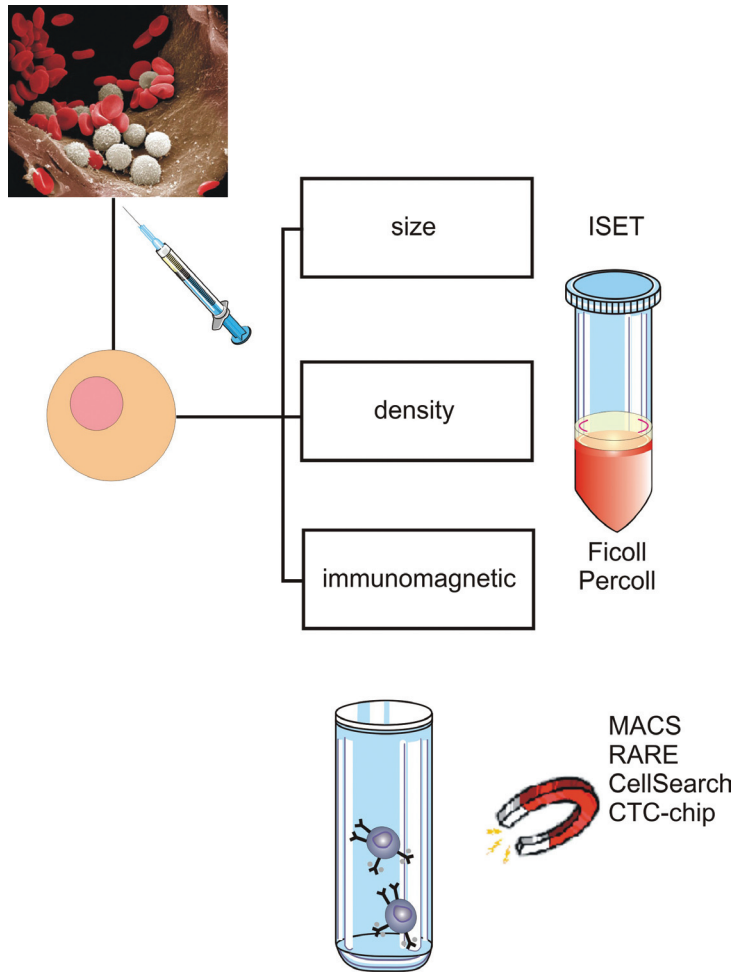


Figure 1

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

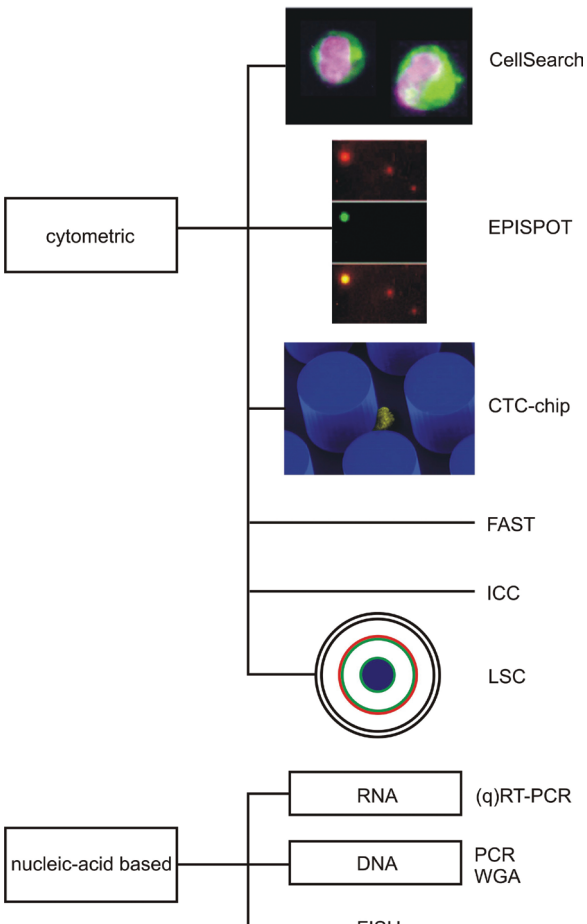


Figure 2

The techniques for detection of CTCs can be cytometric, nucleic-acid based, or a combination of both. The CellSearch system enumerates CTCs based on morphology, Cytokeratin and DAPI positivity and counterstaining with CD45. EPISPOT is an immunological assay based on the enzyme-linked immunosorbent assay (ELISPOT), which detects a number of proteins released by breast cancer CTCs, such as CK19 or Mucin-1. The CTC-Chip uses controlled blood flow conditions across a chip containing EpCAM-coated microposts. Detection of CTCs then follows by counting CD45-/DAPI+/CK+ cells with a camera. The Laser Scan Cytometer (LSC) analyses fluorescence after the cells are contoured using forward scatter as a threshold parameter and corrects for background fluorescence variation dynamically. FAST (Fiber-optic Array Scanning Technology) is a form of automated digital microscopy using a very large field of view, enabling continuous scanning. Nucleic-acid based detection methods can be based on DNA or RNA. Immunocytochemistry (ICC) allows the identification of CTCs based on marker expression and morphologic features. Part of the major drawback of ICC, the sheer volume of cells needing evaluation, can be relieved by automated microscopes such as ACIS and ARIOL.

Nucleic-acid based techniques can be performed on whole cells or on extracted RNA or DNA. (RT)-PCR (Reverse Transcriptase)-Polymerase Chain Reaction) amplifies a specific RNA or DNA sequence. WGA (Whole Genome Amplification) aims to overcome the scarcity of DNA in a sample by non-specifically amplifying the sample. FISH (Fluorescent In Situ Hybridization) detects the presence of absence of specific DNA sequences.

Chapter 3

Detection of circulating tumor cells in breast cancer may improve through enrichment with anti-CD146

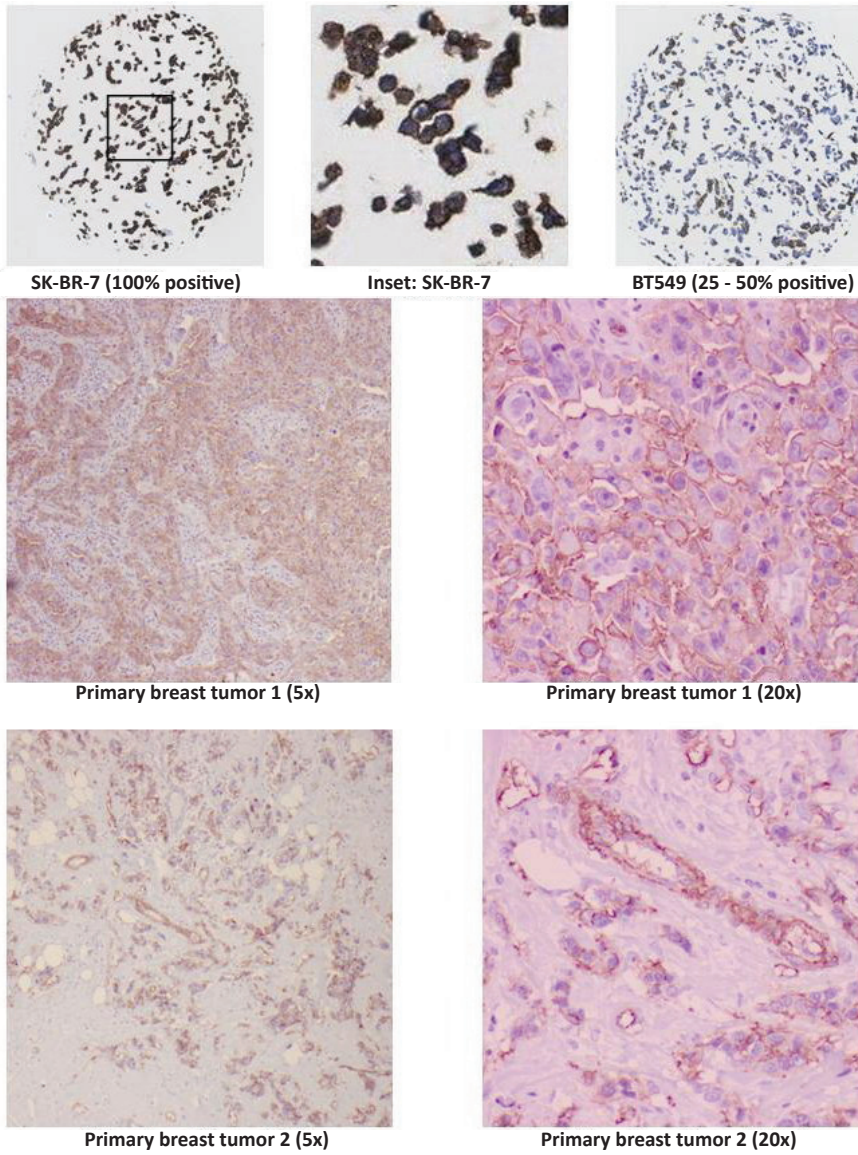


Figure 3

CD146 staining. a Representative pictures of indicated cell lines stained for CD146 (brown). Both cell lines shown are of normal-like molecular subtype **b** Representative pictures of two primary normal-like breast tumors stained for CD146 (brown)

