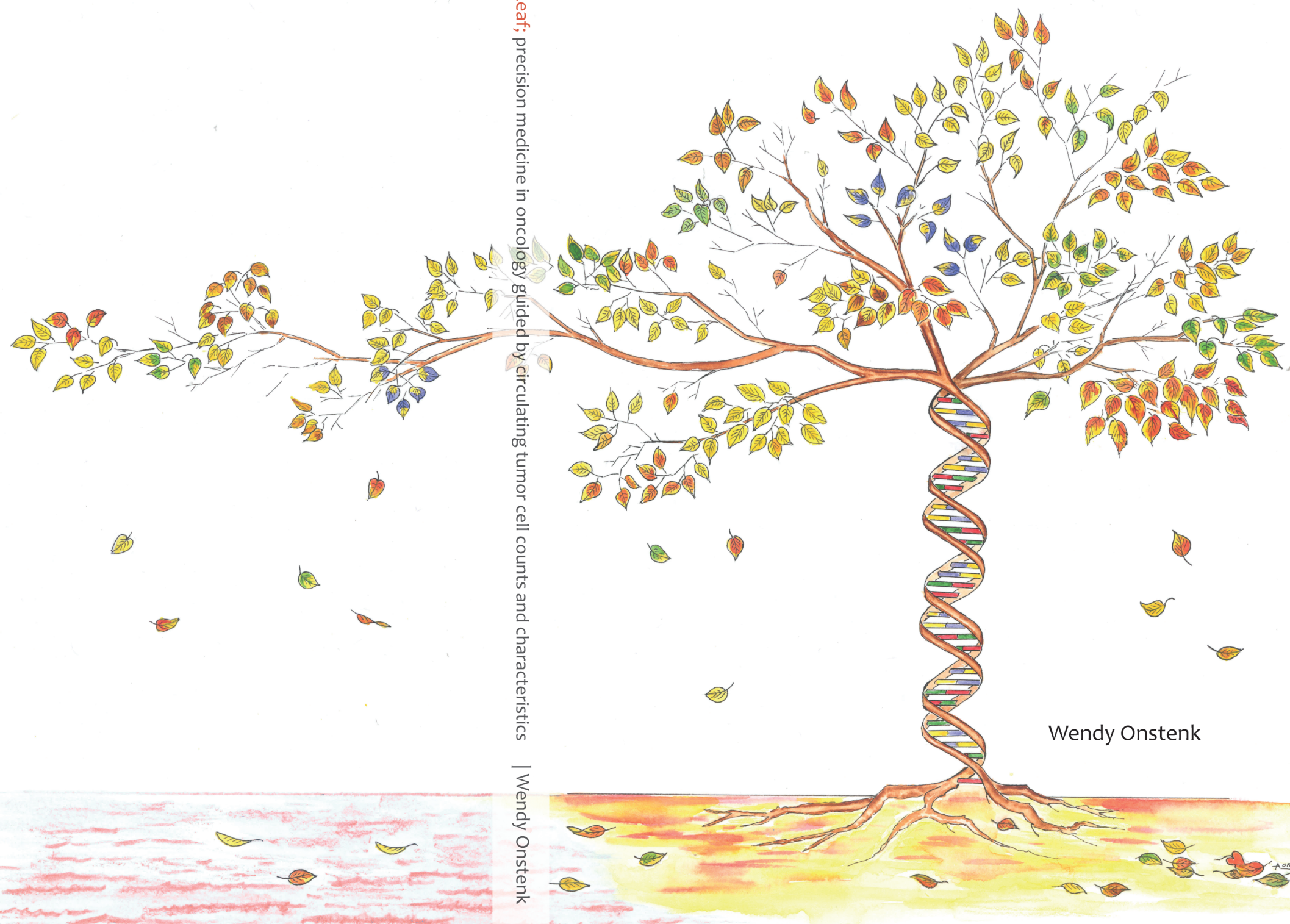
Turning over a New Leaf; precision medicine in oncology guided by circulating tumor cell counts and characteristics | Wendy Onstenk

TURNING OVER A NEW LEAF

Precision medicine in oncology guided by circulating tumor cell counts and characteristics



Wendy Onstenk

TURNING OVER A NEW LEAF;

Precision medicine in oncology guided by circulating tumor cell counts and characteristics

Wendy Onstenk

Molecular Medicine
Postgraduate School

The studies described in this thesis were performed within the framework of the Erasmus MC Molecular Medicine (MolMed) Graduate School at the Department of Medical Oncology, Erasmus MC Cancer Institute, Rotterdam, Netherlands.

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Turning over a New Leaf;

Precision medicine in oncology guided by circulating tumor cell counts and characteristics

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It's all about drive and chance

Tomasetti and Vogelstein

CONTENTS

1

GENERAL INTRODUCTION & OUTLINE OF THIS THESIS

The changing landscape of oncology

Over the recent years, the treatment of patients with solid tumors has been subject to a paradigm shift. Whereas patients have traditionally been treated according to the organ the tumor originated from, new insights into the molecular mechanisms underlying progression and dissemination of cancer have caused us to increasingly relinquish this anatomical cancer subdivision. Specific factors, *e.g.* growth factor receptors and mutations in oncogenes, have been found to be involved in tumori- and mutagenesis, but have also been found to contribute to variable degrees across different tumors. In fact, every tumor seems to have its own genetic signature and no two tumors – within or between patients – are identical [1].

Although the development of small molecules targeting these factors has extended our arsenal of treatment options and has improved outcome for many patients, great challenges still remain. Targeted treatments have only been shown to be efficacious in patients whose tumors are positive for the targeted factor [2, 3], which requires us to incorporate molecular diagnostics into the clinical work-up in order to discriminate patients with a high likelihood of benefiting from a specific targeted treatment from patients unlikely to respond to treatment. However, the presence of a factor in tumor cells may not be stable, but may vary over time and between the different tumor sites within an individual patient (Figure 1) [4, 5]. This so-called temporal and spatial heterogeneity, respectively, results from the genomic instability that is characteristic for tumors and forces us to consider the dynamic changes in the molecular characteristics of a tumor with the interpretation of the molecular work-up.

Already, the assessment of the presence of certain predictive factors, *e.g.* the estrogen receptor (ER) for the treatment of breast cancer patients with aromatase inhibitors or tamoxifen, the Human Epidermal Growth Factor Receptor 2 (HER2) for the treatment of breast cancer patients with trastuzumab or lapatinib [2, 6, 7], and *BRAF* mutations for the treatment of melanoma patients with vemurafenib [3], has been incorporated into clinical care. Notwithstanding clinical guidelines recommending the use of metastatic tissue [8,

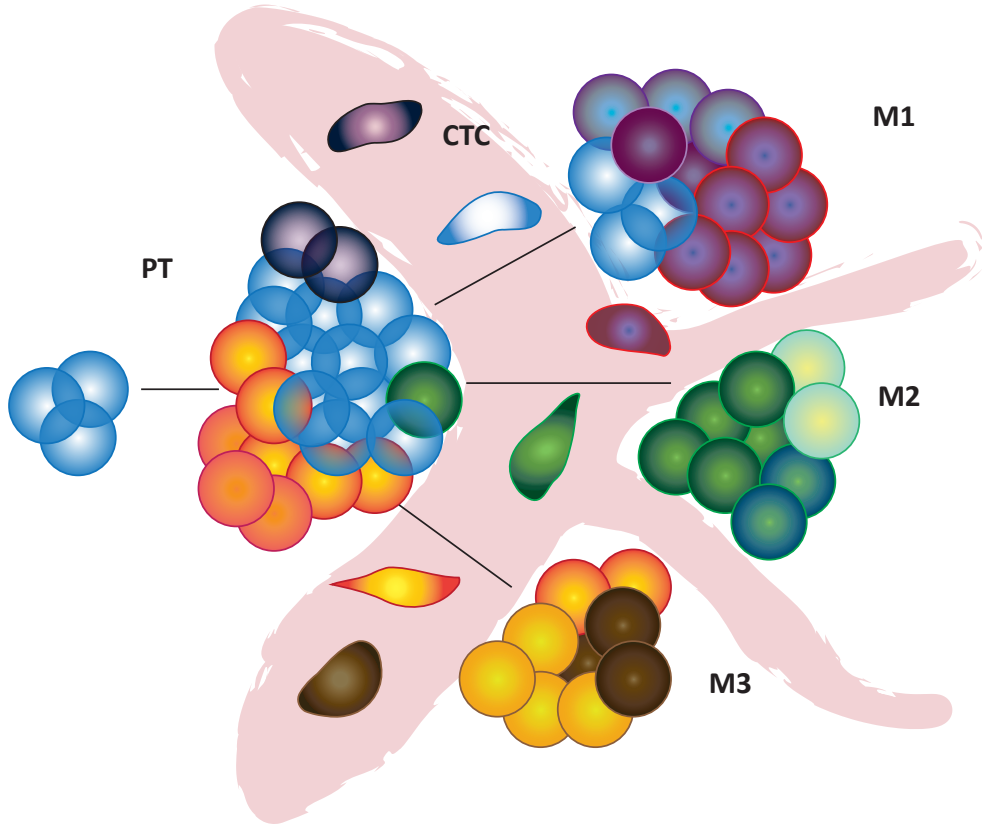

Figure 1. Schematic representation of the development and dissemination of a tumor. The current main hypothesis of clonal evolution states that a primary tumor develops as one homogeneous clone from a single cell (left). Random genetic changes, such as mutations and amplifications, occur in the instable genome and result in genetically and phenotypically different subclones (middle). The primary tumor is now considered spatially heterogeneous. Although not all subclones may acquire the ability to metastasize like the blue, green, and yellow clones, they may release CTCs into the circulation as illustrated by the dark purple clone. The CTCs that survive in the blood stream and are able to colonize distant tissue may grow out to metastases. New genomic changes occurring in the proliferating metastatic cells may cause the characteristics of the metastatic sites to significantly differ from the primary tumor, giving rise to intertumoral or temporal heterogeneity. Also, the metastatic sites themselves can again become spatially heterogeneous due to the formation of different subclones, which all may shed CTCs into the circulation. PT = primary tumor; CTC = circulating tumor cell; M= metastasis.

9], predictive factors are currently mostly determined on the primary tumor; tissue from a primary tumor is routinely available for most patients and taking biopsies of metastatic sites can be painful and is not without risk. However, the dynamic changes that occur in the molecular make-up of a tumor over time and under treatment pressure and the consequent heterogeneity between primary tumors and metastatic sites make primary tumor tissue less suitable for analysis, especially when metastatic disease develops after a latency period of months or even years after the initial presentation. For example, the expression of ER and HER2 – both important treatment targets for patients with metastatic breast cancer (MBC) – have been observed to be discordant between a distant metastasis and the primary tumor in 13-37% and 6-34% of the patients, respectively [10-15]. In prostate cancer, *AR* mutations, amplifications, and splice variants have only rarely been found in primary tumors, but have been detected in the metastases from patients with metastatic castration-resistant prostate cancer (MCRPC) in frequencies of 30-60% [16-18]. Primary tumors and metastases from patients with metastatic colorectal cancer (MCRC) have found to be discordant in the mutational status of the *KRAS* oncogene in 8-23% of the patients [19-21]. These examples underline the urgency for tools enabling to capture the extent of tumor heterogeneity and to monitor the molecular characteristics of a metastatic tumor in real-time.

Circulating tumor cells

The characterization of circulating tumor cells (CTCs) from the peripheral blood has been proposed as a minimally invasive tool to assess tumor characteristics at a specific point in time. After detachment from a solid tumor – either the primary tumor or a metastatic site (Figure 1) – CTCs float freely in the peripheral blood of patients with different forms and stages of epithelial cancer, where they can be captured after a simple venipuncture [22]. Although first described in the publication "*A case of cancer in which cells similar to those in the tumours were seen in the blood after death*" by Thomas Ashworth in 1869 [23], it took over a century to discover the clinical relevance of the drifting CTCs. Technical obstacles due to the rarity of CTCs in the circulation have mainly caused this time lag. Only on average one cell amidst a few billion hematological cells is a CTC, making detection

extremely challenging. The first applied detection methods were reverse transcription polymerase chain reaction (RT-PCR), immunofluorescence (IF) and flow cytometry, but these techniques lacked the sensitivity to discover the clinical value of CTCs [24-26].

A boost in research occurred after the development of the first semi-automated CTC detection and enumeration platform: the CellSearch System (Janssen Diagnostics LLC, Raritan, NJ). Based on the expression of the Epithelial Cell Adhesion Molecule (EpCAM), epithelial cells are immunomagnetically enriched in this system, followed by IF staining to discriminate CTCs from the remaining leukocytes; CTCs stain positive for cytokeratin (CK)-phycoerythrin (PE) and the double-stranded DNA stain 4',6-diamidino-2-phenylindole (DAPI), but negative for cluster of differentiation molecule (CD) 45-allophycocyanin (APC), leukocytes on the other hand stain positive for CD45-APC and DAPI, but negative for CK-PE (Figure 2). All the enriched and stained cells are thereafter transferred to a cartridge, which is scanned by digital fluorescence microscopy. After analysis and processing, a selection of images positive for both CK-PE and DAPI is presented to the operator for further assessment (Figure 3). All cells with a round to oval morphology, a diameter ≥4 µm, an intact cell membrane, and a nucleus which overlaps ≥50% with the cell membrane are counted, leading to the final enumeration of the CTCs present in the sample of 7.5 mL peripheral blood.