Chapter 5

mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients

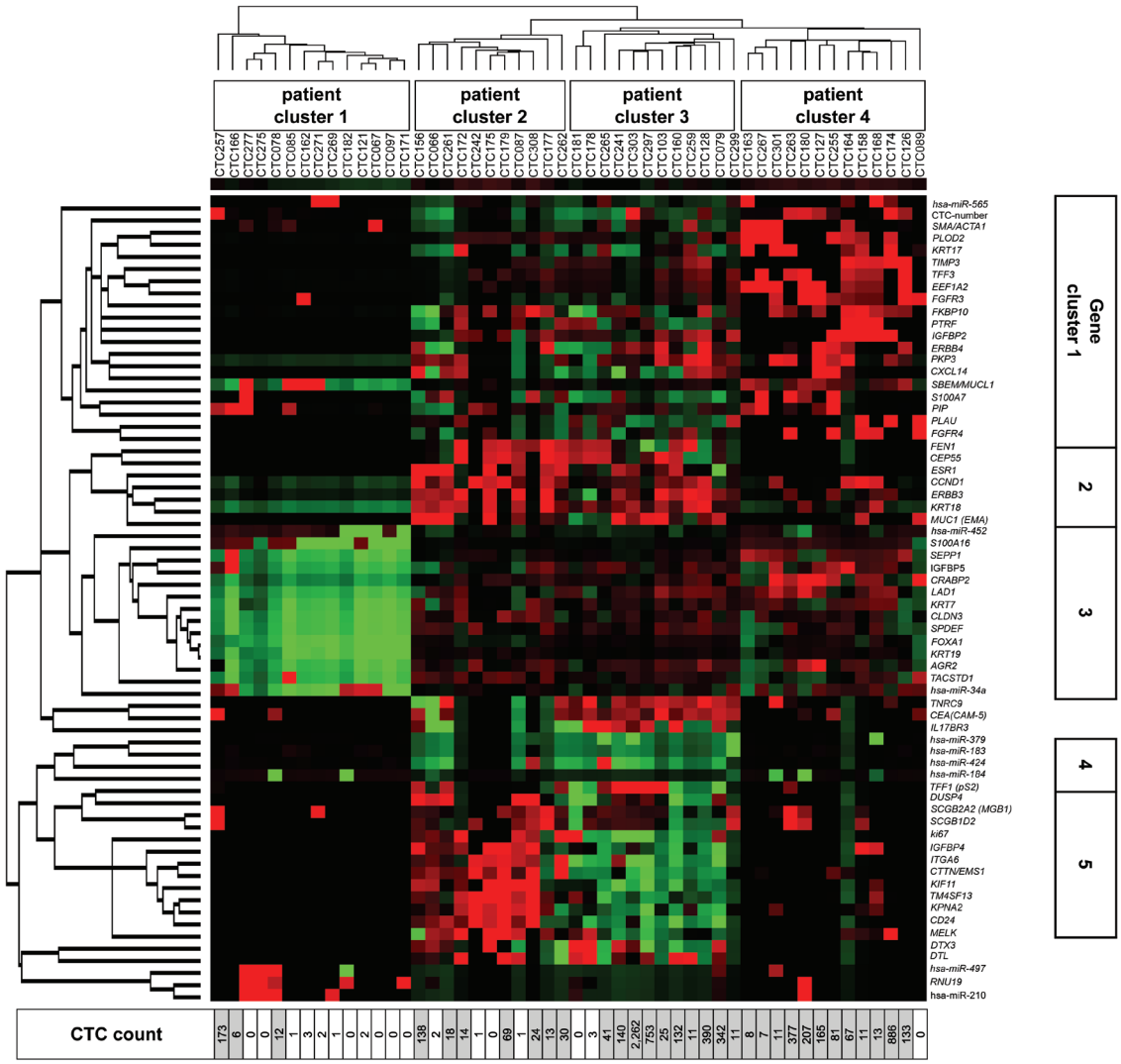


Figure 2

For legend see page 237

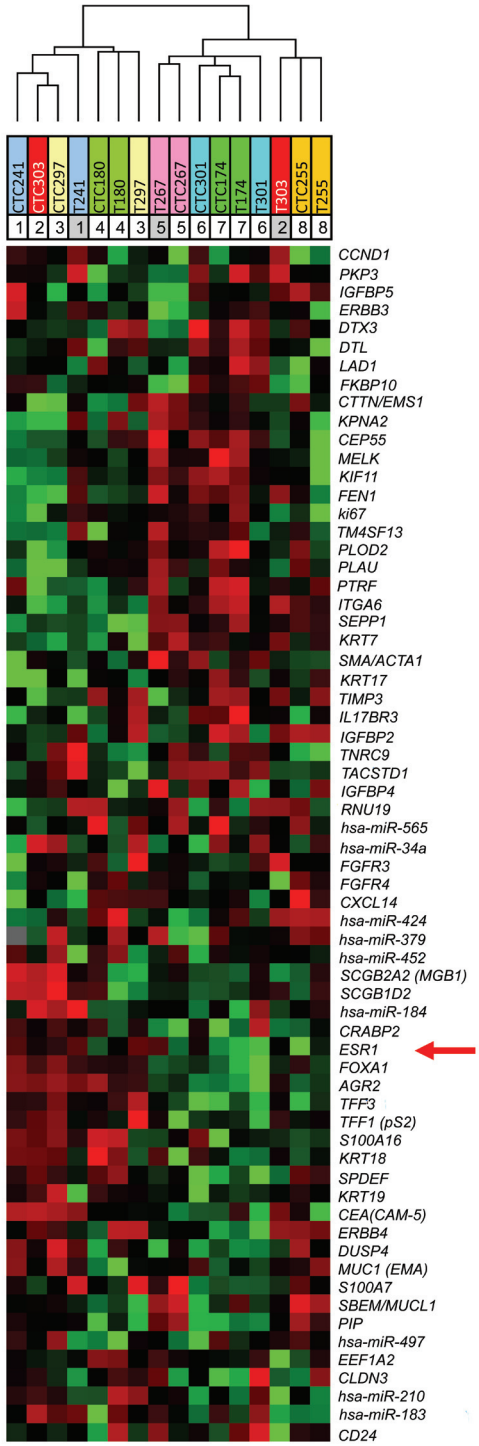


Figure 4

Unsupervised hierarchical cluster analysis comparing gene expression profiles in CTC-enriched blood samples of metastatic breast cancer patients and their corresponding primary tumor. Expression levels were analyzed with real-time RT-PCR with 65 TaqMan Gene Expression Assays in cDNA generated from RNA isolated from the CellSearch-enriched fractions of 8 breast cancer patients with metastatic disease and their corresponding primary tumors. Sample loading and RNA integrity were controlled with 3 additional universal reference genes (*GUSB*, *HPRT1*, and *HMBS*). Prior to real-time PCR, cDNA was pre-amplified in 15 cycles with the PreAmp method from ABI, as described in the Materials and Methods section, by using the same TaqMan Gene Expression Assays that were used for the real-time PCR. Data shown have been subjected to median normalization of each individual gene across all samples followed by median normalization of each individual sample across all genes. Each horizontal row represents a gene, and each vertical column corresponds to a sample (T = tissue). The 3 FFPE primary tumor tissues from patients 1, 2, and 5 are colored gray on top in the graph for easy identification. Red color indicates a transcript level above the median level, black color indicates a median transcript level, and green color indicates a transcript level below the median level of the particular assay as measured in all samples.

Chapter 6

Gene expression profiles in circulating tumor cells predict prognosis in metastatic breast cancer patients