In 2004, the United States Food and Drug Administration (FDA) provided clearance for the clinical use of a CTC count from 7.5 mL of blood by the CellSearch System following the demonstration of a strong, independent prognostic value of CTCs in patients with MBC [27]. In this study, it was found that patients with a favorable CTC count of <5/7.5 mL of blood before the start of systemic therapy as well as during treatment had a longer median progression-free and overall survival (PFS and OS, respectively) than the patients with an unfavorable count of ≥5 CTCs/7.5 mL of blood. Moreover, it was shown that switches from favorable to unfavorable CTC counts or *vice versa* during treatment predicted for treatment resistance or response, respectively, as early as after the first cycle of chemotherapy [28]. The prediction of treatment resistance by changes in CTC counts was shown to be more accurate at an earlier stage than the conventional methods

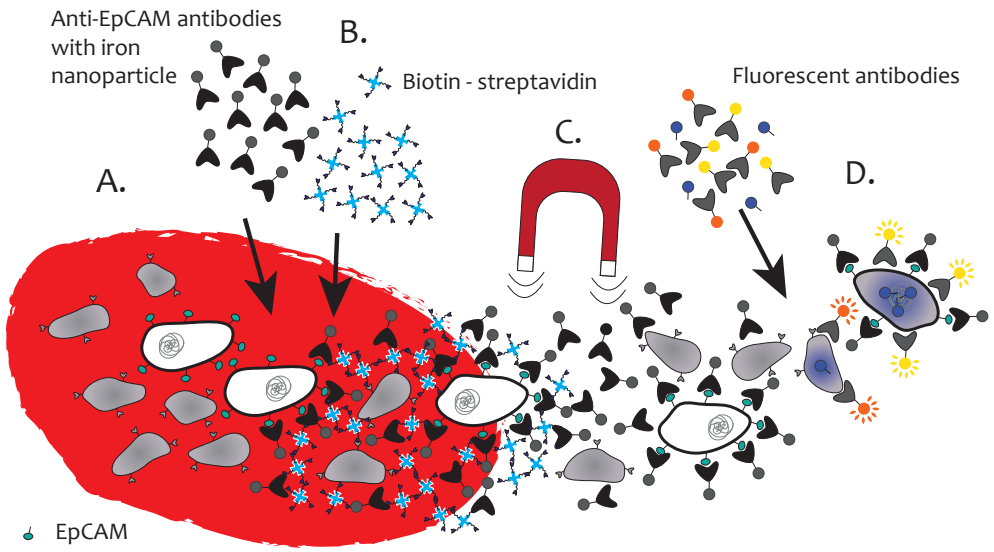

FIGURE 2. The enrichment and detection of CTCs from peripheral blood as done by the CellSearch System. A sample of 7.5 mL whole blood is inserted into the CellSearch AutoPrep System (A). In this system, plasma is aspirated and anti-EpCAM antibodies to which an iron nanoparticle is bound are added together with biotin and streptavidin (B). After binding of the anti-EpCAM antibodies, the EpCAM-positive CTCs present in the blood become magnetic. The biotin-streptavadin network with binding of additional anti-EpCAM ferrofluid antibodies further strengthen the magnetism, which makes the isolation of even 1 CTC in 7.5 mL blood possible. After an incubation step against a strong magnet (C), all magnetic cells plus a surplus of trapped leukocytes remain while the other cells are washed away. To discriminate between CTCs and the remaining leukocytes, fluorescent antibodies are added (D). After scanning by a fluorescence microscope, the CTC count from the initial 7.5 mL blood can be determined.

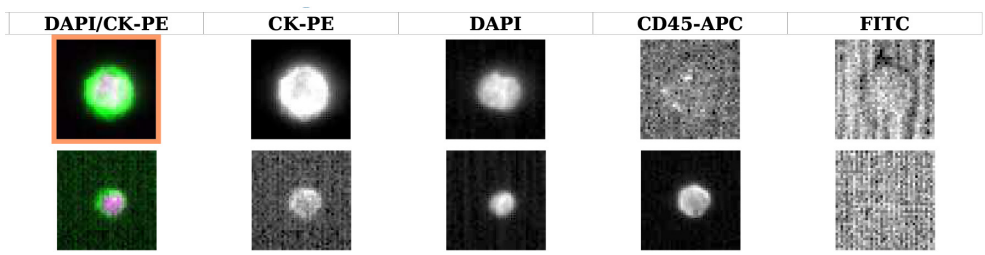

FIGURE 3. Examples of two presented CK-PE and DAPI positive events present in a CellSearch cartridge after CellSearch enrichment, staining and scanning by the CellTracks Analyzer digital fluorescence microscope. The final selection and enumeration of the CTCs from 7.5 mL blood has to be done manually by counting all CK-PE/DAPI positive and CD45-APC negative, intact cells that are larger than 4 µm. The upper row shows an CTC, which has been selected for couting. The lower row shows a cell that stains dimly for CK-PE, but brightly for CD45-APC. This cell is a leukocyte and is therefore not counted. The FITC channel is left open for additional staining and characterization of the CTCs, for example for the expression of HER2.

using radiology or serum cancer antigens [29]. Similar results have been observed in patients with MCRPC, where treatment response evaluation by CTC counts was shown to be more accurate than radiology, bone scintigraphy, and serum levels of prostate-specific antigen (PSA) [30]. Over the years, the prognostic value of CTCs enumerated by the CellSearch System has been confirmed for many other types of epithelial cancers [26], including primary breast cancer (PBC, [31, 32]), MCRC [33]), lung cancer [34, 35], melanoma [36], pancreatic cancer [37], esophageal cancer [38], and hepatocellular cancer [39].

The commercial availability of the CellSearch system has led to an exponential increase in the number of publications on the clinical value of CTCs over the past years (Figure 4). While studies initially focused on the prognostic value of CTCs before and during treatment, this has swiftly shifted to the predictive value and the utility of CTCs for well-considered targeted treatment decisions. With a short circulating half-life of only a few hours [40, 41], CTCs may form an invaluable source of real-time information on metastatic tumor characteristics, thereby potentially offering a minimally invasive alternative for tissue biopsies. Importantly, expanded knowledge of the biological behavior of CTCs will be needed to allow for full appreciation of the clinical relevance of CTCs. For example, after detachment from a solid tumor, CTCs have to survive in the circulation. In order to do so, they are thought to change their epithelial phenotype to a more mesenchymal-like phenotype through a process called epithelial-to-mesenchymal transition (EMT) [42]. In this process, expression levels of cell-adhesion proteins like EpCAM and E-cadherin are downregulated, and mesenchymal proteins like N-cadherin and vimentin are upregulated [43]. Subsequently, before extravasation from the circulation and the formation of new metastases, CTCs are supposed to undergo the reverse process of mesenchymal-to-epithelial transition (MET) [42]. These biological changes in CTCs will have to be taken into consideration with the use of CTCs as "*liquid biopsy*".

Unfortunately, research on the biology of CTCs and the predictive value of CTC characteristics remains hampered by technical issues. The generally low number of

CTCs present in a blood sample and the large surplus of leukocytes that remain even after enrichment require characterization assays to be extremely sensitive and specific. The characterization of CTCs for the presence of proteins [44-46], DNA mutations [19, 47], amplifications [48], and specific gene transcripts [49, 50] has proven feasible, but prospective clinical trials investigating the clinical relevance of the presence of these factors are still scarce. Attempts have been made to increase the CTC capture rate, for example by using other capture markers besides EpCAM [24]. Also, methods have now become available enabling the interrogation of pure CTC fractions or even single CTCs [51, 52]. Such technologies will likely help to further improve our understanding of CTCs and to determine the position of CTCs in clinical care.

Scope of this thesis

The studies described in this thesis focused on the technical aspects of CTC characterization and the clinical significance of CTCs in terms of the prognostic and predictive value. In **chapter 2** a study is described in which a new approach to improve the CTC detection rate of the CellSearch System in patients with locally advanced breast cancer was tested. Also, the associations between CTC counts and outcome to neoadjuvant chemotherapy were investigated. In **chapter 3** an overview is provided of the currently available CTC characterization methods along with their advantages and disadvantages. Besides, the different studies investigating the clinical value of CTC characteristics for patients with breast cancer are summarized as an indication of where we stand in this field. **Chapter 4** contains a study in which the expression levels of tumor-associated genes were determined in the CellSearch-enriched CTCs from patients with MBC. The resulting gene expression profiles were compared to the primary tumors with the aim to investigate to what extent CTCs differ from the primary tumors. The discordance in the expression of the endocrine treatment target ER between the CTCs and the corresponding primary tumor was also analyzed separately given its direct clinical relevance. Lastly, the prognostic value of discordant CTC profiles for OS was investigated. In **chapter 5**, the alterations in the expression of tumor-associated genes in CTCs were explored further, this time in patients with MCRC. The gene expression profiles of matching primary tumors, liver

metastases, and CTCs were compared, allowing us to investigate whether CTCs more closely resembled the metastases than the primary tumor. In **chapter 6**, recent studies investigating the prognostic and predictive value of CTCs for the treatment of patients with MCRPC are summarized. More specifically, recommendations are given how to use CTCs as a tool for optimal sequencing of the new treatment options that have become available over the past decade. In **chapter 7**, an approach is described to measure the presence of the androgen receptor splice variant 7 (*AR-V7*) in the CTCs from patients with MCRPC. The presence of *AR-V7* in CTCs measured by the AdnaTest (Qiagen, Hannover, GE) has been shown to be a prognostic factor under treatment with abiraterone or enzalutamide [53]. The aim of the study described in chapter 7 was to evaluate the prognostic value of the presence of *AR-V7* for response to cabazitaxel and to explore whether CTCs can aid in treatment decision-making in this setting. Lastly, in **chapter 8**, the results obtained from the studies described in this thesis are put into perspective, and recommendations and future directions are given.

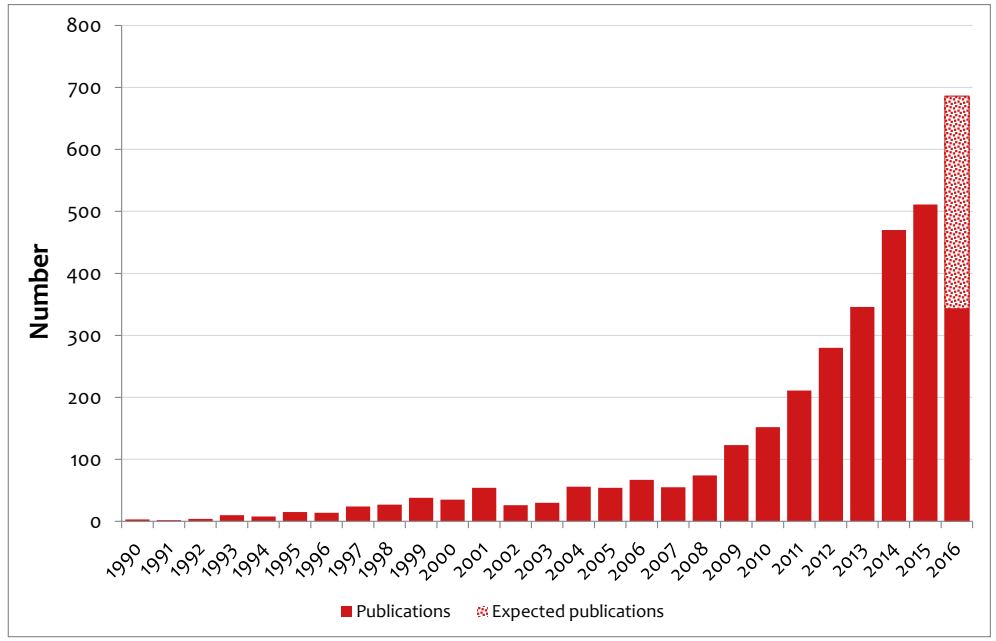

FIGURE 4. The number of publications in PubMed from 1990 until April 2016 as indexed by the terms "circulating epithelial cells" or "circulating tumor cells" at July 1st, 2016. A further increase is expected for the remainder of 2016.

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2

IMPROVED CIRCULATING TUMOR CELL DETECTION BY A COMBINED EPCAM AND MCAM CELLSEARCH ENRICHMENT APPROACH IN PATIENTS WITH BREAST CANCER UNDERGOING NEOADJUVANT CHEMOTHERAPY

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Molecular Cancer Therapeutics, 2015; 14(3): p. 821-7.

ABSTRACT

Circulating tumor cells (CTCs) are detected by the CellSearch System in 20-25% of primary breast cancer (PBC) patients. To improve CTC detection, we investigated melanoma cell adhesion molecule (MCAM) as enrichment marker next to epithelial cell adhesion molecule (EpCAM) and tested the clinical relevance of MCAM-positive CTCs in patients with HER2-negative stage II/III pBC starting neoadjuvant chemotherapy (NAC) in the NEOZOTAC trial. Using the CellSearch System, EpCAM-positive and MCAM-positive CTCs were separately enriched from 7.5 mL blood, at baseline and after the first NAC cycle. Circulating endothelial cells (CECs) were measured using flow cytometry. Primary objective was to improve the CTC detection rate to ≥40% combining EpCAM/MCAM. Correlations of CTC and CEC counts and pathological complete response (pCR) were also explored. At baseline, we detected EpCAM-positive and MCAM-positive CTCs in 12 of 68 (18%) and 8 of 68 (12%) patients, respectively. After one cycle, this was 7 of 44 (16%) and 7 of 44 (16%) patients, respectively. The detection rate improved from 18% at baseline and 16% after one cycle with EpCAM to 25% (*P*=0.08) and 30% (*P*=0.02), respectively, with EpCAM/MCAM. No patients with MCAM-positive CTCs versus 23% of the patients without MCAM-positive CTCs at baseline achieved pCR (*P*=0.13). EpCAM-positive CTCs and CEC counts were not correlated to pCR. Combined EpCAM/MCAM CellSearch enrichment thus increased the CTC detection rate in stage II/III pBC. We found no associations of CTC and CEC counts with pCR to NAC. The clinical relevance MCAM-positive CTCs deserves further study.

Introduction

A circulating tumor cell (CTC) count from peripheral blood as measured by the Food and Drug Administration (FDA)-approved CellSearch System (Janssen Diagnostics, Raritan, NJ) is a strong prognostic factor in both primary and metastatic breast cancers [1]. Although 70% of the patients with metastatic breast cancer (MBC) have ≥1 CTC/7.5 mL of blood, in primary breast cancer (PBC) this proportion is only as low as 20-25% [1-6]. In both cases, the presence of CTCs is associated with poor prognosis. For MBC, patients with ≥5 CTCs/7.5 mL blood have significantly shorter median progression-free survival (PFS) and overall survival (OS) compared with patients with <5 CTCs [1,7,8]. For PBC, patients with ≥1 CTC do significantly worse concerning disease-free survival (DFS) and OS compared with patients without CTCs [1,3,5-7].

Improvements in the detection of CTCs can be made. The CellSearch System relies on the expression of the epithelial cell adhesion molecule (EpCAM; CD326) on CTCs and misses EpCAM-negative CTCs [8-11]. We showed that particularly breast cancer cell lines with epithelial-to-mesenchymal transition (EMT) features lack expression of EpCAM and are therefore not detected by the CellSearch System [9,12]. Because cells that have undergone EMT probably represent an aggressive, clinically relevant subpopulation of CTCs [10], we aimed to detect EpCAM-negative CTCs by alternative approaches. We found melanoma cell adhesion molecule (MCAM; CD146) to be expressed on EpCAM-negative breast cancer cell lines and tested its use as enrichment marker next to EpCAM. In a small series of MBC patients, MCAM-positive CTCs were detected in 9 out of 20 patients (45%), suggesting that CTC detection can be improved using this dual enrichment approach [9].

Besides CTCs, circulating endothelial cells (CECs) have been proposed as a prognostic marker in breast cancer [13]. Being sloughed off the vessel wall, they are a putative marker of angiogenesis and vascular damage. Accordingly, increased CEC counts are found in patients with different solid malignancies, including breast cancer [13]. However, the clinical value of CEC counts before start of and changes during treatment remain to be investigated.

In this study we used an EpCAM/MCAM CellSearch enrichment approach to improve CTC detection in patients with stage II/III breast cancer starting neoadjuvant chemotherapy (NAC). Primary objective was to improve the CTC detection rate from approximately 20% to 40% of patients. Secondary objectives were to determine baseline CEC counts and changes of CTCs and CECs during NAC, and to investigate associations between the presence and dynamics of EpCAM-positive and MCAM-positive CTCs and CECs with pathological complete response (pCR) to NAC.

Patients and methods

Patients

As a side-study to the NEOZOTAC trial – a multicenter, randomized phase III trial initiated by the Dutch Breast Cancer Research Group (BOOG; ref 14) – patients with HER2-negative stage II/III breast cancer who provided additional informed consent for CTC blood sampling were enrolled. Patients were treated with neoadjuvant docetaxel/doxorubicin/cyclophosphamide (TAC) ± zoledronic acid (ZA) and underwent surgery afterwards. Pathological responses on primary tumors and lymph nodes were scored by a pathologist at the Leiden University Medical Center, Leiden, The Netherlands. The definition for pCR was a total absence of invasive tumor cells. This side-study was approved by the Erasmus MC (METC 10-229) and local Institutional Review Boards.

Blood draws and sample processing

Before start of and after the first NAC cycle, 2x10 mL blood was drawn into CellSave preservative tubes (Janssen Diagnostics). All samples were processed within 96 hours at the central laboratory, Erasmus MC Cancer Institute, Rotterdam, The Netherlands. Two CTC enumerations, both from 7.5 mL of blood, were done using the CellSearch System as described before [9]. In brief, EpCAM-positive and MCAM-positive CTCs were enumerated in two separate runs using the CellSearch Epithelial Cell Kit (Janssen Diagnostics). For the MCAM enrichment, anti-MCAM ferrofluid-bound antibodies from the CellSearch Circulating Endothelial Cell kit (Janssen Diagnostics) were used and fluorescein isothiocyanate (FITC)-conjugated CD34 (BD Biosciences, clone 8G12) was added as extra marker to exclude a subset of cytokeratin (CK)-18 expressing CECs [9]. Nucleated, EpCAM or MCAM-enriched cells, positive for CK8/18/19, and negative for CD45 and CD34 for the MCAM-positive cells were considered CTCs. To enable distinction between EpCAM-positive and MCAM-positive CTCs separate EpCAM- and MCAM-enrichments were run. Combined EpCAM/MCAM CTC counts were calculated afterwards, using the sum of both separate enrichments.

The enumeration of CECs was done from 4 mL of blood using a flowcytometric assay with CD34+/DNA+/CD146+/CD45- as CEC phenotype, as described in full detail before [14].

Immunohistochemistry on primary tumor tissue

Expression of EpCAM and MCAM was evaluated on diagnostic core needle biopsies of primary tumors taken before NAC. Slides were incubated with anti-MCAM (1:100, clone N1238; Abcam, Cambridge, UK) or anti-EpCAM (1:500, clone VU1D9; Cell Signaling Technologies, Beverly, MA), followed by the Envision System (DAKO, Glostrup, Denmark) and counterstaining with hematoxylin. Scoring of staining intensity (negative/weak/moderate/strong) and estimation of the percentage of positive tumor cells were done by a well-trained technician and pathologist.

Statistics

The primary objective of this study was to improve the CTC detection rate in patients with PBC using the EpCAM/MCAM enrichment approach. The overall relapse rate in breast cancer approximates 40% [15]. Since this study included patients with a poor prognosis, we deemed an improvement in CTC detection rate from 20 to 25% with EpCAM [2,3,6] to ≥40% using the extended approach clinically relevant. In patients with stage II/III PBC starting NAC, EpCAM-positive CTCs have before been detected in 46/213 (22%) [2] and 22/97 (23%) [5]. Using these numbers, with an alpha of 0.05 and power 0.80, we had to enroll 57 patients. Secondary objectives included (i) assessment of CEC counts at baseline and changes thereof during NAC, and (ii) associations of CTCs and CEC numbers and changes thereof with pCR to NAC.

Standard statistical tests were applied: binomial tests for percentages, Pearson's

Chi-square tests for categorical variables, and Student t or Mann-Whitney U tests for continuous variables. Changes in CEC counts were tested by the Wilcoxon signed rank test. Binomial tests were one-sided, all other tests were two-sided. We considered a $P<0.05$ as statistically significant. Analyses were performed using SPSS 21 (IBM, Armonk, NY, USA).

Results

From December 2010 until May 2012, 81 patients were recruited from the NEOZOTAC trial [16]. Only patients with both EpCAM and MCAM enumerations available were used in the analyses, leaving 68 evaluable patients at baseline – thus meeting our power calculations – and 39 patients after one NAC cycle (Figure 1). The characteristics of the 68 patients are summarized in Table 1 and specified per patient in Supplementary Table S1.

CTC counts

At baseline, 12 patients (18%) had ≥1 EpCAM-positive CTC(s) (median 1, range 1 – 4) and 8 (12%) had ≥1 MCAM-positive CTC(s) (median 1, range 1 – 5). Five patients (7%) had MCAM-positive CTCs only. The CTC detection rate increased from 18% with EpCAM alone to 25% when considering all EpCAM-positive and/or MCAM-positive CTCs (P=0.08). The comparison between EpCAM-positive and MCAM-positive CTCs is shown in Table 2A.

After the first NAC cycle, we detected EpCAM-positive CTCs in 6 patients (15%; median 1, range 1 – 7) and MCAM-positive CTCs in 6 patients (15%; median 1, range 1 – 4). Only one patient had both five EpCAM-positive CTCs and one MCAM-positive CTC. The CTC detection rate at this time point significantly increased from 16% with EpCAM only, to 30% using EpCAM/MCAM combined (P=0.02; Table 2B).

Comparing CTC counts at baseline and after the first cycle, 5 patients (13%) switched from CTC-negative to positive when considering EpCAM-positive CTCs. Three of these patients did not have any MCAM-positive CTCs at both time points, whereas 2 had one MCAM-positive CTC after the first NAC cycle, of whom 1 had no MCAM-positive CTCs at baseline. In 5 other patients we detected MCAM-positive CTCs after the first NAC cycle, whereas

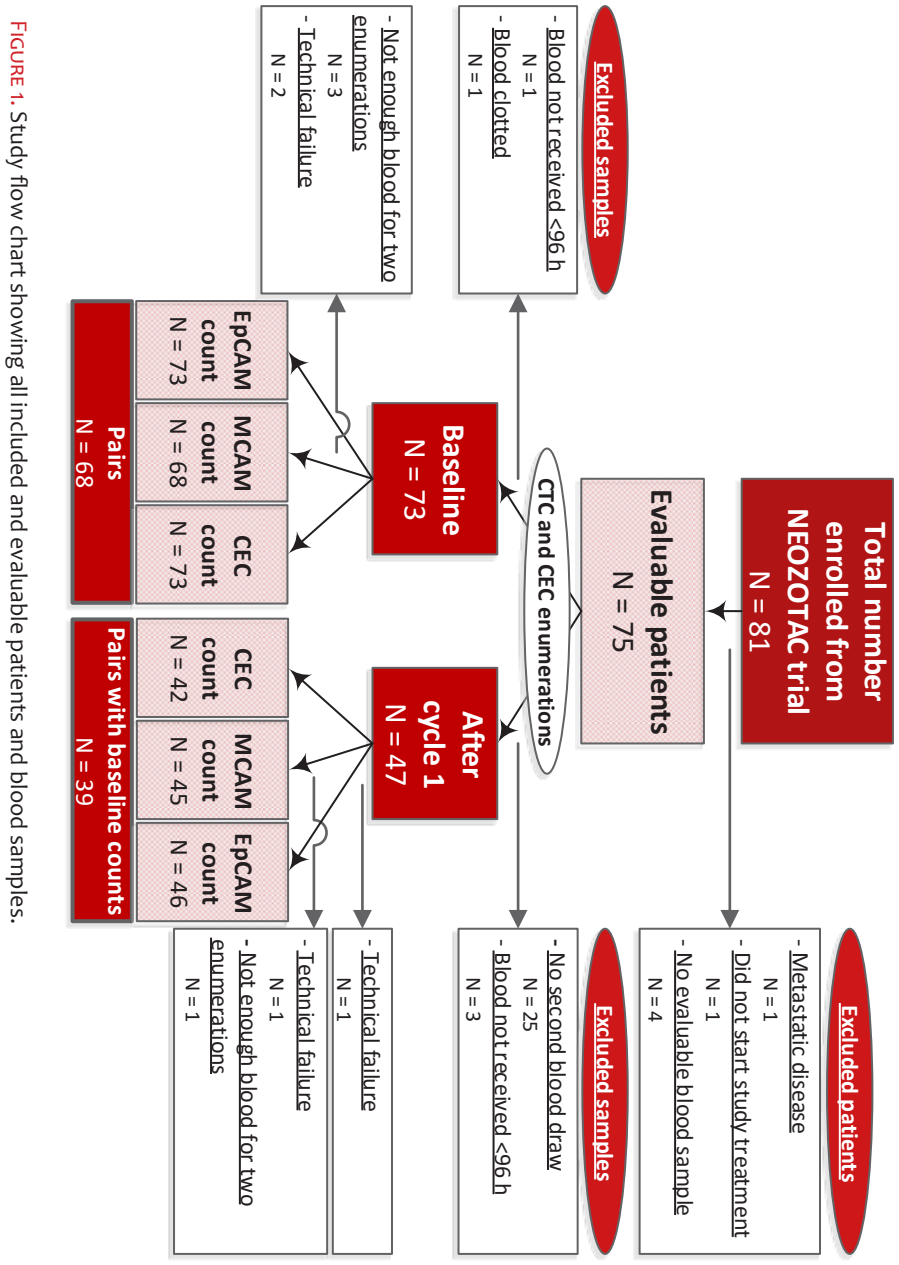

FIGURE 1. Study flow chart showing all included and evaluable patients and blood samples.

TABLE 1. Patient characteristics and comparison of the characteristics and outcome to NAC between patients with and without EpCAM-positive CTC(s) and patients with and without MCAM-positive CTC(s).

		EpCAM-positive CTCs at baseline			MCAM-positive CTCs at baseline			EpCAM and/or MCAM-positive CTCs at baseline		
Clinicopathological variable at diagnosis	All patients	No CTCs	≥1 CTC	*P* value	No CTCs	≥1 CTC	*P* value	No CTCs	≥1 CTC	*P* value
N	68	56	12		60	8		51	17	
Age at diagnosis (years ± sd)	51 ± 8	51 ± 8	50 ± 7	0.54	51 ± 8	50 ± 8	0.59	52 ± 8	50 ± 7	0.55
Clinical tumor classification before NAC										
cT2	44 (65%)	36 (64%)	8 (67%)	0.88	40 (67%)	4 (50%)	0.35	35 (69%)	9 (53%)	0.24
cT3 or cT4	24 (35%)	20 (36%)	4 (33%)		20 (33%)	4 (50%)		16 (31%)	8 (47%)	
Clinical lymph node classification before NAC										
cN0	39 (57%)	33 (59%)	6 (50%)	0.57	35 (58%)	4 (50%)	0.65	31 (61%)	8 (47%)	0.32
cN+	29 (43%)	23 (41%)	6 (50%)		25 (42%)	4 (50%)		20 (39%)	9 (53%)	
Histological subtype										
Ductal	37 (54%)	28 (50%)	9 (75%)	0.57	30 (50%)	7 (88%)	0.38	24 (47%)	13 (76%)	0.26
Lobular	13 (19%)	12 (21%)	1 (8%)		13 (22%)	0		12 (23%)	1 (6%)	
Other	7 (10%)	6 (11%)	1 (8%)		6 (10%)	1 (12%)		5 (10%)	2 (12%)	
Unknown*	11 (16%)	10 (18%)	1 (8%)		11 (18%)	0		10 (20%)	1 (6%)	
Hormone receptor expression										
Estrogen receptor										
Positive	57 (84%)	46 (82%)	11 (92%)	0.42	50 (83%)	7 (88%)	0.76	42 (82%)	15 (88%)	0.57
Progesterone receptor										
Positive	45 (66%)	36 (64%)	9 (75%)	0.48	38 (63%)	7 (88%)	0.18	32 (63%)	13 (77%)	0.30
Menopausal status										
Pre/perimenopausal	35 (52%)	29 (52%)	6 (50%)	0.88	31 (52%)	4 (50%)	0.93	27 (53%)	8 (47%)	0.75
Postmenopausal	32 (47%)	26 (46%)	6 (50%)		28 (47%)	4 (50%)		23 (45%)	9 (53%)	
Unknown	1 (1%)	1 (2%)	0		1 (2%)	0		1 (2%)	0	
Treatment received										
TAC	33 (49%)	27 (48%)	6 (50%)	0.91	30 (50%)	3 (38%)	0.51	25 (49%)	8 (47%)	0.89
TAC + ZA	35 (51%)	29 (52%)	6 (50%)		30 (50%)	5 (62%)		26 (51%)	9 (53%)	
Pathological complete response to NAC										
Yes	14 (21%)	12 (21%)	2 (17%)	0.71	14 (23%)	0	0.13	12 (24%)	2 (12%)	0.30
No	54 (79%)	44 (79%)	10 (83%)		46 (77%)	8 (100%)		39 (76%)	15 (88%)	

* Not enough tumor cells left after neoadjuvant chemotherapy for reliable histological subtyping.
Reported *P* values are from Student t tests for age and Pearson $\chi 2$ for all other categorical variables.

A. BASELINE

MCAM \ EpCAM	Negative	Positive	
Negative	51	9	60
Positive	5	3	8
	56	12	68

B. AFTER CYCLE 1

MCAM \ EpCAM	Negative	Positive	
Negative	31	6	37
Positive	6	1	7
	37	7	44

TABLE 2. Observed CTC counts after EpCAM versus MCAM enrichment in patients with both enumerations available at baseline (A.), and after the first cycle of NAC (B.). Both enrichments were done from 7.5 mL of blood in separate runs and compared afterwards. A positive CTC count means ≥1 CTC/7.5 mL.

there were none detectable at baseline. None of these patients had EpCAM-positive CTCs at baseline or after the first cycle. One patient (3%) had MCAM-positive CTCs at both time points and also turned positive for EpCAM-positive CTCs during NAC. Figure 2 (left three bars) shows the observed directions of changes in CTC counts during NAC.

Endothelial cell counts

At baseline and after the first cycle, CECs were enumerated in 68 and 42 patients, respectively (Figure 1). Median CEC counts were 44.5/4 mL blood (range 3-1,475) at baseline and 144.5/4 mL blood (range 9-807) after the first cycle. In the 42 patients with CEC counts at both time points available we observed a significant median increase during the first NAC cycle from 31.5 to 144.5 CECs ($P<0.001$; Figure 3). In 10 patients (24%), CECs decreased during treatment.