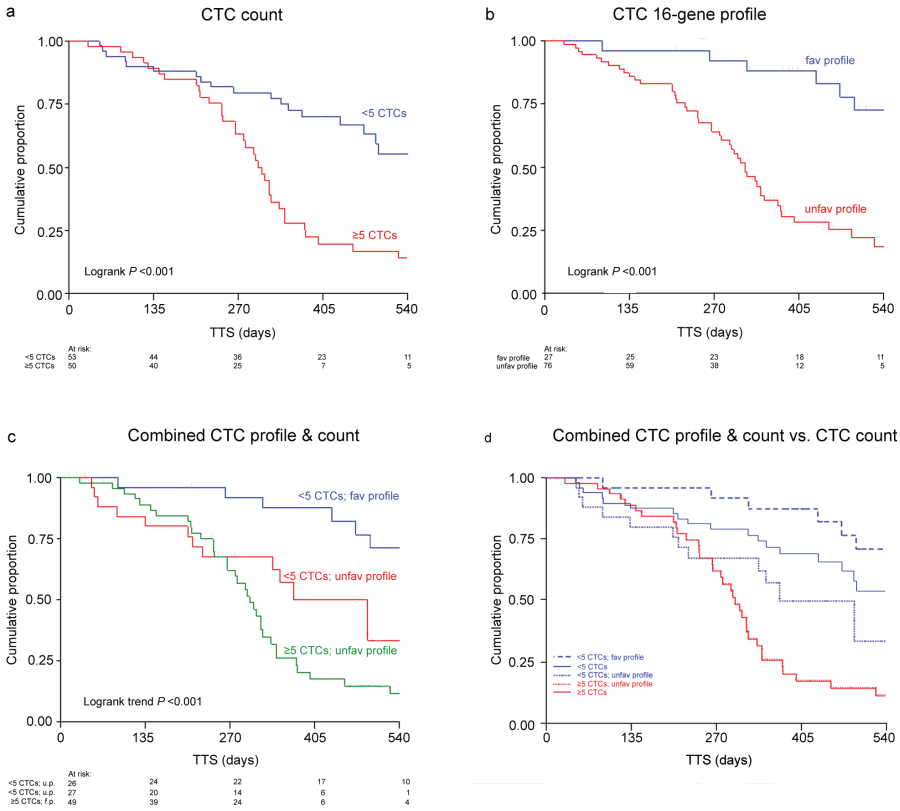


Figure 3

Kaplan-Meier plots for patient subgroups as defined by CTC count (**panel a**), the 16-gene CTC-profile (**panel b**) and the combination of CTC count and 16-gene CTC profile (**panel c**). Only one patient had more than 5 CTCs and a favorable 16-gene CTC profile, therefore no curve is depicted for this subgroup. **Panel d** combines panels a and c and shows that the 16-gene CTC panel is able to distinguish a truly good and an intermediate prognosis group among patients with <5 CTCs, while no added value is seen in patients with ≥5 CTCs. unfav; unfavorable profile, fav; favorable profile

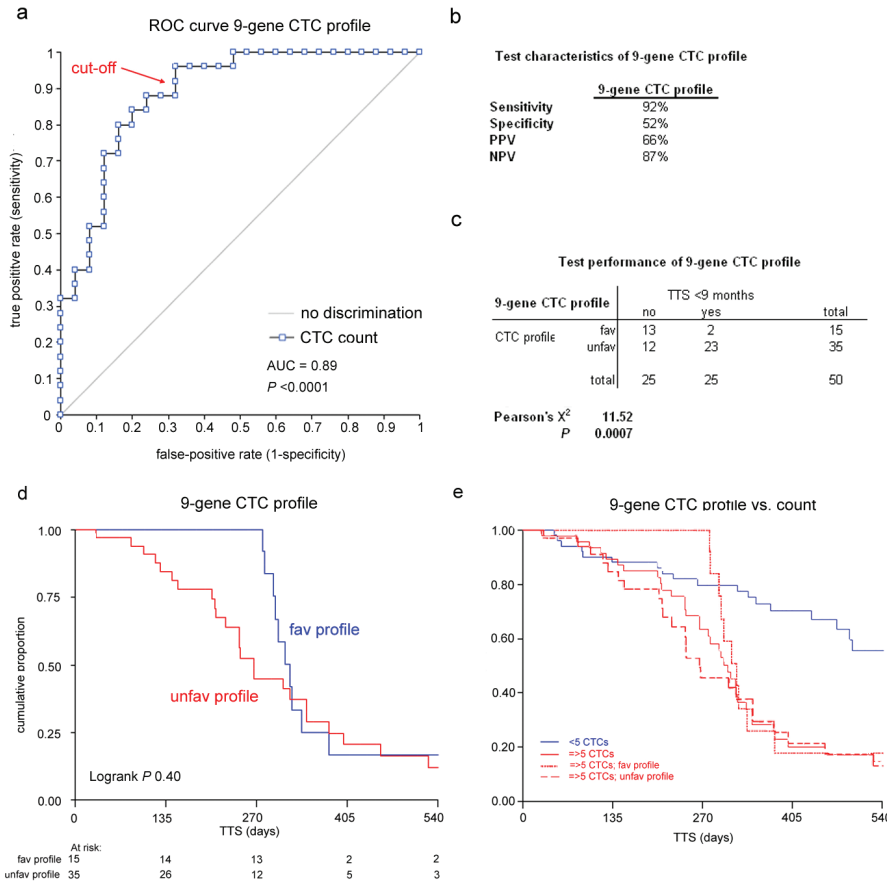


Figure 4

ROC-curve (**panel a**), test performance (**panel b**) and test characteristics (**panel c**) of the 9-gene CTC profile as generated in 50 patients with ≥5 CTCs. **Panel d** and **e** depict Kaplan-Meier plots for patient subgroups as defined by the 9-gene CTC profile (**panel d**) and a combined graph of the curves according to CTC count (solid lines blue and red lines) and 9-gene profile (dashed red lines, **panel e**). AUC; area under the curve, PPV; positive predictive value, NPV; negative predictive value

Chapter 7

mRNA and miRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients

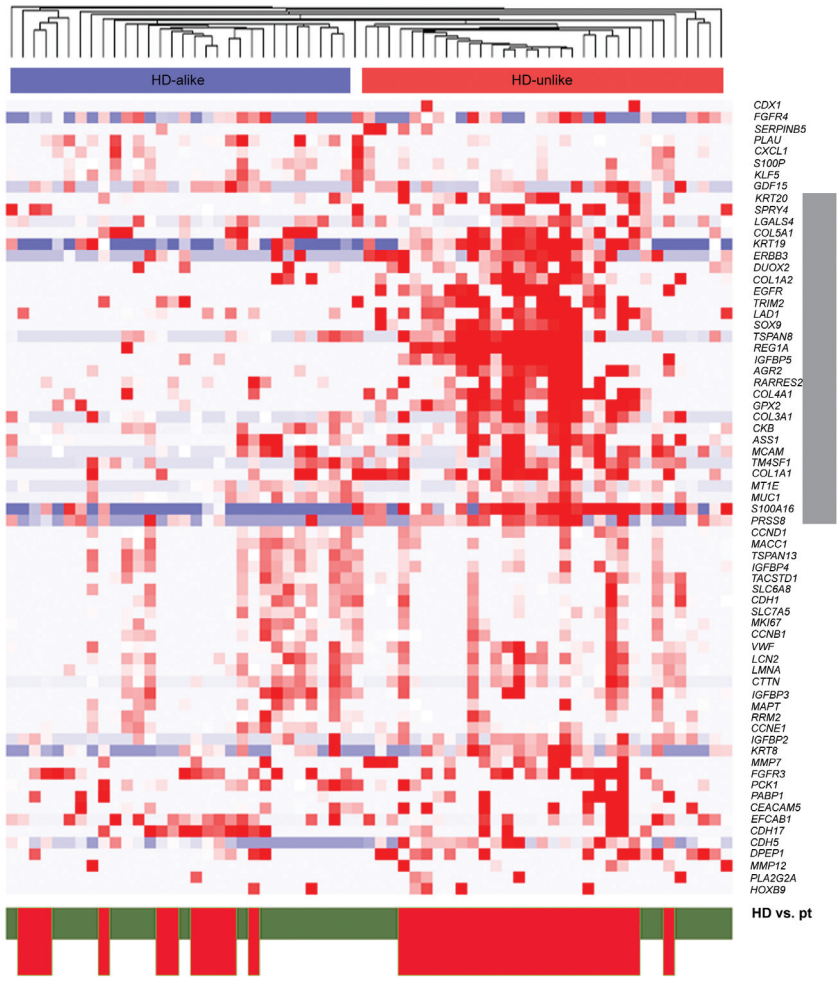


Figure 1: Clustering of HDs and patients without detectable CTCs

Unsupervised hierarchical cluster analysis comparing mRNA gene expression profiles in colorectal CTC-enriched fractions from 33 patients without detectable CTCs and QQ mRNA data available and 30 HDs. Data shown have been subjected to median normalization of each individual sample across all genes followed by median normalization of each individual gene across all samples. Columns represent patient samples, rows represent genes. Red color indicates a transcript level above the median level, white color indicates a median transcript level, and blue color indicates a transcript level below the median level of the particular mRNA in all samples. Depicted gene clusters were identified at an average linkage correlation greater than 0.2. At the top, the HD-unlike patient cluster is indicated in red; the HD-alike patient cluster in blue. Highly expressed genes that characterize the HD-unlike patient cluster are signified by the gray rectangle (at the right). Healthy donors (HD, green) and patients (pt, red) are depicted on the bottom of the graph.