Associations with clinical parameters

We found no associations between the presence of EpCAM-positive and/or MCAM-positive CTC(s) at baseline and clinical characteristics (Table 1). Fourteen of the 68 patients (21%) achieved a pCR to NAC. The presence of EpCAM-positive CTC(s) at baseline was not correlated with pCR. Interestingly, none of the 8 patients with ≥1 MCAM-positive CTC(s) at baseline achieved pCR compared to 14 of the 60 patients (23%) without MCAM-positive CTCs ($P=0.13$). Changes of either EpCAM-positive or MCAM-positive CTCs during NAC were not associated with pCR (Figure 2, middle and right bars).

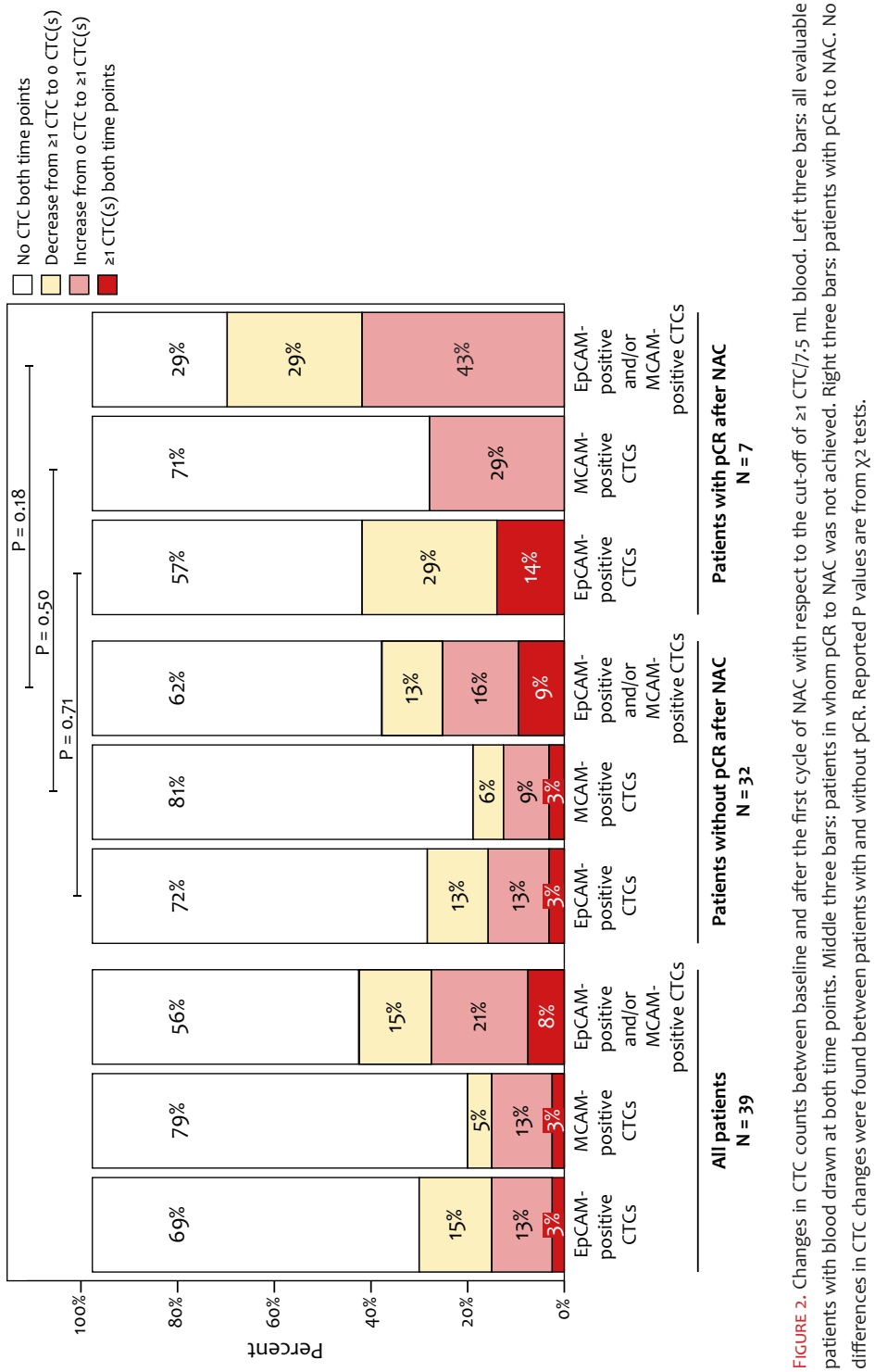

FIGURE 2. Changes in CTC counts between baseline and after the first cycle of NAC with respect to the cut-off of ≥1 CTC/7.5 mL blood. Left three bars: all evaluable patients with blood drawn at both time points. Middle three bars: patients in whom pCR to NAC was not achieved. Right three bars: patients with pCR to NAC. No differences in CTC changes were found between patients with and without pCR. Reported P values are from χ^2 tests.

Median CEC counts at baseline were 61.5/4 mL in the 14 patients with pCR compared with 40.5 in the 54 patients without pCR (*P*=0.37). In the 42 patients with both CEC counts available, comparable median increases were observed between patients with and without pCR to NAC (Figure 3). The pCR rate in patients with decreasing CEC counts was 2/10 (20%), which was not different from the 7 of the 32 patients (22%; *P*=0.90) with pCR and increasing CEC counts.

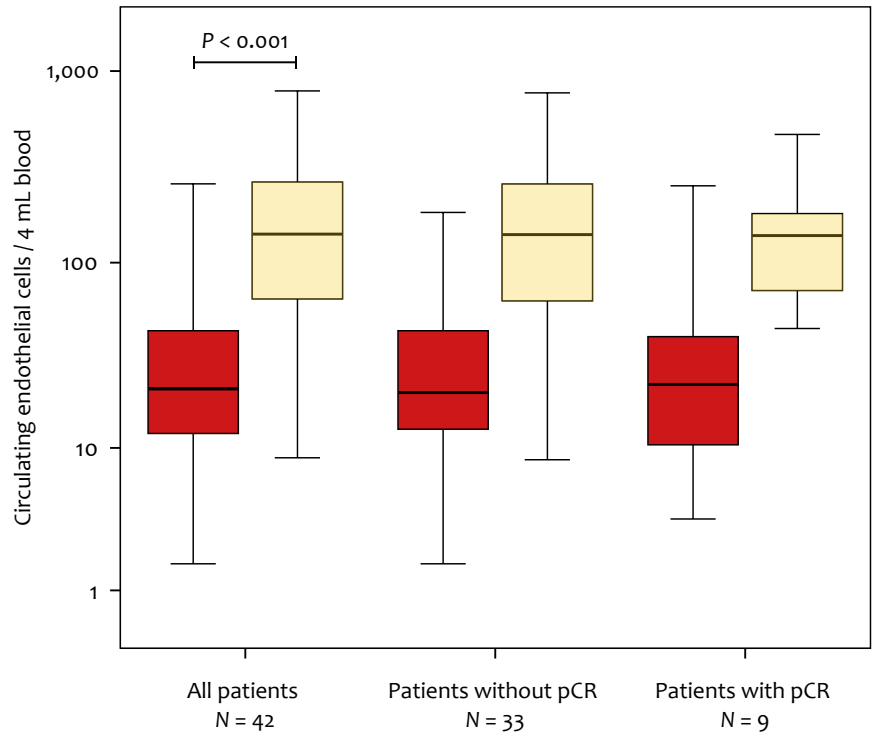

FIGURE 3. Box-and-whisker plots showing observed CEC counts from 4 mL blood at baseline (dark red boxes) and after the first cycle of NAC (yellow boxes) in all evaluable patients with blood drawn at both time points (*N* = 42; left), patients who did not achieve pCR (middle) and those who did achieve pCR (right). A significant median increase in CECs during NAC was found, which was not different for patients with or without pCR. Boxes show the medians (middle line) and interquartile ranges (IQR), whiskers extend from the median + 1.5 x IQR to median – 1.5 x IQR. The reported *P* value is from a Wilcoxon signed ranks test.

Expression of EpCAM and MCAM in primary tumors

Core needle biopsies taken before NAC were collected from 65 patients. In 5 patients, no invasive tumor or too few tumor cells were present for reliable evaluation, leaving 60 tumors for the evaluation of EpCAM expression. All tumors were positive for EpCAM, but seven tumors showed an EpCAM-negative focus and six had an EpCAM-weak focus. Expression of MCAM could be assessed in 59 tumors and was found positive in 11 (19%; Supplementary figure S1, Supplementary Table S1). The expression of EpCAM/MCAM in primary tumors was not correlated to the presence of MCAM-positive CTCs at baseline. No MCAM-positive CTCs were detected in patients with MCAM-positive tumors. We detected MCAM-positive CTCs in 14% of the patients with an EpCAM-negative focus in the primary tumor compared with 33% of the patients with an EpCAM-weak focus and 6% of the patients with homogeneously EpCAM-positive tumors.

Discussion

In this study, we investigated MCAM as additional CellSearch enrichment marker next to EpCAM to improve the CTC capture rate in stage II/III breast cancer. At baseline, the CTC detection rate increased from 18% using EpCAM only to 25% using both MCAM and EpCAM. After one NAC cycle we observed a significant increase from 16% to 30%. Nevertheless, the primary goal to improve the detection rate to ≥40%, at beforehand defined as clinically relevant, was not met.

Neither the presence of EpCAM-positive or MCAM-positive CTCs at baseline, nor changes of CTCs after the first NAC cycle correlated with clinicopathological parameters. Interestingly, none of the patients with MCAM-positive CTCs at baseline achieved pCR compared with 23% of the patients without MCAM-positive CTCs. Although not statistically significant, this difference may point to a prognostic unfavorable value of MCAM-positive CTCs and deserves further study. The pCR rate between patients with and without EpCAM-positive CTCs was similar. Baseline CEC counts and changes of either CECs or CTCs during NAC were not associated with pCR in our patient group. Associations with clinical outcome in terms of DFS and OS will have to be awaited.

Three other studies investigated the predictive and/or prognostic values of CTCs in the neoadjuvant setting (Table 3; refs. [2,5-7,17]). The 11 to 23% of the patients found CTC-positive by EpCAM enrichment in these trials compares well with the 18% we found using the EpCAM enrichment only. Also in agreement with our findings, neither the presence of CTCs before or after NAC, nor changes during treatment correlated with pCR [2,5,17]. Importantly, in the REMAGUS02 trial, pCR was no prognostic factor for distant metastasis-free survival (DMFS) and OS, whereas the baseline CTC count was [6,7]. The presence of CTCs thus might outperform pCR as prognostic factor in patients treated with NAC, possibly as indicator of the presence of micrometastases.

Increasing the CTC capture rate from peripheral blood will probably improve the prognostic and predictive value of CTC enumeration. Because MCAM is an EMT-inducer [18,19], it might be a valuable enrichment marker for mesenchymal CTCs. Epithelial and mesenchymal CTCs were found to co-occur in patients with MBC, but mesenchymal cells

Reference	Trial	Detection platform	Blood volume (mL)	N	CTC positive patients		Correlation with pCR	DMFS	OS
					pre NAC	post NAC			
Pierga *et al.* (2008) [5]	REMAGUS02	CellSearch	7.5	115	23%	17%	No	HR 5.0 (95% CI 1.4-17; *P*=0.01) 36 mo FU	HR 9.0 (95% CI 1.8-45; *P*=0.007) 36 mo FU
Bidard *et al.* (2009, 2013) [6,7]								- HR 2.4 (95% CI 0.9-6, *P*=0.06) 70 mo FU	- HR 3.0 (95 CI 1.0 - 9.5; *P*=0.05) 70 mo FU
Riethdorf *et al.* (2010) [2]	GeparQuattro	CellSearch	7.5	287	22%	11%	No	NR	NR
Azim *et al.* (2013) [17]	NeoALLTO	CellSearch after Ficoll density gradient separation	22.5	51	11%	13%	No	NR	NR

Table 3. Overview of relevant literature concerning the prognostic value of CTCs in patients with PBC treated with NAC.

showed to be better capable in predicting treatment failure [10]. Previously, we showed that the CellSearch System misses EpCAM-negative breast cancer cell lines with EMT features and that recovery of these cell lines improves using MCAM, which is frequently expressed on these cell lines [9,12]. We investigated the dual EpCAM/MCAM enrichment approach in patients with MBC and detected MCAM-positive CTCs in 9 of 20 patients (45%) [9]. Although associations with clinical outcome were not investigated, we hypothesized that MCAM-positive CTCs represent the mesenchymal, more aggressive subtype of CTCs. An upregulation of EMT-related transcription factors in CTCs during NAC has also been reported, possibly as a survival mechanism for CTCs during chemotherapy [20]. More insight into the process of EMT and the phenotype of mesenchymal CTCs will be required to investigate the clinical relevance of mesenchymal CTCs. Besides a loss of EpCAM, we found a downregulation of cytokeratins. Instead we found CD49f to be upregulated. Combining cytokeratin staining with CD49f in the CellSearch System resulted in improved recovery of cell lines with EMT features [21]. The value of CD49f on the recovery of MCAM-positive CTCs and the clinical value thereof will be subject in a future study.

Little is known about the prognostic value of CECs in breast cancer. Research in this field is greatly hampered by the lack of consensus on CEC phenotype. Consequently, different CEC definitions are handled and observed CEC counts using the different techniques are a 1,000-fold apart. Nonvalidated methods also showed to count macroparticles and large platelets as CECs, leading to incomparable results [13,22]. Technical obstacles have to be taken before concluding on the clinical value of CEC counts. Using a thoroughly validated flowcytometric method to measure CECs in 4 mL of peripheral blood [15], we found increasing CEC numbers during NAC, but no associations with pCR to NAC. The increase in CECs probably represent vascular damage due to NAC [13]. Whether this is associated with long-term vascular complications warrants additional studies.

In conclusion, using MCAM as additional enrichment marker next to EpCAM in the CellSearch System might improve the detection of CTCs in stage II/III breast cancer. Whether the detection of MCAM-positive CTCs and changes thereof during treatment of localized or metastatic breast cancer are of clinical relevance in terms of clinical outcome deserves further investigation.

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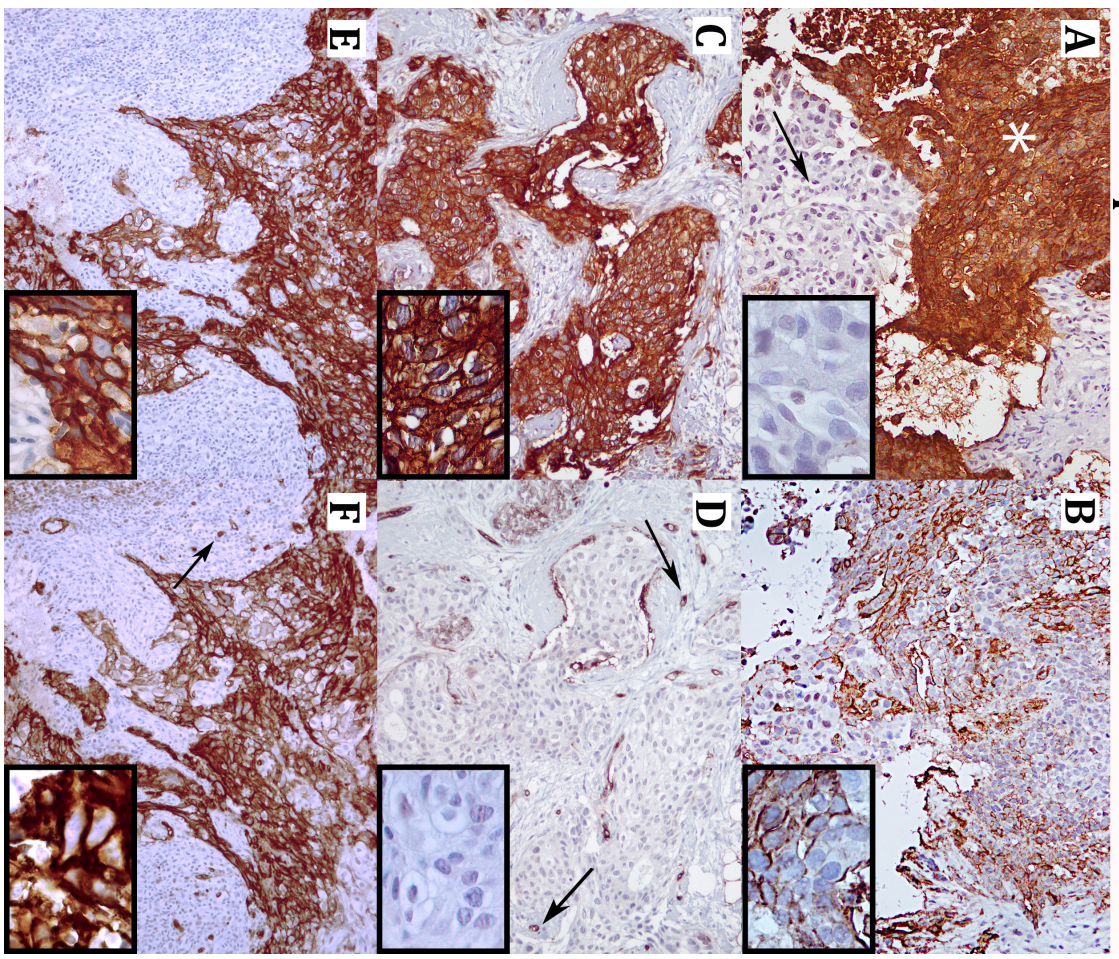
SUPPLEMENTARY TABLE S1. Clinicopathological characteristics, CTC, and CEC counts specified per patient.

Pt	Age	Meno-pausal status	Allocated treat-ment	cT	cN	ER	PR	ypT	ypN	pCR	Ep-CAM+ CTCs	MCAM+ CTCs	CECs	Ep-CAM+ CTCs	MCAM+ CTCs	CECs	EpCAM intensity	Est % Ep-CAM-neg / weak	MCAM intensity	Est % MCAM-pos
1	43	pre/peri	TAC + ZA	2	0	+	+	1	1	-	1	0	17	1	0	298	Strong		Negative	
2	55	post	TAC + ZA	3/4	+	+	-	3	3	-	0	0	13	0	0	78	Strong		Strong	70%
3	41	pre/peri	TAC + ZA	2	0	+	+	1	1	-	0	0	27	0	0	9	Moderate to strong		Negative	
4	62	post	TAC + ZA	3/4	+	-	-	0	0	+	0	0	61	0	1	73	Moderate to strong		Negative	
5	46	pre/peri	TAC	2	+	+	-	0	0	+	0	0	1475				Strong		Strong	70%
6	59	post	TAC + ZA	3/4	0	+	+	1	0	-	0	1	38	5	1	106	Moderate to strong		Negative	
7	54	post	TAC	3/4	0	+	+	2	2	-	0	0	23	0	0	155	Moderate to strong		Negative	
8	55	post	TAC + ZA	2	+	+	-	3	1 (mi)	-	1	0	218				Strong		Negative	
9	39	pre/peri	TAC	3/4	+	+	+	2	1	-	1	0	4	0	0	23				
10	66	post	TAC + ZA	2	0	+	-	2	0	-	0	0	784				Moderate to strong		Negative	
11	51	pre/peri	TAC	3/4	+	+	-	0	0	+	1	0	49	0	0	419	Strong		Negative	
12	57	post	TAC + ZA	2	0	+	+	2	1	-	4	5	890				No invasive tumor		Not enough tumor cells	
13	55	post	TAC	3/4	+	+	+	2	1	-	0	1	128				Heteroge-neous	5%	Negative	
14	49	pre/peri	TAC	2	0	+	+	2	1	-	0	0	33	0	1	32	Strong		Moderate	10%
15	53		TAC	2	+	+	+	1	1	-	0	0	18	0	0	290	Strong		Negative	
16	52	pre/peri	TAC	2	+	+	-	0	1	+	0	0	6			479	Moderate		Moderate	60%
17	49	pre/peri	TAC + ZA	2	0	+	+	1	0	-	0	0	8	0	0	54	Strong		Negative	
18	47	pre/peri	TAC + ZA	3/4	0	+	+	3	3	-	0	0	60	0	0	23	Moderate to strong		Negative	
19	66	post	TAC	2	+	-	-	2	1	-	0	0	127	0	0	12	Heteroge-neous	tumor 2: 100%	Weak to moderate	50%
20	50	post	TAC	2	0	+	+	1	1	-	0	0	267	0	0	66	Not enough tumor cells		Negative	
21	49	pre/peri	TAC + ZA	3/4	0	+	+	2	0 (i+)	-	0	0	26				Moderate		Negative	
22	51	pre/peri	TAC	3/4	0	+	+	2	0	-	0	0	136	0	0	129	Moderate to strong		Negative	
23	51	post	TAC + ZA	3/4	+	+	+	2	2	-	0	0	22	0	1	270	Strong		Negative	
24	53	pre/peri	TAC	2	0	+	+	0	0	+	0	0	62				Strong		Negative	
25	47	pre/peri	TAC	3/4	+	-	-	1	1	-	0	1	23							
26	40	pre/peri	TAC + ZA	2	0	+	+	1	0		0	0	139	0	0	473	Moderate to strong		Negative	
27	63	post	TAC + ZA	2	+	+	+	1	1 (mi)	-	0	0	81				Heteroge-neous	10%	Negative	
28	56	post	TAC	2	0	+	-	2	0	-	0	0	114				Moderate to strong		Negative	
29	65	post	TAC	2	+	+	+	1	0	-	0	0	17				Not enough tumor cells		Not enough tumor cells	
30	55	post	TAC + ZA	3/4	+	+	+	1	2	-	0	1	19	1	0	326	Strong		Negative	
31	58	post	TAC	3/4	0	-	-	2	0	-	1	0	43				Heteroge-neous	tumor 2: 100%	Moderate to strong	50%
32	58	post	TAC	2	0	+	+	1	0	+	1	0	16	0	0	99	Strong		Negative	
33	45	pre/peri	TAC + ZA	3/4	0	+	+	2	0	-	0	0	234				Strong		Negative	
34	55	pre/peri	TAC + ZA	3/4	0	+	+	2	0	-	0	0	25				Moderate		Negative	
35	57	post	TAC	2	0	-	-	2	0	-	0	0	24	0	0	172	Strong		Not enough tumor cells	
36	43	post	TAC	2	0	+	+	1	0	-	0	0	532				Strong		Weak	<5%
37	36	pre/peri	TAC + ZA	2	0	+	+	1	0	-	0	0	15				Strong		Negative	
38	43	pre/peri	TAC + ZA	2	+	+	+	1	1	-	1	1	25			147	Heteroge-neous	5%	Negative	
39	48	post	TAC + ZA	2	0	+	+	2	0	-	1	0	30	0	0	807	Strong		Negative	
40	56	post	TAC	3/4	+	+	+	2	0	-	1	0	295				Strong		Not enough tumor cells	
41	45	pre/peri	TAC	2	+	+	+	2	1	-	0	0	6				Moderate to strong		Negative	
42	48	post	TAC + ZA	2	+	+	-	2	2	-	0	0	13	0	0	109	Strong		Negative	
43	62	post	TAC + ZA	2	0	+	-	1	0	-	0	0	93				Heteroge-neous	60%	Negative	
44	47	pre/peri	TAC	2	0	-	+	0	0	+	0	0	187				Strong		Moderate	90%
45	45	pre/peri	TAC	2	+	+	-	2	0	-	0	0	59	0	0	269	Strong		Negative	
46	61	post	TAC	3/4	0	+	-	1	0		0	0	46				Strong		Negative	

Continued on next page

SUPPLEMENTARY TABLE S1 (CONTINUED).

Pt	Age	Meno-pausal status	Allocated treat-ment	cT	cN	ER	PR	ypT	ypN	pCR	Ep-CAM+ CTCs	MCAM+ CTCs	CECs	Ep-CAM+ CTCs	MCAM+ CTCs	CECs	EpCAM intensity	Est % Ep-CAM-neg / weak	MCAM intensity	Est % MCAM-pos
47	61	post	TAC + ZA	2	0	+	+	1	1 (mi)	-	0	0	3	0	0	134	Heteroge-neous	5%	Negative	
48	48	pre/peri	TAC + ZA	3/4	0	+	+	2	1	-	0	0	43	0	1	112	Strong		Negative	
49	50	post	TAC + ZA	3/4	+	+	+	2	2	-	0	0	48	0	0	215	Moderate to strong		Negative	
50	43	pre/peri	TAC	3/4	+	+	+	1	3	-	0	0	88	1	0	49	Strong		Negative	
51	49	pre/peri	TAC + ZA	2	+	-	-	1	0	-	0	0	116	0	0	618	Heteroge-neous	<1% neg; 5% weak	Moderate	30%
52	51	pre/peri	TAC	2	0	+	+	1	0	-	0	0	23	0	0	306	Heteroge-neous	5% neg; 10% weak	Negative	
53	41	pre/peri	TAC	2	0	-	-	0	0	+	0	0	190			185	Strong		Moderate	30%
54	38	pre/peri	TAC + ZA	2	0	+	+	1	1	-	0	1	4				Strong		Negative	
55	40	pre/peri	TAC	2	+	+	+	1	1	-	0	0	147	0	0	166				
56	63	post	TAC + ZA	2	+	+	+			+	0	0	96				Strong		Negative	
57	46	pre/peri	TAC + ZA	3/4	0	+	+	1	0	-	0	0	64	0	0	210	Moderate to strong		Negative	
58	58	post	TAC + ZA	2	0	+	+	1	1 (mi)	-	0	0	168	1	0	343	Heteroge-neous	10%	Negative	
59	46	pre/peri	TAC + ZA	2	0	+	-	0	0	+	0	0	13	0	0	185	Heteroge-neous	10%	Negative	
60	64	post	TAC	2	0	+	-	0	0	+	0	0	77				Heteroge-neous	10% neg; 30% weak	Negative	
61	47	pre/peri	TAC + ZA	2	+	+	+	2	2	-	2	0	50	0	0	83	Moderate to strong		Negative	
62	34	pre/peri	TAC + ZA	3/4	0	+	-	2	0	+	0	0	34	0	4	142	Not enough tumor cells		Not enough tumor cells	
63	58	post	TAC + ZA	2	+	-	-	1	2	-	0	0	29				Strong		Negative	
64	44	pre/peri	TAC	2	0	+	+	1	0	-	1	3	17	0	0	256	Heteroge-neous	10%	Negative	
65	46	pre/peri	TAC	2	+	-	+	0	x	+	0	0	25	0	0	62	Not enough tumor cells		Moderate	40%
66	54	pre/peri	TAC + ZA	2	0	+	+	1	0	+	0	0	376	7	0	46	Strong		Negative	
67	60	post	TAC	2	0	-	-	2	0	-	0	0	52				Strong		Not enough tumor cells	
68	54	post	TAC	3/4	+	+	+	2	2	-	0	0	29	0	0	14	Strong		Negative	



SUPPLEMENTARY FIGURE S1. Examples of EpCAM and MCAM staining in primary tumor tissues from core needle biopsies taken before start of NAC at 200x and 600x (inserts) magnification. A AND B. Tumor with EpCAM-positive (asterisk) and EpCAM-negative (arrow) foci. Both foci show moderate positivity for MCAM as can be deduced from the brown membrane staining. C AND D. Strongly EpCAM-positive tumor surrounded by EpCAM-negative stroma. This tumor shows no staining for MCAM. As a positive control, staining can be found in MCAM-positive endothelium of small vasculature in the surrounding stroma (arrows). E AND F. Strongly EpCAM-positive tumor, also positive for MCAM. The surrounding infiltrate is negative for both EpCAM and MCAM. Vasculature surrounded by infiltrate (arrow as example) is positive for MCAM.

3

TOWARDS A PERSONALIZED BREAST CANCER TREATMENT APPROACH GUIDED BY CIRCULATING TUMOR CELL CHARACTERISTICS

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ABSTRACT

Circulating tumor cells (CTCs) can be found in the peripheral blood of patients with different solid tumors, including breast cancer. A CTC count is a strong established prognostic factor in various stages in several tumor types. Besides that, characterization of CTCs is expected to become an invaluable tool to predict treatment response and personalize cancer treatments. Likely, CTCs are shed by different tumor lesions and may therefore provide a comprehensive view of tumor characteristics at a certain time-point, including inter- and intratumoral heterogeneity. Obtained through a simple venipuncture, CTCs could this way serve as a "liquid biopsy". However, isolation and subsequent characterization of CTCs is technically extremely challenging, mainly due to the small number of cells amidst a large majority of leukocytes. A wide range of assays have been developed, but only the CellSearch System (Veridex, Raritan, NJ, USA) has obtained FDA clearance for CTC enumeration so far. For characterization purposes, no assay has been validated at all. Nevertheless, the first studies investigating the clinical value of CTC characteristics have been performed. Here, we review these clinical studies. The various techniques used to interrogate CTCs are briefly described and an overview of the clinical relevance of CTC characterization in breast cancer is given.

Introduction

In today's clinical oncology the dogma is shifting to personalizing treatments. Here, treatments are deliberately chosen based on tumor cell characteristics, thereby selecting agents that specifically target factors essential in the biology of the tumor. Successful examples of this approach include the use of imatinib in gastro-intestinal stromal tumors (GIST), vemurafenib in BRAFV600-mutated melanoma, and crizotinib in non-small cell lung cancer with EML4-ALK translocations. However, eventually resistance to targeted agents emerges due to the genomic instability of cancer and inter- and intratumoral heterogeneity. This eventually leads to survival and growth of resistant tumor cell clones under the pressure of treatment [1,2]. Consequently, differences arise in the molecular make-up of a tumor over time. Treatment decisions thus must be made on the basis of tumor cell characteristics, not only just prior to treatment initiation, but also repetitively during treatment to adapt systemic therapy when necessary.