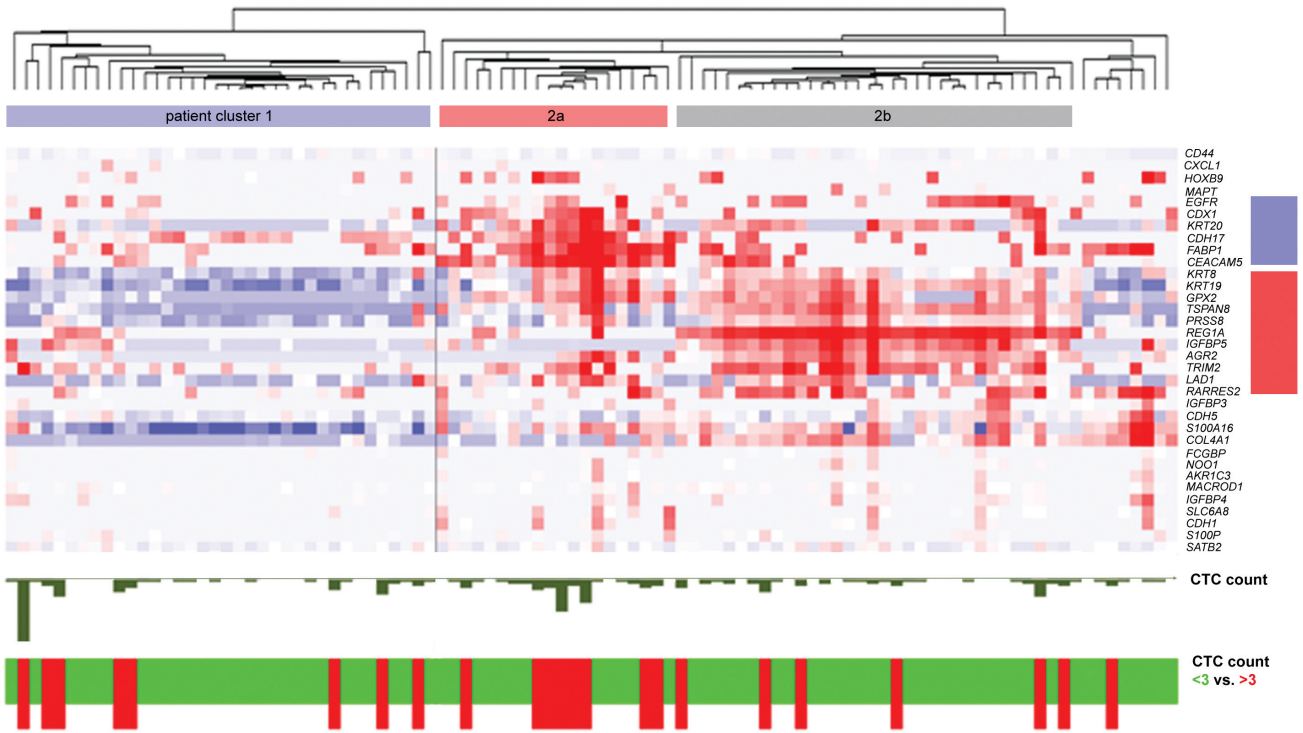


Figure 3: Cluster analysis of mRNA data

Unsupervised hierarchical clustering analysis comparing mRNA expression profiles in CTC-enriched blood samples of 98 colorectal breast cancer patients with QQ mRNA data. Each horizontal line corresponds to a gene; each vertical line represents a patient. Red color signifies a transcript level above the median level, white color represents an intermediate level and blue color a transcript level below the median level of the particular mRNA in all samples. Three patient clusters (1 and 2a and b) can be distinguished. Clusters 2a and b were characterized by two gene clusters (depicted by the blue and red rectangles, respectively). CTC count as enumerated by CellSearch is depicted on the bottom of the graph; in the 'CTC count' panel, the number of CTCs is represented by the size of the green bars (range 0 – 35). In the "CTC count <3 vs. ≥3" panel, a green bar corresponds to a CTC count <3 and a red bar to a CTC count ≥3 per 30 mL blood.

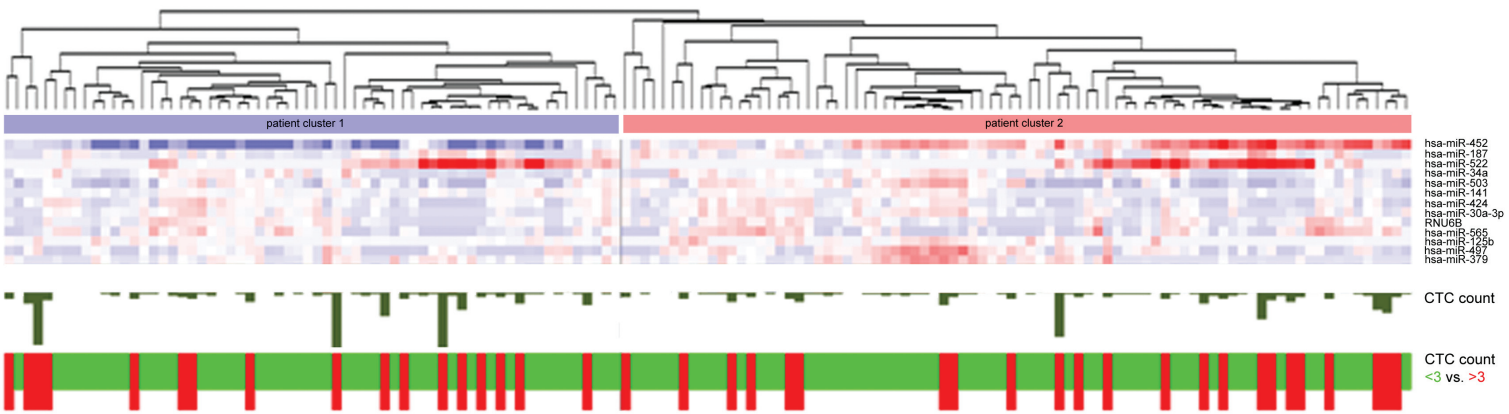


Figure 4: Cluster analysis of miRNA data

Unsupervised hierarchical clustering analysis comparing miRNA expression profiles in CTC-enriched blood samples of 146 colorectal breast cancer patients with QQ miRNA data. Each horizontal line corresponds to a miRNA; each vertical line represents a patient. Red color signifies a transcript level above the median level, white color represents an intermediate level and blue color a transcript level below the median level of the particular miRNA in all samples. Two patient clusters (1 and 2) were identified, which were mainly characterized by high expression of *hsa-miR-452* and *hsa-miR-187* in cluster 2. CTC count as enumerated by CellSearch is depicted on the bottom of the graph; in the 'CTC count' panel, the number of CTCs is represented by the size of the green bars (range 0 – 35). In the "CTC count <3 vs. ≥3" panel, a green bar corresponds to a CTC count <3 and a red bar to a CTC count ≥3 per 30 mL blood.

Chapter 8

***KRAS* and *BRAF* mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue**

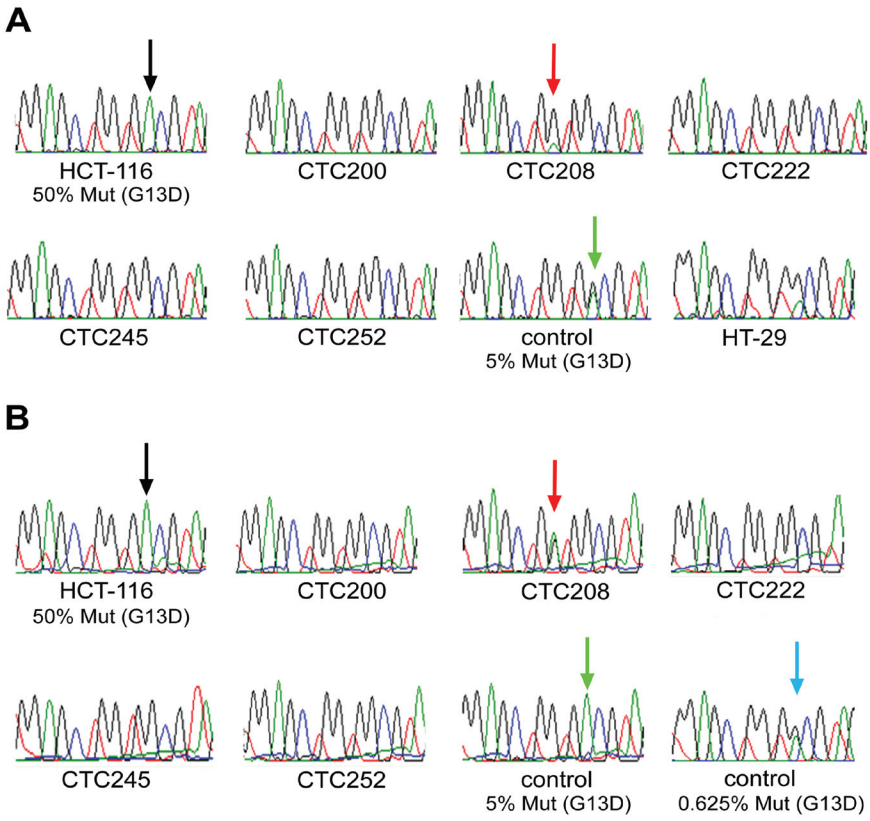


Figure 1

Sequencing electropherograms of first round (panel a) and second round (panel b) enrichment PCR products. Panel a: Black arrow indicates the location of a G13D mutation (Mut) present in pure cells of the colorectal cancer cell line HCT116 (50% G>A mutant); black peak corresponds to the wild-type G and green peak to mutated A. Green arrow indicates the location of a G13D mutation present in 5% HCT116 cell line cells mixed with 95% *KRASwt* SK-BR-3 cell lines cells. Red arrow indicates location of G13D mutation present in patient sample CTC208. HT29 was the wild-type control. CTC200, CTC222, CTC245 and CTC252 all had *KRASmt* tissue, but no mutation in their CTCs detected with COLD-PCR (see **Table 2** and Results section)