In metastatic breast cancer (MBC), two proteins are essential for treatment decision making: the human epidermal growth factor receptor-2 (HER2) and the estrogen receptor (ER). Presence of these predictive factors is usually assessed on primary tumor tissue in standard daily practice. However, it is increasingly recognized that primary tumors and the different metastatic lesions can greatly differ in molecular characteristics, including differences in HER2 and ER expression [1,3]. In the metastatic setting such predictive factors thus should be determined in metastatic tissue rather than falling back on the primary tumor. Since taking biopsies from metastases is often painful and frequently technically not possible, circulating tumor cells (CTCs) from peripheral blood form an attractive alternative for the assessment of predictive factors. A CTC count is a strong prognostic factor at all time-points during treatment in MBC [4] and characterization of CTCs could even be of greater importance. As CTCs may be released by several separate tumor lesions, they possibly provide a comprehensive view of tumor characteristics, including inter- and intratumoral heterogeneity. Furthermore, CTCs are obtained through a simple venipuncture, which enables repetitive and real-time monitoring of a tumor's characteristics, thereby serving as a "liquid biopsy".

Isolation of CTCs from peripheral blood is still technically extremely challenging (reviewed in [5]). With respect to CTC enumeration, the CellSearch System (Veridex LLC, Raritan, NJ) is the only Food and Drug Administration (FDA)-cleared technique, whereas characterization assays have not yet been validated at all. But despite this lack of validated characterization assays, the first studies investigating the clinical value of CTC characterization have already been performed. In this review, advances in the field of CTC characterization in breast cancer are discussed. It is beyond the scope of this review to address all the available characterization techniques and the accompanying technical issues. Instead, we focus on the clinical relevance of CTC characterization in breast cancer and provide a short overview of the techniques used so far to investigate the clinical value of CTC characteristics.

CTC characterization methods

Characterization of CTCs is technically challenging for two main reasons: the low number of cells and the leukocyte background. Using the CellSearch System, CTCs are detectable in 70% of MBC patients, but only in 50% a number of ≥5 CTCs/7.5 mL blood is found [6]. In primary breast cancer (PBC), ≥1 CTC(s)/7.5 mL of blood can be found in only 24% of the patients [7]. Part of the explanation for the low numbers of detectable CTCs is that most isolation assays still rely on an epithelial cell adhesion molecule (EpCAM)-based enrichment step. However, EpCAM-negative CTCs exist, for example due to epithelial-to-mesenchymal-transition (EMT). In this process, CTCs lose their epithelial phenotype, including the expression of EpCAM and cytokeratins (CKs) [8,9]. These CTCs may thus be missed by currently available assays, which all are based on EpCAM-positivity of the tumor cells. Furthermore, the enriched CTCs that are isolated are outnumbered by a 1,000 - 10,000-fold excess of "contaminating" leukocytes. Highly sensitive assays are thus needed to characterize small numbers of CTCs in enriched samples, and the presence of leukocytes may yield false-positive findings in such assays. Over the years, many new methods have been developed to characterize CTCs on the level of protein expression, mRNA expression and chromosomal abnormalities. However, none of these assays have yet been validated and they all have their advantages and disadvantages

(Table 1). Below the techniques are described that have been used to investigate the clinical value of CTCs. It is beyond the scope of this review to provide a full overview of all possible characterization techniques.

TABLE 1. Summary of currently used CTC characterization methods.

Method	Advantages	Disadvantages
Protein expression		
Immunofluorescence	CTC count can be obtained in the same assay	No cut-off at the sample level available due to heterogeneity between CTCs in one sample
	Heterogeneity between cells can be assessed	Limited possibilities for multiplexing
	Cut-off defined at the cell-level by comparison with cell line cells for HER2	
	Interactions between proteins can be studied	
mRNA expression		
Polymerase chain reaction (PCR)	Multiplexing possible up to a large number of genes	No information on CTC cell count in a sample
	Only small reaction volumes required	Information on heterogeneity is lost
		Severely hampered by contaminating leukocytes
Chromosomal abnormalities		
Fluorescence in situ hybridization (FISH)	CTC count can be obtained in the same assay	Lower sensitivity for small genes due to the large size of FISH probes
	Heterogeneity between cells can be assessed	Knowledge needed of possibly altered genes for design of probes
		Limited possibilities for multiplexing

Protein expression

The most validated, and the only currently commercially available characterization assay, is immunofluorescence staining of HER2 using the CellSearch System, using the fourth spare filter on the CellTracks Analyzer digital microscope. Fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies directed against HER2 are added simultaneously with anti-CK, anti-CD45 and DAPI - used for the discrimination of CTCs from leukocytes- and CTC enumeration and characterization for HER2 expression are simultaneously

carried out on the microscope. Using this assay, overexpression on the protein level has shown to correlate well with gene amplification when assessed in parallel by fluorescence in situ hybridization (FISH) on cytospun CTCs obtained from cell lines and patient samples [10-14]. The direct comparison of immunocytochemistry with FISH led to a 0-3 immunofluorescence intensity score, where 0 and 1+ stand for HER2-negative CTCs and 2+ and 3+ indicate HER2-positivity [13,14]. Recently, an automated approach has been proposed using a dynamic cut-off that differs per sample. For this, an algorithm is applied that measures the intensity of HER2 immunofluorescence staining of both the CTCs and the leukocytes in a sample. Only CTCs with an HER2 intensity staining greater than the 91st percentile of the intensity staining of all leukocytes in that same sample are considered HER2-positive [15]. However, heterogeneity in HER2 expression exists between the different CTCs within one sample, likewise tumor cells within primary tumors and metastases.[1-3] This heterogeneity makes it difficult to assess HER2-positivity for the whole sample [10,13-18]. Different cut-offs for HER2-positivity have been proposed on the sample-level, including ≥1 CTC 2+ or 3+ [14,16], ≥50% or ≥75% of CTCs positive for HER2 in a sample[11,15] and a calculated score using immunofluorescence intensity and the percentage of positive cells [10]. Importantly, studies to compare and validate the proposed sample-level cut-offs with respect to clinical outcomes have not been carried out so far.

Next to HER2, other proteins can be stained using the CellSearch system, *e.g.* the human epidermal growth factor receptor-1 (EGFR/HER1).[19] A protocol to develop and optimize a user-defined assay has recently been published [20]. Other methods have also been used to stain CTCs by immunofluorescence, mostly on cytospins after density gradient centrifugation or immunomagnetical isolation [12,17,18,21-24].

Instead of measuring total protein levels, newly developed assays claim to only measure the activated, phosphorylated portion [25,26]. Only the Collaborative Enzyme Enhanced Reactive (CEER)-immunoassay has been applied on CTCs [26]. This assay was used to measure phosphorylated HER2 (pHER2) using two detection antibodies, one against HER2 and the other against the phosphorylation site. Both antibodies are labeled with enzymes that only create a fluorescent signal when in close proximity, thus only creating a signal when a phosphotyrosine molecule is bound to HER2. However, only a proof-of-principle study on CTCs has been performed thus far [26].

mRNA expression

Many studies characterized CTCs by looking at mRNA expression using reverse transcription polymerase chain reaction (RT-PCR) [16-18,21,24,27-42]. Most of them used the commercially available AdnaTest (AdnaGen AG, Langenhagen, Germany) [16,24,31,35-40], where HER2 mRNA is measured as one of the three tumor-associated transcripts for the detection of CTCs together with EpCAM and mucin 1 (MUC1). At the same time presence of HER2 transcripts can be used for characterization, qualifying a sample HER2-positive above the cut-off of >0.15 ng/µL. It is also possible to measure the expression of other genes, for example those coding for ER and phosphoinositide 3-kinase (PI3K) [31]. Gene-expression profiling of CTCs after CellSearch or other enrichment approaches is also possible. Our group has developed an assay to measure the expression of 96 genes in as little as 1 CTC after CellSearch processing [32,33]. Given the large surplus of leukocytes, which is still left after enrichment, we selected genes that are not or only at low levels expressed in leukocytes. Among the selected genes in our panel are the genes coding for ER, EGFR, HER2, HER3 and the fibroblast growth factor receptor-4 (FGFR4). Several other assays have been designed to measure mRNA levels, mostly using multiplex quantitative RT-PCR after immunomagnetical enrichment using anti-EpCAM-labeled capture beads [27-30,34] or a density-gradient separation step [13,17,18,21,40-42].

Chromosomal abnormalities

On the DNA level, FISH on CTCs can show chromosomal aberrations, such as gene amplifications and translocations. One of the first studies on CTCs used FISH to evaluate aneusomy and prove the malignant origin of CTCs [43]. Subsequently, other studies used FISH on CTCs, mainly to investigate amplifications of the *ERBB2* gene, coding for HER2 [10-14,17,23,24,40,41,44-47]. Most of these studies used cytospins after either enrichment

through CellSearch, immunomagnetical beads or density gradient isolation. However, since CTCs are lost during the spinning process, fixation and FISH inside the CellSearch cartridge might be preferred [10,48]. A protocol to fix cells and perform FISH inside a CellSearch cartridge has been published [48].

Optimal characterization assay?

Obtaining a comprehensive view of the characteristics of the few CTCs present in a sample is feasible. However, choices have to be made when it comes to the assays that can be applied, as only a limited amount of cells is available and not all assays work well together or successively. It is possible to combine immunofluorescence-based assays for detection of protein expression and FISH, but one has to consider that only a limited number of fluorophores can be used due to the spectral overlap. Using PCR for multiplexing is easier, but since a sample has to be lysed, other assays can no longer be applied. Besides that, PCR-based assays are severely hampered by the contribution of the large background of leukocytes present after any enrichment approach. With the current state of the art, the combination of an immunomagnetical enrichment followed by immunofluorescence and image cytometry seems to be the most informative assay for CTC characterization. The most complete picture is obtained of both the number of cells present in a sample and the characteristics of these cells, including intensity of staining and heterogeneity between different CTCs. However, in the near future single cell genomic profiling assays will become available [49]. These will probably further boost CTC characterization options as they are able to provide lots of information on for example mutational status, copy number variations and heterogeneity at once.

Clinical significance of CTC characterization

Human Epidermal Growth Factor Receptor-2 (HER2)

In approximately 10-15% of the primary breast tumors HER2 is overexpressed, thereby offering an important treatment target both in patients with PBC and MBC. For the latter, HER2 status is still assessed on primary tumor tissue, even though the primary tumor often has surgically been removed years before the diagnosis of metastatic disease, and in spite of the fact that studies have shown that significant discrepancies can exist between primary tumors and metastases [1-3]. Probably, CTC characteristics resemble the characteristics of the metastases better than the primary tumor does [12,50,51]. Significant differences exist in the expression of targetable receptors between the primary tumor and CTCs, among which HER2 [1-3], and therefore, the presence of HER2-positive CTCs could be a better indication for anti-HER2 treatments, irrespective of the HER2-status of the primary tumor.

Reported HER2-positivity rates in CTCs lie between 19-90%, an imprecise estimate due to differences in isolation and characterization methods and applied cut-offs (Table 2) [10-12,14-18,21,22,24,26,27,29,30,33,41,52-56]. The fact that positivity rates for HER2 on CTCs exceed 15% in all studies suggests discrepant expression of HER2 compared to the primary tumor in a proportion of patients. Indeed, such discrepancies have been reported. Meng *et al*. [12] selected 29 patients with HER2-negative PBC or MBC and detectable CTCs after an EpCAM-based immunomagnetical enrichment. Amplification of HER2 was assessed by FISH and actually found in 9/24 patients (38%), suggesting an acquisition during tumor progression. Ever since, discordances between primary tumors and CTCs have been reported by multiple groups using different assays and seem to occur both ways, *i.e.* HER2-positive primary tumors with HER2-negative CTCs, and HER2-negative primary tumors with HER2-positive CTCs (Table 2) [10-17,22,26,31,33,37,53,56,57].

In contrast to most other studies, Mayer *et al*. [45] found a high overall HER2 concordance rate between primary tumor and CTCs of 93%. Using 10 different capture markers, immunofluorescence staining for several CKs and FISH, they observed a proportion of

TABLE 2. Overview of reported HER2 expression on CTCs.

Reference	Enrichment method	Characterization method	PBC / MBC	Patients with detectable CTCs positive for HER2	Cut-off applied	Positive primary tumor with negative CTC	Negative primary tumor with positive CTC	Overall discrepancy rate
Meng *et al.* (2004) [12]	EpCAM-based immunomagnetical capture of CTCs	IF / FISH on slides	Both	9/24 (38%)	No cut-off applied	Only patients with negative tumor included	9/24 (38%)	NR
Wülfing *et al.* (2006) [56]	Density gradient centrifugation and immunomagnetical separation of CK-pos cells	IF on cytospins	Both	PBC: 17/35 (49%); MBC: 7/7 (100%)	No cut-off applied	PBC: 1/3 (33%); MBC: NR	PBC: 12/24 (50%); MBC: NR	PBC: 13/27 (48%); MBC: NR
Ignatiadis *et al.* (2008) [21]	Density gradient centrifugation	RT-PCR / IF on cytospins	PBC	50/175 (29%)	No cut-off applied	37/48 (77%)	38/124 (31%)	75/172 (44%)
Fehm *et al.* (2009) [35]	AdnaTest	RT-PCR	PBC	22/58 (38%)	Expression signal intensity ≥ 0,15 ng/uL	9/9 (100%)	22/49 (45%)	31/58 (53%)
Pestrin *et al.* (2009) [11]	CellSearch	IF / FISH on cytospin	Both	15/40 (38%)	Any staining in ≥50% of CTCs if total of ≥2 CTC	5/12 (42%)	8/28 (29%)	13/40 (33%)
Tewes *et al.* (2009) [31]	AdnaTest	RT-PCR	MBC	7/22 (32%)	Expression signal intensity ≥ 0,15 ng/uL	3/5 (60%)	5/17 (29%)	8/22 (36%)
Fehm et al. (2010) [16]	AdnaTest CellSearch	IF / RT-PCR	MBC	CellSearch: 50/122 (41%); AdnaTest: 42/90 (47%)	AdnaTest: expression signal intensity ≥ 0.15 ng/μL; CellSearch: ≥ 5 CTC and ≥ 1 CTC with score 3+ according to Riethdorf et al.	CellSearch: 13/31 (42%); AdnaTest: 13/22 (59%)	CellSearch: 25/76 (33%); AdnaTest : 28/57 (49%)	CellSearch: 38/107 (36%); AdnaTest: 41/79 (52%)
Kim et al. (2011) [26]	CellSearch	Collaborative Enzyme Enhanced Reactive (CEER)-immunoassay	Both	13/27 (48%)	Background mean from healthy controls plus 2.3 x SD	4/10 (40%)	7/17 (41%)	11/27 (41%)
Munzone *et al.* (2010) [53]	CellSearch	IF	MBC	19/57 (33%)	No cut-off applied	Before treatment: 2/15 (13%); During treatment: 3/16 (19%)	Before treatment: 6/42 (14%); During treatment: 8/45 (18%)	Before treatment: 8/57 (14%); During treatment: 11/61 (18%)
Punnoose *et al.* (2010) [10]	CellSearch	IF / FISH in cartridge	MBC	12/29 (41%)	H-score >200 (calculation based on the percentage of positive CTCs and the intensity of staining)	2/11 (18%)	3/18 (17%)	5/29 (17%)
Riethdorf *et al.* (2010) [14]	CellSearch	IF	PBC; locally advanced tumors	Before treatment: 8/37 (22%) After treatment: 6/21 (29%)	at least 1 CTC with a 2+ or 3+ staining, based on staining and amplifications of different cell lines	Before treatment: 5/11 (45%); After treatment: 6/10 (60%)	Before treatment: 7/26 (27%); After treatment: 3/11 (27%)	Before treatment: 12/26 (46%); After treatment: 9/21 (43%)
Gradilone *et al.* (2011) [27]	Anti-EpCAM-coated capture beads	RT-PCR	MBC	15/28 (54%)	No cut-off applied	4/13 (31%)	6/15 (40%)	10/28 (36%)
Ignatiadis *et al.* (2011) [13]	CellSearch	IF	Both	PBC: 11/18 (61%); MBC: 14/23 (61%)	Staining 2.5x higher than the sample background	PBC: 0/5 (0%); MBC: not reported	PBC: 5/12 (42%); MBC: 13/33 (39%)	PBC: 5/17 (29%); MBC: not reported
Kallergi *et al.* (2008) [22]	Density gradient centrifugation	IF on cytospins	Both	PBC: 8/16 (50%) MBC: 10/16 (63%)	No cut-off applied	PBC: 1/3 (33%) MBC: 1/5 (20%)	PBC: 6/12 (50%) MBC: 6/11 (55%)	PBC: 7/15 (47%) MBC: 7/16 (44%)

NR = not reported; all other abbbreviations have been listed on page 218-21.

Continued on next page

TABLE 2 (CONTINUED).

Reference	Enrichment method	Characterization method	PBC / MBC	Patients with detectable CTCs positive for HER2	Cut-off applied	Positive primary tumor with negative CTC	Negative primary tumor with positive CTC	Overall discrepancy rate
Sieuwerts *et al.* (2011) [33]	CellSearch	quantitative RT-PCR	MBC	17/35 (49%)	Higher expression than the median in 31 enriched healthy donor blood	2/10 (20%)	8/25 (32%)	10/35 (29%)
Somlo *et al.* (2011) [57]	Depletion of CD45-positive cells using beads	IF on slides	Both	PBC: 1/8 (13%); MBC: 5/18 (28%)	Intensity sample score ≥ 3 (calculation based on the percentage of positive CTCs and the intensity of staining)	PBC: 3/3 (100%); MBC: 3/5 (60%)	PBC: 1/5 (20%); MBC: 3/13 (23%)	PBC: 4/8 (50%); MBC: 6/18 (33%)
Andreopoulou *et al.* (2012) [37]	AdnaTest	RT-PCR	MBC	19/29 (66%)	Expression signal intensity ≥ 0,15 ng/uL	NR	11/19 (58%)	NR
Georgoulias *et al.* (2012) [18]	Density gradient centrifugation	RT-PCR IF on cytospins	PBC	51/57 (90%)	RT-PCR: no cut-off applied; IF: Any staining	Only patients with negative tumor included	51/57 (90%)	NR
Hayashi *et al.* (2012) [47]	CellSearch	IF / FISH on slides	MBC	Before treatment: 8/31 (26%); After 3-4 weeks during treatment: 7/21 (33%)	IF :strong staining comparable to SK-BR-3 cells; FISH: ratio HER2:CEP17 ≥ 2.0	4/9 (44%)	3/22 (14%)	7/31 (21%)
Mayer *et al.* (2011) [45]	OncoCEE micropost device	FISH	Both	NR	HER2:CEP 17 ratio ≥ 2.2	1/19 (2%)	2/24 (5%)	3/43 (7%)
Ligthart *et al.* (2012) [15]	CellSearch	IF	Both	22/90 (24%)	Dynamic cut-off based on background intensity staining of all leukocytes in a sample	PBC: 0/5 (0%) MBC: 5/28 (18%) (only patients with ≥5 CTCs included)	PBC: 0/8 (0%); MBC: 5/69 (7%) (only patients with ≥5 CTCs included)	PBC: 0/13 (0%); MBC: 10/90 (11%) (only patients with ≥5 CTCs included)

NR = not reported; all other abbbreviations have been listed on page 218-21.

cells that had the morphology of a CTC, but were negative for both CK and the pan-leukocyte marker CD45. These cells were considered CK-negative CTCs since they showed a similar HER2 amplification patterns compared to the CK-positive portion of CTCs. Loss of HER2 on CTCs could thus also be caused by assays relying on the expression of CK, and missing CK-negative CTCs.

Only a few studies investigated the clinical value of HER2 expression on CTCs. Several groups found that presence of HER2-positive CTCs, irrespective of the HER2-status of the primary tumor, is an adverse prognostic factor for disease-free survival (DFS) and OS in PBC [41,56] and MBC [47] (Table 3). Bozionellou *et al.* [40] selected 30 PBC/MBC patients that had just completed a treatment line and still had detectable CK-19-positive CTCs and/or bone marrow disseminated tumor cells (DTCs) as assessed by RT-PCR after density gradient centrifugation. Irrespective of the HER2-status of the primary tumor, they treated all patients with trastuzumab, a monoclonal antibody that targets HER2. While HER2 mRNA was detected in the enriched samples in 25/30 patients (83%) before start of trastuzumab, afterwards this was only in 33%. Trastuzumab thus possibly eliminated CTCs in 50% of the patients, although the significance with respect to clinical outcome parameters, such as progression or DFS, had not been investigated. Georgoulias *et al.* [18] continued on this concept and randomized 75 patients with HER2-negative PBC and persisting CK-19-positive CTCs after adjuvant treatment between six cycles of trastuzumab monotherapy (N=36) or observation (N=39). At the end of the trastuzumab treatment, 23/32 (72%) of patients with a successful CTC enumeration had turned CTC-negative compared to only 7/27 (26%) in the observational arm. A good quality cytospin for HER2 immunofluorescence was available of 57 patients and in 51 patients (90%) HER2-positive CTCs were detected. Patients treated with trastuzumab had a significantly better DFS, with 11% relapses compared to 38% in the observational group. This study suggests that trastuzumab has clinically relevant anti-tumor activity in patients with a HER2-negative primary tumor.

TABLE 3. Associations of HER2 overexpression on CTCs with clinical outcome.

Reference	Enrichment method	Characterization method	PBC/MBC	N	Time of blood draw	Median survival (months) HER2- versus HER2+ CTCs				HR (95% CI)			
						DFS / PFS	P value	OS	P value	DFS / PFS	P value	OS	P value
Wülfing *et al.* (2006) [56]	Density gradient centrifugation and immunomagnetical separation	IF	PBC	35	Before surgery and adjuvant treatment	124 versus 60	0.007	125 versus 89	0.02	-	-	-	-
Apostolaki *et al.* (2007) [41]	Density gradient centrifugation	RT-PCR	PBC	214	After completion of adjuvant chemotherapy	>120 versus 87	0.006	>120 versus 108	0.24	3.2 (1.8-5.9)	<0.001	2.1 (0.9-4.6)	0.08
Hayashi *et al.* (2012) [47]	CellSearch	IF FISH	MBC	52	At first follow-up visit	29 versus 5	0.001	12 versus 5	0.01	3.0	0.03	2.0	0.18
						HER2-positive CTCs at baseline were not associated with either PFS or OS							

In another prospective trial, Pestrin *et al.* [46] selected 96 patients with HER2-negative MBC and detectable CTCs by the CellSearch system. Only 7 patients had HER2-positive CTCs and were treated with single-agent lapatinib to evaluate efficacy of HER2 inhibition in this patient group; one patient showed disease stabilization and the rest progressed. Unfortunately, due to the small number of patients, no efficacy analysis could be done.

Early attempts have been made to measure pHER2 on CTCs, since this might be a more accurate predictor of sensitivity to anti-HER2 treatments than HER2 overexpression alone [58]. Kim *et al.* [26] used a CEER-immunoassay to measure HER2 and pHER2 on CellSearch isolated CTCs from 27 patients with PBC/MBC. In 7/7 patients with HER2-positive CTCs and a HER2-negative primary tumor, CTCs were positive for pHER2. On the other hand, in only 4/6 patients with HER2-positive CTCs and a HER2-positive primary tumor pHER2 was detected. Besides, in 3/17 (18%) of the patients with HER2-negative CTCs and a HER2-negative primary tumor, pHER2 was detected on CTCs. Interestingly, Kim's work shows that HER2 apparently can be activated despite the fact that it is not overexpressed and can be overexpressed but not activated.

In conclusion, discrepancies exist in the expression of HER2 on CTCs compared to the primary tumor and involve losses and gains of HER2 in similar probabilities. These discrepancies may partially be due to different techniques to measure HER2 in primary tumors and CTCs and the lack of highly sensitive and specific methods to reliably measure the HER2-status of CTCs. However, at least part of the discrepancies is due to biological reasons, justifying further research as this could have large clinical implications. We are currently investigating differences in gene expression profiles between the primary tumor, CTCs and lymph node metastases in patients with metastatic breast cancer to further address this issue.

So far, only three relatively small, prospective clinical trials investigated the efficacy of anti-HER2-treatments against HER2-positive CTCs [18,40,46]. However, given the small number of patients and the differences in patient groups, study design, and used techniques, no firm conclusions can be drawn. To do so, more, larger prospective trials are needed, some of which have already started. The DETECT III and the TREAT-CTC trials are examples here; the first investigating efficacy of lapatinib added to standard first-line chemotherapy in patients with HER2-negative MBC and HER2-positive CTCs as assessed by the CellSearch system (trial number NCT01619111), and the latter investigating efficacy of trastuzumab versus observation in HER2-negative PBC with detectable CTCs by the CellSearch system after (neo)adjuvant chemotherapy (trial number NCT01548677). To further address the question whether the HER2-status of CTCs can aid us in the personalization of therapy, we will have to await the results from these and other well-designed studies to come.

TABLE 4. Overview of reported ER expression on CTCs.

Reference	Enrichment method	Characterization method	PBC/ MBC	Patients with detectable CTCs positive for ER	Cut-off applied	Positive primary tumor with negative CTC	Negative primary tumor with positive CTC	Overall discrepancy rate
Fehm *et al.* (2009) [35]	AdnaTest	RT-PCR	PBC	12/48 (25%)	Expression signal intensity ≥ 0,60 ng/uL	NR	NR	71%
Tewes *et al.* (2009) [31]	AdnaTest	RT-PCR	MBC	6/17 (35%)	Expression signal intensity ≥ 0,60 ng/uL	5/11 (45%)	0/6 (0%)	5/17 (29%)
Aktas *et al.* (2011) [38]	AdnaTest	RT-PCR	MBC	17/87 (20%)	Expression signal intensity ≥ 0,15 ng/uL	48/62 (77%)	3/25 (12%)	51/87 (59%)
Gradilone *et al.* (2011) [27]	Anti-EpCAM-coated capture beads	RT-PCR	MBC	6/28 (21%)	NR	10/16 (63%)	0/12 (0%)	10/28 (36%)
Raimondi *et al.* (2011) [29]	Anti-EpCAM-coated capture beads	RT-PCR	Both	11/61 (18%)	NR	NR	NR	NR
Sieuwerts *et al.* (2011) [33]	CellSearch	quantitative RT-PCR	MBC	20/36 (56%)	Higher expression than the median in 31 enriched healthy donor blood	11/30 (37%)	1/6 (17%)	12/36 (33%)
Somlo *et al.* (2011) [57]	Depletion of CD45-positive cells using beads	Immunofluorescence of slides	Both	PBC: 4/8 (50%) MBC: 8/14 (57%)	Intensity sample score ≥ 3 (calculation based on the percentage of positive CTCs and the intensity of staining)	PBC: 2/3 (67%) MBC: 3/9 (33%)	PBC: 3/5 (60%) MBC: 2/5 (40%)	PBC: 5/8 (63%) MBC: 5/14 (36%)
Banys *et al.* (2012) [39]	AdnaTest	RT-PCR	PBC	8/43 (19%)	Expression signal intensity ≥ 0,60 ng/uL	33/41 (80%)	1/2 (50%)	34/43 (79%)

NR = not reported

Estrogen receptor (ER)

As the target of hormonal treatment, accurate information on the expression of ER on metastases is important. Similar to HER2, the presence of ER-positive CTCs could be an indication for hormonal treatments, irrespective of the ER-status of the primary tumor. However, less is known about the expression of ER in CTCs; only a few exploratory studies, using both immunofluorescence and PCR-based methods, have been carried out (Table 4). In these studies, positivity rates for the expression of ER in patients with detectable CTCs lie between 18-57% [27,29,31,33,35,38,39,57]. On average, this proportion is lower than the approximate 70% of primary tumors that is positive for ER, suggesting discrepant ER expression patterns between primary tumors and CTCs. While loss and gain of HER2 amplification seem both as likely to occur, for ER this is different. In all but one study, loss of ER from an ER-positive primary tumor to ER-negative CTCs is more frequently observed than a gain of ER on CTCs in patients with ER-negative primary tumors. In MBC, compared to the primary tumor, a loss of ER in CTCs was observed in 33-77% of the patients, whereas a gain of ER in CTCs was only found in 0-40% [27,31,33,38,57]. In PBC, this difference is less obvious, with reported rates of 67-80% for a loss and 50-60% for a gain of ER, respectively.[39,57] In a study performed by our group, we found a loss of ER in 11/30 (37%) MBC patients, while in a single patient (1/6, 17%) there was a gain [33]. However, as in most studies, the obtained discrepancy rates resulted from a comparison of two different assays to measure the expression of ER: mRNA expression in CTCs by RT-PCR versus protein expression in the primary tumor assessed by immunohistochemistry. To make a fair comparison we next measured the expression of ER on the mRNA level in both CTCs and corresponding primary tumors in 8 patients [33]. Again, a loss of ER in the CTCs was seen in 3/8 patients (38%), whereas a gain was only seen in one patient (13%). Our results appear to correspond with the results obtained by others, supporting the hypothesis that expression of ER in CTCs can be discrepant and is mainly lost over time. Studies on the clinical consequences of ER-status conversion in CTCs are still lacking. Only one study so far investigated changes in the expression of ER in CTCs under the pressure of treatment. In this study, 30 mL of blood was drawn from 98 patients with PBC at baseline and either after completion of neoadjuvant or 3 cycles of adjuvant

chemotherapy. In all samples, CTCs were isolated and characterized by density gradient centrifugation, immunomagnetical cell sorting using anti-CK-7/8/18/19-antibodies and immunofluorescence [23]. Comparing the pre- and after treatment samples, in 2/8 patients with an ER-positive tumor and ER-positive CTCs at baseline, a conversion to ER-negative CTCs was observed. Although the method of isolation is not optimal - the authors describe recovery rates of only up to 60% - and although this study only included a small number of patients, the observation of loss of ER under pressure of chemotherapy warrants further investigation.