Panel b: Sequencing electropherograms after a second round of enrichment; the same results are obtained for controls HCT116 (black arrow) and mixed cell line control (green arrow). Blue arrow indicates the location of a G13D mutation present in mixed *KRASmt* cancer cell lines diluted to 0.625% mutation frequency. Red arrow indicates a now more pronounced green A peak in mutated patients sample CTC208. Red peak; T, blue peak; C

Chapter 9

Diagnostic applications of cell-free and circulating tumor cell-associated miRNAs in cancer patients

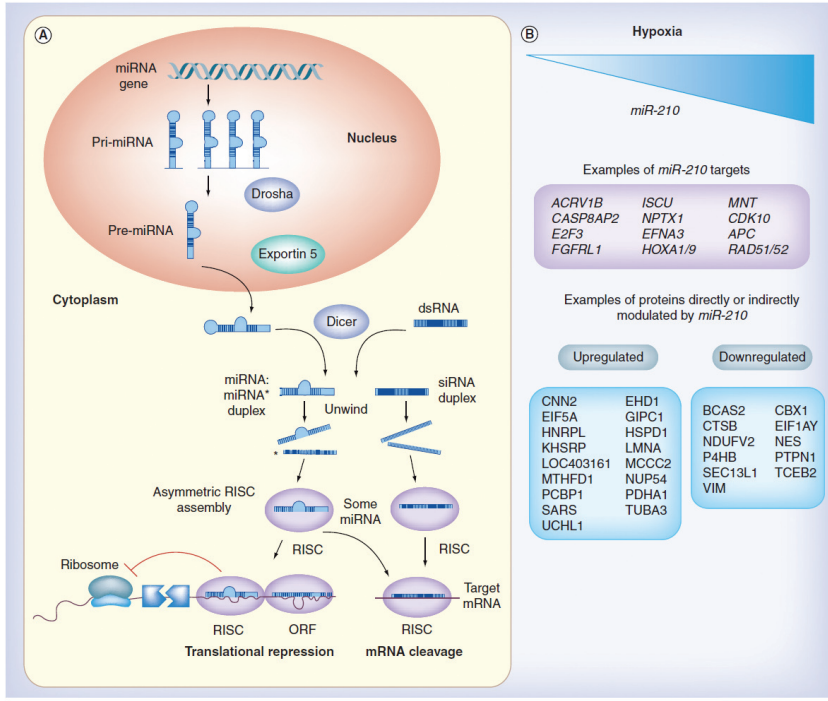


Figure 1

miRNA processing resulting in altered protein expression. Left panel: miRNA processing; In the nucleus, miRNAs are transcribed into primary miRNA transcripts (pri-miRNAs). These pri-miRNAs are subsequently cleaved into precursor miRNAs (pre-miRNAs) by *Drosha*. These pre-miRNAs, 70 to 90 nucleotides in length, are transported into the cytoplasm by *Exportin 5* where the hairpin precursors are cleaved by *Dicer*, resulting in a small dsRNA duplex that contains both the mature miRNA strand and its complementary strand. The 20 to 25 nucleotides in length mature miRNA strand is then incorporated into a RNA-induced silencing complex (*RISC*), which inhibits the function of its target mRNA by mRNA degradation or, most commonly, by translational repression[393,472] (Figure freely adapted from http://www.marligen.com and[473]).

Right panel: an example of the multiple functions of *miR-210* in the for cancer important process of hypoxia, which allows cancer cells to adapt to a low oxygen environment. Increased *miR-210* expression has been linked to increased metastatic capability and increased hypoxia signaling in lymph node-negative estrogen receptor-positive human breast cancer[286]. *MiR-210* is regulated by hypoxia-inducible factor 1-alpha (HIF-1alpha), and the expression of both is increased in response to hypoxia. The upregulated

expression of *miR-210* expression during hypoxia results in the repressed translation of its multiple target genes. Through these *miR-210* targeted genes a large number of proteins are up and down regulated in response to increased *miR-210* expression[474]. The direct or indirect up and down regulation of these downstream proteins enables cells to adjust and adapt to a hypoxic environment, sustaining their rapid growth rate despite insufficient angiogenesis to match their proliferation rate.

Figure 2 (see page 228)

Unsupervised hierarchical cluster analysis comparing gene expression profiles in CTC-enriched blood samples of metastatic breast cancer patients. Expression levels were analyzed with real-time RT-PCR with 65 TaqMan Gene Expression Assays in cDNA generated from RNA isolated from the CTC-enriched fractions of 50 metastatic breast cancer patients. Sample loading and RNA integrity were controlled with 3 additional universal reference genes (*GUSB*, *HPRT1*, and *HMBS*). Prior to real-time PCR, cDNA was pre-amplified in 15 cycles with the PreAmp method from ABI, as described in the Materials and Methods section, by using the same TaqMan Gene Expression Assays that were used for the real-time PCR. Data shown have been subjected to median normalization of each individual gene across all samples followed by median normalization of each individual sample across all genes. Each horizontal row represents a gene, and each vertical column corresponds to a sample. Red color indicates a transcript level above the median level, black color indicates a median transcript level, and green color indicates a transcript level below the median level of the particular assay as measured in all samples. Depicted gene clusters were identified at an average linkage correlation greater than 0.2. The number of CTCs as established by the CellSearch Epithelial Kit is given below the graph, with the samples with at least 5 CTCs according these cell counts marked gray for easy identification.

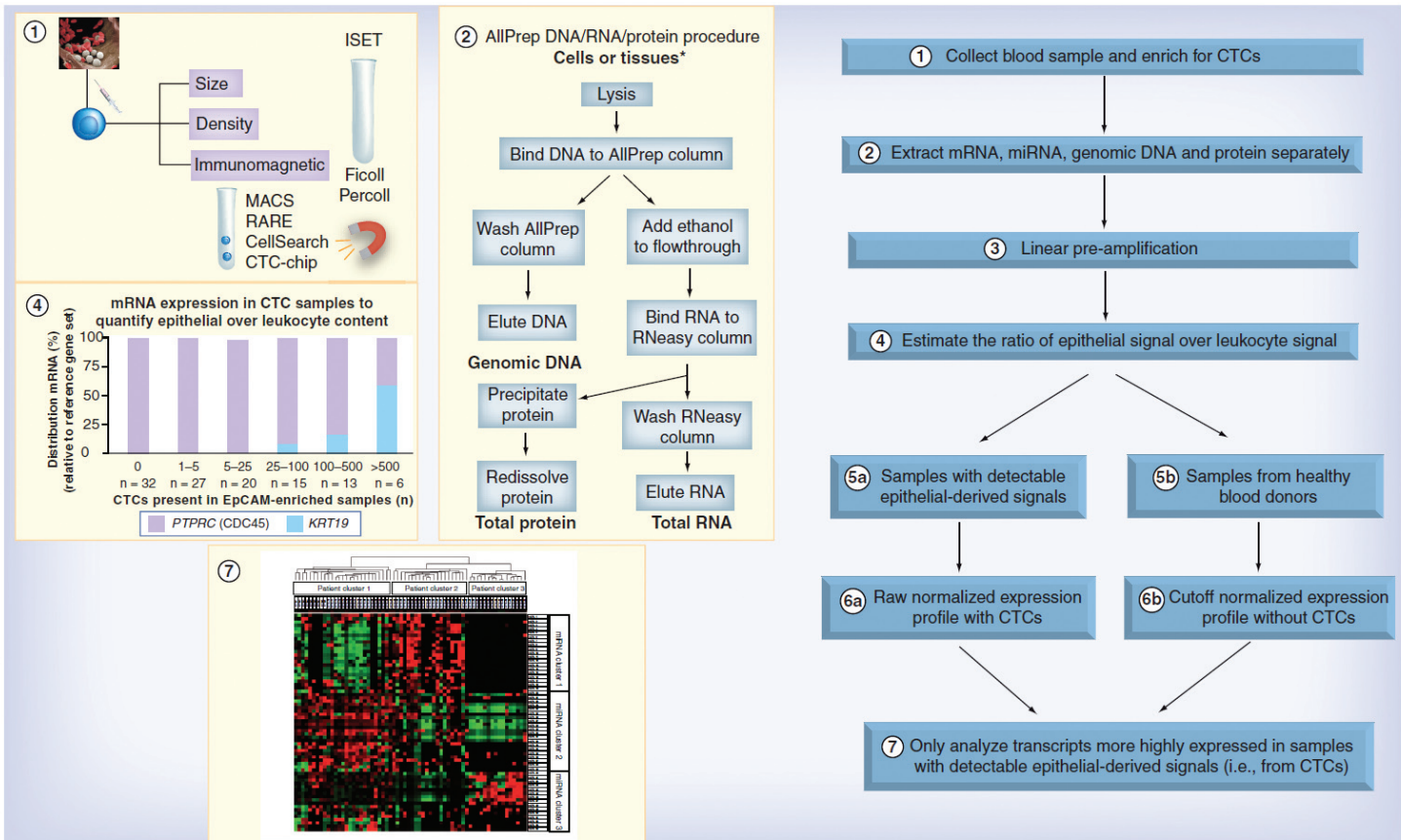

1
ISET
Size
Density
Immunomagnetic
Ficoll
Percoll
MACS
RARE
CellSearch
CTC-chip
2 AllPrep DNA/RNA/protein procedure
Cells or tissues*
Lysis
Bind DNA to AllPrep column
Wash AllPrep column
Add ethanol to flowthrough
Elute DNA
Bind RNA to RNeasy column
Genomic DNA
Precipitate protein
Wash RNeasy column
Redissolve protein
Elute RNA
Total protein
Total RNA
4
mRNA expression in CTC samples to quantify epithelial over leukocyte content
Distribution mRNA (%) (relative to reference gene set)
100
75
50
25
0
0 1–5 5–25 25–100 100–500 >500
n = 32 n = 27 n = 20 n = 15 n = 13 n = 6
CTCs present in EpCAM-enriched samples (n)
PTPRC (CDC45)
KRT19
7
Patient cluster 1
Patient cluster 2
Patient cluster 3
miRNA cluster 1
miRNA cluster 2
miRNA cluster 3
1 Collect blood sample and enrich for CTCs
2 Extract mRNA, miRNA, genomic DNA and protein separately
3 Linear pre-amplification
4 Estimate the ratio of epithelial signal over leukocyte signal
5a Samples with detectable epithelial-derived signals
5b Samples from healthy blood donors
6a Raw normalized expression profile with CTCs
6b Cutoff normalized expression profile without CTCs
7 Only analyze transcripts more highly expressed in samples with detectable epithelial-derived signals (i.e., from CTCs)

Figure 2

Step-by-step scheme for reliable measurement of CTC-associated miRNAs. After collecting blood samples in EDTA tubes to preserve RNA integrity and enrichment for CTCs (**1**), a sensitive isolation technique – preferably one that is able to isolate genomic DNA, mRNA, miRNA and protein in separate fractions (**2**, an example from qiagen.com) – and linear pre-amplification steps (**3**) are needed to enable detection of molecules in material from as little as one cell.

Next, CTC- and leukocyte-specific signals are used to estimate the ratio of the tumor cell-specific signal over leukocyte-derived signal (**4**). Now, samples can be grouped into those with detectable epithelial-derived signals (**5a**) and those without detectable epithelial signals (**5b**), with the latter group comprising both patient samples without detectable epithelial signals and samples from healthy donors. Until consensus has been established on a robust reference miRNA set, normalizing on the mean expression of all expressed miRNAs in both groups (**6a** and **6b**) is probably the optimal method when multiple miRNA transcripts are measured at the same time.

Finally, to ensure epithelial tumor cell-specific gene expression profiling of CTCs, levels measured in the samples without detectable epithelial signals (**6b**) are used as cut-off for the samples with detectable epithelial signals (**6a**) to calculate the remaining CTC-specific signals (**7**).

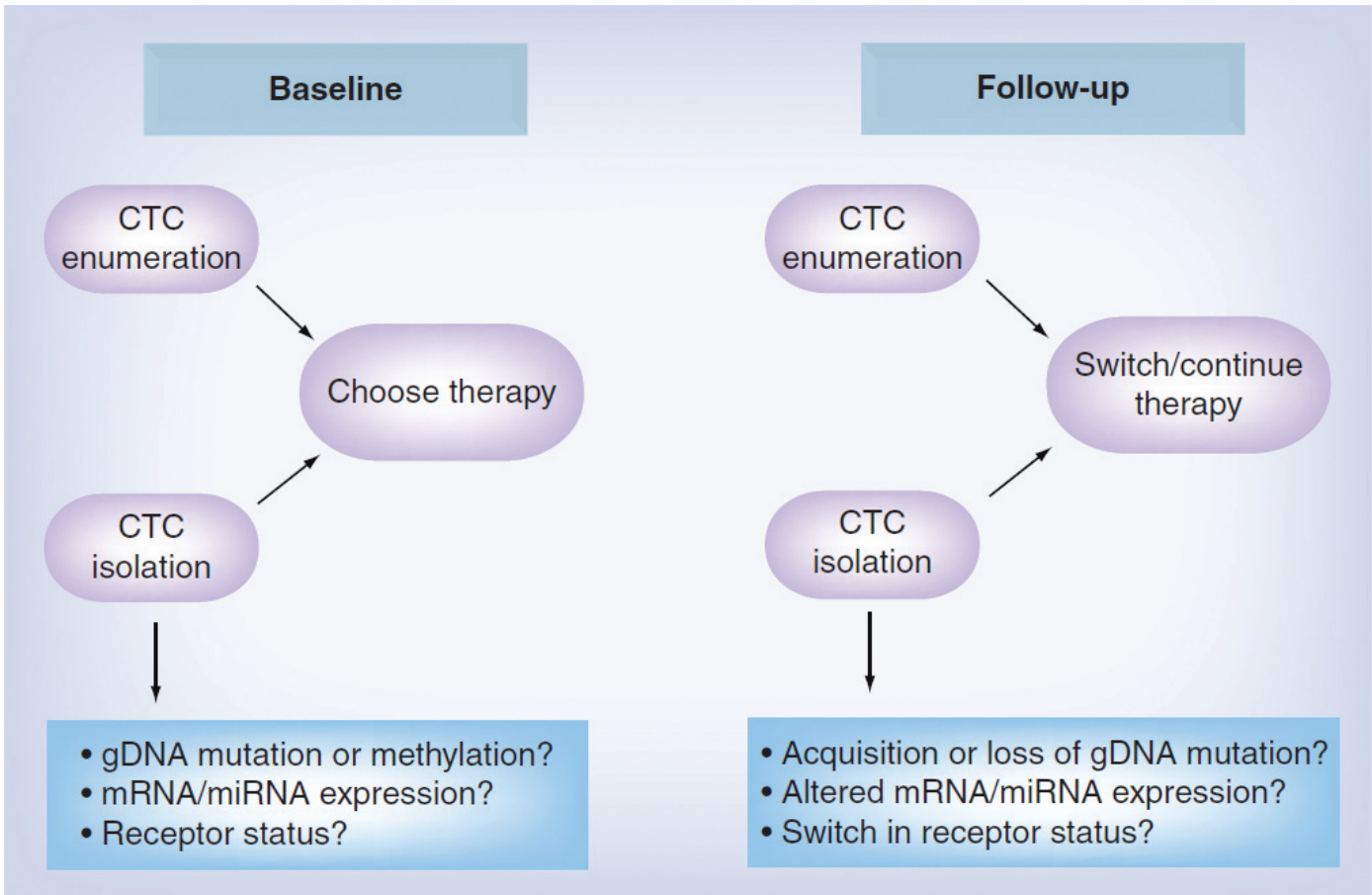

Baseline
Follow-up
CTC enumeration
Choose therapy
CTC isolation
• gDNA mutation or methylation?
• mRNA/miRNA expression?
• Receptor status?
CTC enumeration
Switch/continue therapy
CTC isolation
• Acquisition or loss of gDNA mutation?
• Altered mRNA/miRNA expression?
• Switch in receptor status?

Figure 3

Implementation of CTC enumeration and characterization into the clinic. Here, we explain how we envision the place of CTC enumeration and characterization in the cancer management of the future. Before starting a new line of systemic treatment for metastatic cancer patients, CTC enumeration and isolation can be performed. A CTC count will clarify the prognosis of the patient, and an up-to-date characterization of gDNA mutations and methylation, mRNA and miRNA expression and receptor status helps the clinician to select the most effective tumor therapy. After the first cycle of chemotherapy, CTC enumeration and isolation will be repeated; a rise in CTC count can be an argument to switch therapy, as is a switch in receptor status, acquired or loss of mutations and altered mRNA or miRNA expression. Drug targets, predictive and prognostic factors can be continuously reassessed during the course of treatment and at the time of relapse in a non-invasive manner, resulting in truly patient-tailored treatment.

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List of abbreviations

7AAD	7-Aminoactinomycin D
AA	African American
APC	allophycocyanin
AUC	area under the curve
CA	Caucasian american
CCP	compound covariate predictor
CD	cluster of differentiation
cDNA	complementary DNA
CECs	circulating endothelial cells
CK	cytokeratin
CRC	colorectal cancer
Ct	threshold cycle
CTCs	circulating tumor cells
CV	coefficient of variation
DAPI	4’,6-diamidino-2-phenylindole
DAVID	database for annotation, visualization, and integrated discovery
ddH2O	double-distilled water
dim	dim expression
dsRNA	double-stranded RNA
DTCs	disseminated tumor cells
EDTA	ethyleendiamine tetra-acetate
EGFR	epithelial growth factor receptor
EMT	Epithelial-to-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ER	estrogen receptor
FDA	Food and Drug Administration
FDR	false discovery rate
FF	fresh frozen
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescence in situ hybridization
FITC	fluorescein isothiocyanate
FU	follow-up
gDNA	genomic DNA
HCC	hepatocellular carcinoma
HER2	human epidermal growth factor receptor 2
H(B)D	healthy (blood) donor

HR	hazard ratio
ICC	immunocytochemistry
Ig	immunoglobulin
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid
NA	not available
NPV	negative predictive value
NSCLC	non small cell lung cancer
nt	nucleotide
OS	overall survival
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PD	progressive disease
PE	phycoerythrin
PFS	progression-free survival
PPV	positive predictive value
PR	progesterone receptor
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
QQ	of sufficient quality and quantity
qRT-PCR	quantitative reverse transcribed polymerase chain reaction
RISC	RNA-induced silencing complex
RMS	rhabdomyosarcoma
RNU/SNOR	small nucleolar RNAs
RT	reverse transribed
SCC	squamous cell carcinoma
s/n	signal-to-noise
TMA	tissue microarray
TTS	time to treatment switch
vs.	versus
WB	whole blood

Dankwoord

Een proefschrift wordt nooit alleen gemaakt, maar de studies in dit proefschrift zijn bij uitstek het resultaat van een nauwe samenwerking tussen verschillende afdelingen en disciplines. Ik ben dan ook heel veel mensen oprecht dankbaar voor hun inzet en de bereidheid om hun kennis en ervaring in te zetten voor dit project, alsmede voor hun geloof in het slagen van dit voor het Erasmus MC nieuwe samenwerkingsverband. Het starten van de CTC-werkgroep betekende de eerste samenwerking tussen de afdeling Medische Tumorimmunologie in de Daniel den Hoed Kliniek en de afdeling Breast Cancer Genomics and Proteomics van het Josephine Nefkens Instituut. Twee afdelingen die elkaar maar nauwelijks kenden en bovendien expertise hadden in toch behoorlijk verschillende onderzoeksgebieden. Onder de bezielende leiding van Stefan Sleijfer en John Foekens werd er een CTC-werkgroep gevormd, die mede door een heen- en weer fietsende promovenda een gesmeerd lopende machine moest worden. De bereidheid aan beide kanten van de Maas om de deuren van hun lab open te zetten, eindeloos vragen te beantwoorden en buiten de hokjes te denken, hebben ervoor gezorgd dat nu, lang voordat de fysieke nabijheid van de 2 afdelingen gerealiseerd is, niet alleen de onderlinge afstand overbrugd is maar er ook een wederzijdse waardering en interesse gegroeid is voor elkaars vakgebied. Ik hoop dat deze goede samenwerking in de komende jaren vastgehouden en uitgebreid kan worden.

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Daarnaast ben ik veel dank verschuldigd aan alle patiënten en gezonde bloeddonoren die belangeloos hebben deelgenomen aan de CTC-studies. De inclusie in de verschillende studies was ook niet mogelijk geweest zonder de hulp van de oncologen in de perifere deelnemende centra: Jacqueline Stouthard in het Maasstad Ziekenhuis, Paul Hamberg en Dr. Zuetenhorst in het Sint Franciscus Gasthuis, Felix de Jongh en Dr. Baggen in het Ikazia, Dr. Kehrer in het IJsselland en Dr. Dirix in het Sint-Augustinus Ziekenhuis. Ook de researchverpleegkundigen

(Brenda van Prooijen, Janny Salomé, Anita van der Poel, Corry Leunis en Nicoline Schuur) en de laboratoriummedewerkers die de inclusie van die patiënten hebben gefaciliteerd wil ik graag hartelijk bedanken.

Mijn lieve paranimfen Jantine Posthuma de Boer en Daniëlla Oom: ik ben heel trots dat jullie naast me staan tijdens de verdediging van mijn proefschrift. Ik heb bewondering voor jullie beider uitzonderlijke prestaties, maar waardeer bovenal jullie vriendschap. Ontzettend bedankt dat jullie mijn paranimfen wilden zijn, ik voel me vereerd!

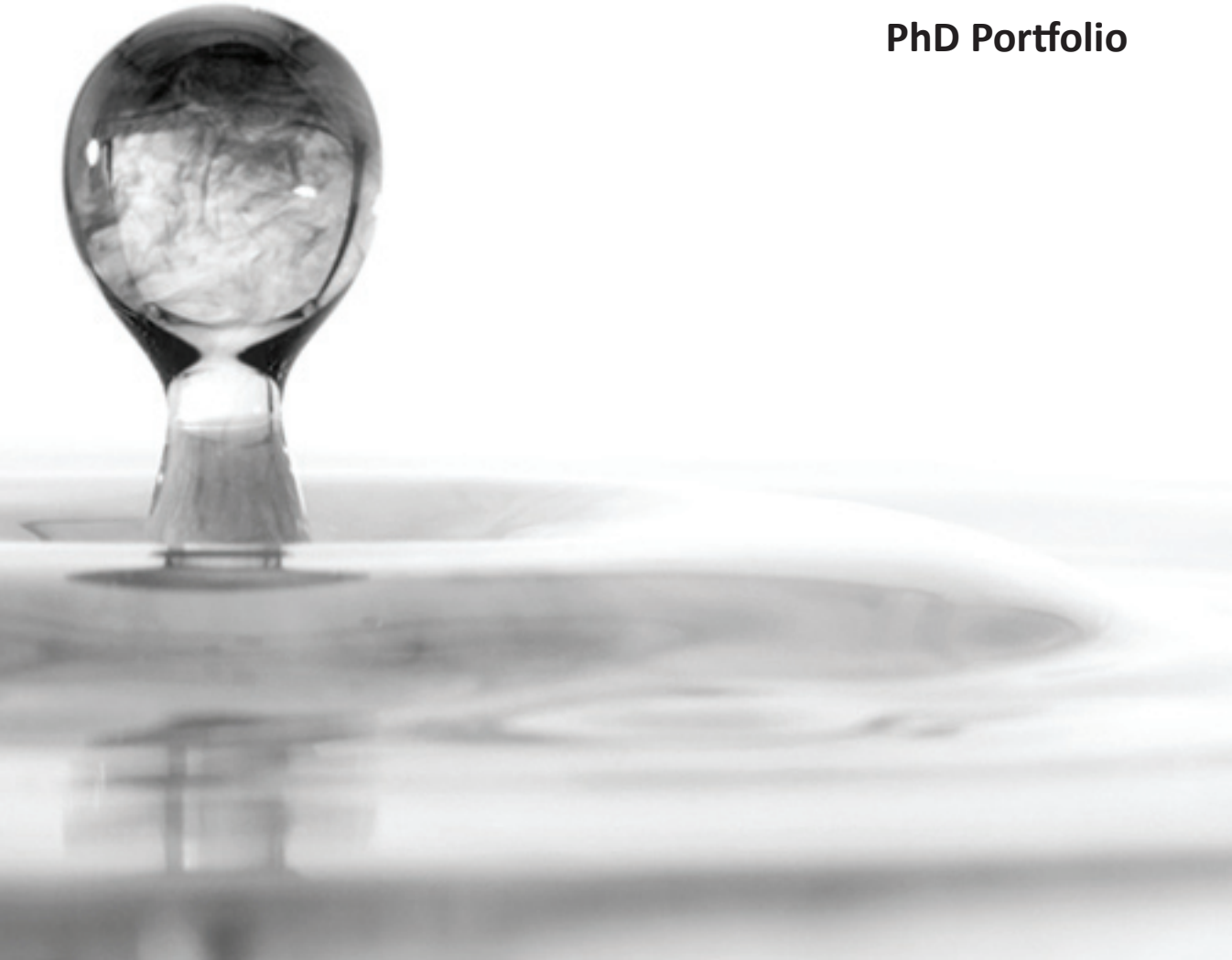
Ook mijn andere lieve vrienden wil ik bedanken voor hun humor, relativeringsvermogen, en optimisme. Een wijntje, etentje of even sporten, altijd goed voor nieuwe energie en ik ben heel blij dat ik zoveel bijzondere mensen om me heen heb!

Marjolein, jou moet ik ook mijn grote dank betuigen voor je hulp bij het lay-out en drukproces, hadden destijds we niet kunnen voorzien in de Flintstone-tent!

Allerliefste familie, de vader en de moeder, Li en Bart, ooms en tantes, nichten en neven; ik ben dankbaar voor mijn grote, hechte familie! In tijden van drukte en stress vind ik bij jullie rust en afleiding, en in tijden van verdriet of vreugde in de familie worden de zorgen van het onderzoek op gepaste wijze gerelativeerd. Ik mag niet zeggen dat het zonder jullie niet was gelukt om te promoveren, maar wellicht was ik er dan niet eens aan begonnen...

Martijn, ik doe je denk ik niets tekort als ik zeg dat je weinig hebt bijgedragen aan de totstandkoming van dit boekje, maar wél aan mijn geluk de afgelopen tijd. Ik ben oprecht blij met je!

PhD Portfolio



PHD TRAINING	Year	Workload (ECTS)
General academic skills		
– Biomedical English writing and communication	2009	4.0
– Advanced imaging techniques for MDs	2009	0.2
– Photoshop and Illustrator CS5 workshop	2011	0.3
– Integrity in Research	2011	2.0
Research skills		
– Basic course regulations and organization for clinical researchers (Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers [BROK])	2009	
– Clinical farmacological research in oncology (Klinisch geneesmiddelonderzoek in de Oncologie)	2009	
– Biostatistics for clinicians	2009	1.0
– Methodology of patient-involving research and preparation of grant writing	2009	
– Biomedical Research Techniques	2009	
– Introduction to clinical research	2010	
In-depth courses		
– Basic and Translational Oncology	2008	1.8
– Molecular Medicine	2008	1.9
– 1st European Breast cancer course at Institut Curie Paris	2008	
– Oncogenesis and tumor biology	2008	1.5
– Molecular Diagnostics IV	2009	1.0
– Diagnostic Development Tutorial at the EORTC-NCI-ASCO Annual meeting on “Molecular Markers in cancer”	2011	

	Year	Workload (ECTS)
Presentations		
– Oral presentation at Daniel Den Hoed Clinic for nurses	2008	
– Bi-annual oral presentation at MTI Journal Club	2008 – 2011	
– Oral presentation Junior Med School	2008 – 2009	
– Oral presentation at the Medical Oncology Science Meeting	2009; 2011	
– Annual oral presentation at the Scientific JNI Lab Meeting	2009 – 2011	0.9
– Oral presentation at 7th International symposium on minimal residual cancer, Athens, Greece	2009	
– Oral presentation at IKR-IKW Breast Cancer Meeting	2009	
– Poster presentation at the annual Molecular Medicine Day	2009	0.3
– Oral presentation at 6th Clinical Course of the ESCCA "Cellular biomarkers of cancer', Valencia, Spain	2010	
– Oral presentation for TU Delft students doing a minor in oncology	2011	
– Oral presentation at Diagnostic Development Tutorial at the EORTC-NCI-ASCO Annual meeting on "Molecular markers in cancer"	2011	
– Oral presentation for Erasmus MC Internal Medicine residents and attending	2012	
– Poster presentation at AACR Annual Meeting	2012	
– Oral presentation at ASCO Annual Meeting	2012	
International conferences		
– 7th International symposium on Minimal Residual Cancer, Athens, Greece	2009	
– Joint meeting of the Belgian Society for Analytical Cytology and the Dutch Society for Cytometry	2009	
– 6th Clinical Course of the ESCCA 'Cellular biomarker of cancer", Valencia, Spain	2010	
– 10th Euroconference of the ESCCA, Valencia, Spain	2010	
– 2011 European Multidisciplinary Cancer Congress, Stockholm, Sweden	2011	
– EORTC-NCI-ASCO Annual Meeting on "Molecular Markers in Cancer", Brussels, Belgium	2011	

	Year	Workload (ECTS)
– AACR Annual Meeting, Chicago, Illinois, US	2012	
– ASCO Annual Meeting, Chicago, Illinois, US	2012	
Seminars and workshops		
– Weekly MTI Journal Club	2008 – 2010	
– Monthly JNI Oncology lectures	2008 – 2012	1.2
– Monthly Bridge Meetings Molecular Medicine	2008 – 2012	
– Monthly Breast Cancer meeting Erasmus MC	2008 – 2012	
– Annual Molecular Medicine Day Rotterdam	2009	0.3
– Cancer Genomics Centre Annual Scientific Meeting	2009 – 2011	
– Monthly OMBO course Erasmus MC	2009 – 2012	
– Borstkanker Behandeling Beter	2010 – 2011	
TEACHING ACTIVITIES		
Supervising students and bachelor's thesis		
– Junior Med School: supervising 3 high school students	2008	
– Supervising 21-week research project for 4th year medical student	2009	
– Junior Med School: supervising 2 high school students	2011	

Curriculum Vitae

Bianca Mostert werd op 22 januari 1983 geboren in Vlaardingen. De lagere school doorliep zij op de ds J.J. Buskesschool te Rotterdam. In 2000 behaalde zij haar Gymnasium diploma cum laude aan het Comenius College te Capelle aan den IJssel. In datzelfde jaar begon zij aan de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Tijdens haar studie was zij actief in de Medische Faculteits Vereniging Rotterdam (MFVR). Haar afstudeeronderzoek deed zij op de afdeling Congenitale Cardiologie van het Erasmus MC te Rotterdam, waar zij onderzoek deed naar het risico op complicaties tijdens de zwangerschap in vrouwen met een congenitale aortaklepstenose, onder supervisie van Prof.dr. J.W. Roos-Hesselink en Prof.dr. F.J. Meijboom. In september 2004 behaalde zij het Doctoraalexamen Geneeskunde.

Na haar afstudeeronderzoek en in de wachttijd voor aanvang van haar coschappen studeerde zij Griekse en Latijnse taal en cultuur aan de Universiteit van Leiden en volgde zij een minor Spaans aan Columbia University te New York City, NY, USA. In september 2005 begon zij met haar coschappen, en behaalde na een oudste coschap op de afdeling Hemato-Oncologie in het Hospital Universitario La Paz te Madrid, Spanje in september 2007 cum laude het artsexamen. Aansluitend werkte zij als ANIOS (assistent-geneeskundige-niet-in-opleiding-tot-specialist) op de afdeling Inwendige Geneeskunde van het Ikazia Ziekenhuis te Rotterdam (opleider Dr. A. Dees). In mei 2008 startte zij een promotie-onderzoek op de afdeling Interne Oncologie van het Erasmus MC te Rotterdam op de locatie Daniel den Hoed kliniek, onder supervisie van Prof.dr. J.A. Foekens, Prof.dr. S. Sleijfer en Dr. J.W. Gratama, met dit proefschrift als resultaat. Tijdens haar promotie-onderzoek was zij door het winnen van de Pieter de Mulder Award 2009 in staat een onderzoeksstage te lopen bij Veridex LLC te Raritan, NJ, USA. In mei 2012 is zij begonnen met de opleiding Inwendige Geneeskunde van het Erasmus MC (opleider Prof. dr. J.L.C.M. van Saase) in het Albert Schweitzer Ziekenhuis te Dordrecht (opleider Dr. E.F.H. van Bommel).

List op publications

Mostert B, Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, van Galen A, Peeters D, Dirix LY, Seynaeve CM, Jager A, de Jongh FE, Hamberg P, Stouthard JML, Kehrer DFS, Look MP, Smid M, Jiang Y, Wang Y, Gratama JW, Foekens JA, Martens JWM, Sleijfer S. Gene expression profiles in circulating tumor cells predict prognosis in metastatic breast cancer patients. *Manuscript in preparation*.

Mostert B, Sieuwerts AM, Bolt-de Vries J, Kraan J, Lalmahomed Z, van Galen A, van der Spoel P, de Weerd V, Ramírez-Moreno R, Smid M, Look MP, Jiang Y, Wang Y, Verhoef C, IJzermans JNM, Gratama JW, Sleijfer S, Martens JWM, Foekens JA. mRNA and miRNA expression profiles in circulating tumor cells of metastatic colorectal cancer patients. *Manuscript submitted for publication*

Mostert B, Jiang Y, Sieuwerts AM, Wang H, Bolt-de Vries J, Biermann K, Kraan J, Lalmahomed Z, van Galen A, de Weerd V, van der Spoel P, Ramírez-Moreno R, Verhoef C, IJzermans JNM, Wang Y, Gratama JW, Foekens JA, Sleijfer S, Martens JWM,. KRAS and BRAF mutation status in circulating colorectal tumor cells and their correlation with primary and metastatic tumor tissue. *Manuscript submitted for publication*

Mostert B, Kraan J, Sieuwerts AM, van der Spoel P, Bolt-de Vries J, Prager-van der Smissen WJC, Smid M, Timmermans AM, Martens JWM, Gratama JW, Foekens JA, Sleijfer S. CD49f-based selection of circulating tumor cells (CTCs) improves detection across breast cancer subtypes. *Cancer Lett 2011; 319(1): 49-55*

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Sieuwerts AM, **Mostert B**, Bolt-de Vries J, Peeters D, de Jongh FE, Stouthard JM, Dirix LY, van Dam PA, Van Galen A, de Weerd V, Kraan J, van der Spoel P, Ramírez-Moreno R, van Deurzen CH, Smid M, Yu JX, Jiang J, Wang Y, Gratama JW, Sleijfer S, Foekens JA, Martens JW. mRNA and microRNA expression profiles in circulating tumor cells and primary tumors of metastatic breast cancer patients. *Clin Cancer Res. 2011 Jun 1;17(11):3600-18.*

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Mostert B, Sieuwerts AM, Martens JW, Sleijfer S. Diagnostic applications of cell-free and circulating tumor cell-associated miRNAs in cancer patients. *Expert Rev Mol Diagn. 2011 Apr;11(3):259-75.*

Lalmahomed ZS, Kraan J, Gratama JW, **Mostert B**, Sleijfer S, Verhoef C. Circulating tumor cells and sample size: the more, the better. *J Clin Oncol. 2010 Jun 10;28(17):e288-9; author reply e290.*

Sieuwerts AM, Kraan J, Bolt-de Vries J, van der Spoel P, **Mostert B**, Martens JW, Gratama JW, Sleijfer S, Foekens JA. Molecular characterization of circulating tumor cells in large quantities of contaminating leukocytes by a multiplex real-time PCR. *Breast Cancer Res Treat. 2009 Dec;118(3):455-68.*

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