Before conducting clinical studies on the predictive value of ER-expression in CTCs, important technical issues should be solved first. No assay, either immunofluorescence or PCR-based, has been validated in any way and nothing is known about a cut-off value for ER-positivity on the cell-level let alone on the sample-level. Proposed cut-offs are any staining in >10% of all CTCs in a sample on the protein-level or >0.6 ng/µL of ER transcripts on the mRNA-level [31,57]. However, heterogeneity in the expression of ER in CTCs within a single patient has been observed, similar to the situation in primary tumors and metastatic lesions where ER positivity can also be heterogeneous [3,23]. It will therefore be necessary to establish a valid and clinically relevant cut-off level.

New predictive factors

Currently, HER2 and ER are the only validated predictive factors used to tailor treatments in breast cancer. However, not all patients respond well to anti-HER2 and hormonal treatments, even when clinically indicated. To better predict resistance to these treatments, other predictive factors are needed, which are sought in parallel signaling pathways, such as the EGFR-pathway, or proteins downstream of these pathways, such as PI3K.

Expression of EGFR has been detected on 38-86% of the CTCs in patients with both PBC and MBC [19,22,23]. Furthermore, Kallergi *et al*. [22] found evidence for activation of the EGFR pathway in a proportion of CTCs. After density gradient centrifugation and

cytospinning, CTCs were stained for the expression of EGFR, phosphorylated EGFR (pEGFR) and the downstream proteins phosphorylated PI3K (pPI3K) and phosphorylated Akt (pAkt) by immunofluorescence. They found EGFR and pEGFR-positive CTCs in 6/16 PBC patients and 7/16 MBC patients with detectable CTCs; 2/6 and 6/7 patients with EGFR-positive CTCs, respectively, were also positive for pEGFR. When EGFR expression of CTCs was compared with that of the primary tumor, a loss of EGFR-expression was seen in 1/3 of PBC patients and 1/1 MBC patient; in increment in EGFR expression was seen in 2/9 patients and 3/9 patients, respectively. In >80% of patients also pPI3K and pAkt were positive. Altogether, the authors concluded that expression of EGFR in combination with pEGFR, pPI3K and pAKT suggests an activated pathway and a possible functional role in the biology of cancer cells.

Several other factors that may be helpful for treatment decision-making in breast cancer include factors activated by chemotherapy-induced cell damage. Examples are excision repair cross-complementing protein 1 (ERCC1), which is involved in the repair of DNA-adducts formed by platinum-based chemotherapy and has been associated with cisplatin resistance [59], and gamma-H2AX, which is involved in the repair of DNA double-strand breaks (DSB) and has been associated with resistance to chemotherapy-agents inducing DSB [60]. Both proteins can be detected in CTCs [57,60]. In the case of irreparable DNA damage, apoptosis pathways are activated and caspases start to cleave CK. Fragments of CK-18 can be measured as the M30 protein and have been associated with response to chemotherapy [61]. Staining of CTCs for M30 using the CellSearch system is possible [20,61]. All three proteins, ERCC1, gamma-H2AX and M30, are upregulated in response to DNA damage, thus after administration of chemotherapy. Although these factors are not genuine predictive factors that could predict sensitivity at forehand, they could still increase cancer treatment efficacy by detecting ineffective regimens at an early time-point. Whether this will provide additional information over simply the changes in CTC-counts during treatment, which also indicate treatment response or resistance, will have to be proven.

Discussion

We increasingly appreciate that cancer is a heterogeneous and dynamic disease that, due to clonal heterogeneity [1,2], continuously changes its genetic and molecular make-up over time and under the pressure of treatment. For a genuine personalized treatment approach it will therefore be crucial to identify accurate predictive factors to inform us at any time-point which pathways are activated, how to inhibit them and whether this is successful or if other pathways are being upregulated thereby causing resistance.

For several reasons CTCs provide an excellent basis for the assessment of predictive factors; at first, they are easily accessible via a simple blood draw as opposed to painful and cumbersome tissue biopsies. Thus, they provide the possibility for repetitive measurements and enable real-time monitoring of tumor characteristics. Second, CTCs are likely shed from the different tumor lesions present within a patient and the heterogeneity found between the different CTCs in a sample supposedly represents an individual's inter- and intratumoral heterogeneity [10,14,16-18,21,23,37,57]. Third, CTCs provide a basis to obtain a complete molecular picture. Analysis on circulating cell free DNA (cfDNA) can reveal the presence of actionable mutations and can even predict treatment resistance months before radiological progression of disease in the case of resistance-causing mutations [62,63]. But as discussed in this review, CTCs offer the great advantage over cfDNA approaches that the expression of proteins and mRNA as well as chromosomal aberrations, such as mutations and amplifications, can be assessed at the single-cell level in parallel. Before CTCs can be applied to guide treatment decisions, major technical hurdles have to be overcome first, of which the most important and urgent one is the lack of a reliable isolation method. In only 70% of the patients with MBC we are able to detect CTCs and in most cases only in very small numbers [6]. Nevertheless, the first studies investigating predictive factors on CTCs have already been conducted. At first these focused solely on technical issues how to characterize CTCs, but now also the first prospective clinical trials have been initiated. Most studies investigated HER2 expression and almost all found discrepancies between the CTCs and the primary tumor in a proportion of patients, losses and gains at similar rates. Fewer studies focused on the expression of ER in CTCs. It seems that loss of ER expression in CTCs in patients with ER-positive primary tumors is quite common, though its clinical relevance remains to be unraveled. Technical difficulties can partly underlie the observed discrepancies. However, a plausible biological explanation would be that ER-negative CTCs are shed by ER-negative metastases that have been selected under pressure of hormonal treatment, and that CTCs this way truly reflect the characteristics of the ER-negative metastases. Early attempts have also been made to investigate new, potentially predictive factors in CTCs, for example EGFR and proteins involved in DNA damage repair, but these are still proof-of-principle studies.

Due to the many different isolation and characterization techniques employed in the different studies performed so far, obtained results can hardly be compared and it is impossible to draw firm conclusions. Clearly, consensus on the techniques to be used in clinical trials is needed. Characterization of CTCs is technically very challenging due to the low detectable numbers among an abundance of leukocytes; to overcome these problems numerous assays have been developed. Immunocytochemistry for now probably is the most usable assay as it provides the most comprehensive picture: on CTC count, intensity of staining and heterogeneity in expression between the different CTCs in one sample; this in contrast to PCR, where cells are lysed and all information on CTC count and heterogeneity is lost. For HER2, immunocytochemistry has been validated by comparing in parallel protein overexpression with gene amplification as assessed by FISH in both cell lines and patient samples [10-14]. This led to a 0-3+ scoring for CTCs based on the intensity of staining, but due to the heterogeneity observed between CTCs a sample-level cut-off is needed next to a cell-level cut-off [10,13,14]. The need for cut-offs also applies to ER, although in this case we will have to begin by defining and validating a cell-level cut-off.

In conclusion, characterization of CTCs as a tool for a personalized cancer treatment approach is still in its infancy. Importantly, technical issues have to be solved first, meaning that validated assays and validated cut-offs are urgently needed before predictive factors on CTCs can reliably be studied. Despite that, promising results have

already been obtained, which show that HER2 can be both lost and gained on CTCs and ER is mainly lost during disease progression. Whether patients with metastatic disease should be treated based on the expression of predictive factors on CTCs irrespective of the status of the primary tumor remains to be investigated. Possibly, in the near future CTCs will prove to be inestimable tools that help improve the prognosis of cancer patients by telling when patients need to be treated with which targeted agents and when to switch to another agent.

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4

GENE EXPRESSION PROFILES OF CIRCULATING TUMOR CELLS VERSUS PRIMARY TUMORS IN METASTATIC BREAST CANCER

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ABSTRACT

Before using circulating tumor cells (CTCs) as liquid biopsy, insight into molecular discrepancies between CTCs and primary tumors is essential. We characterized CellSearch-enriched CTCs from 62 metastatic breast cancer (MBC) patients with ≥5 CTCs starting first-line systemic treatment. Expression levels of 35 tumor-associated, CTC-specific genes, including *ESR1,* coding for the estrogen receptor (ER), were measured by reverse transcription quantitative polymerase chain reaction and correlated to corresponding primary tumors. In 30 patients (48%), gene expression profiles of 35 genes were discrepant between CTCs and the primary tumor, but this had no prognostic consequences. In 15 patients (24%), the expression of ER was discrepant. Patients with ER-negative primary tumors and ER-positive CTCs had a longer median TTS compared to those with concordantly ER-negative CTCs (8.5 versus 2.1 months, $P = 0.05$). From seven patients, an axillary lymph node metastasis was available. In two patients, the CTC profiles better resembled the lymph node metastasis than the primary tumor. Our findings suggest that molecular discordances between CTCs and primary tumors frequently occur, but that this bears no prognostic consequences. Alterations in ER-status between primary tumors and CTCs might have prognostic implications.

Introduction

Over the past decade, the concept of tumor heterogeneity between primary tumors and metastases has increasingly been acknowledged. Under the influence of time and treatment, tumor cell characteristics, including the expression of treatment targets such as the estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) in breast cancer, can vary between the primary tumor and distant metastatic sites [1-6]. Besides intertumor or temporal heterogeneity, even cell clones within one tumor site can differ in characteristics, giving rise to intratumor or spatial heterogeneity. Tumor heterogeneity may form the basis of treatment resistance and is therefore important to take into account in treatment decision-making.

Nevertheless, the choice for palliative treatments in metastatic breast cancer (MBC) is still generally based on primary tumor characteristics. Although a re-evaluation of ER and HER2 expression on a tumor tissue biopsy at the time of metastatic disease is recommended in guidelines [7], this is frequently omitted as obtaining tissue from metastases can be challenging or even impossible. Therefore, better and more patient-friendly tools are urgently needed to analyze characteristics of metastases before start of and repetitively during treatment.

Analysis of circulating tumor cells (CTCs) might be an attractive means to assess the characteristics of metastases. Being present in the peripheral blood, CTCs can easily be obtained through a venipuncture and as such form a promising alternative for biopsies from metastatic lesions [8,9]. However, before we can fully appreciate the potential clinical value of CTC characterization, we need to learn more about the biology and to what extent CTCs – as suggested representation of metastatic cells – differ in their characteristics from primary tumors. In this study, we used the CellSearch System (Janssen Diagnostics, Raritan, NJ) to isolate CTCs from MBC patients followed by gene expression profiling of 35 epithelial, tumor-associated, and CTC-specific genes [10]. The main objective of this study was to compare the overall molecular CTC profile to the corresponding primary tumor profile and to assess the proportion of patients with

discordant molecular make-up. A profile from an axillary lymph node metastasis taken at the time of primary tumor resection was also available for comparison in a subset of patients. The expression of ER in CTCs and discordances with the primary tumor were investigated separately. Additionally, we explored the prognostic significance of observed discrepancies between primary tumor and CTC profiles.

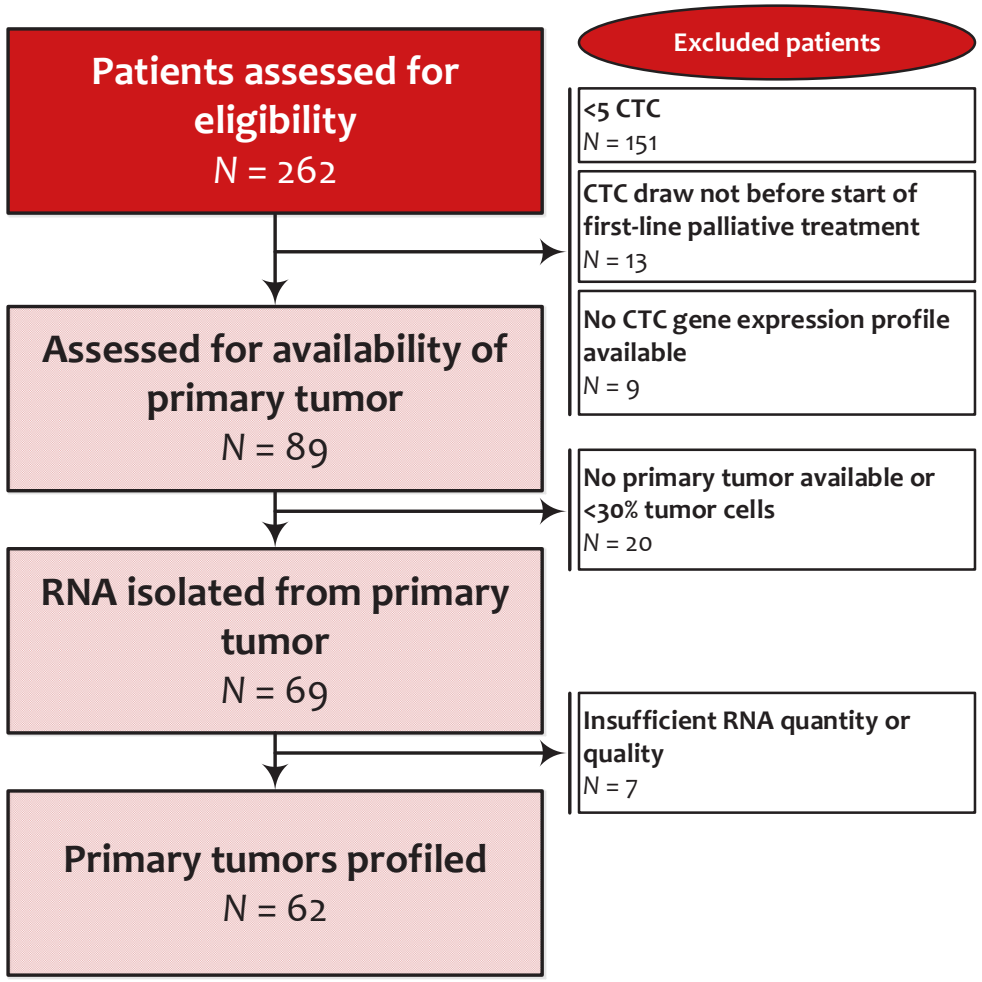

FIGURE 1. Study flowchart. In total 262 patients from an ongoing prospective clinical trial were evaluated for eligibility for this study. After excluding patients not meeting our inclusion criteria (right boxes), 62 pairs of CTC and FFPE primary tumor profiles remained for the subsequent analyses.

MATERIALS AND METHODS

Wherever possible, the data are reported conform to the reporting recommendations for tumor marker prognostic studies (REMARK; [11]). A study flowchart is presented in Figure 1.

Patients

We retrospectively selected patients from a clinical trial enrolling MBC patients starting first-line systemic treatment, either endocrine or chemotherapy according to the physician's decision [10,12]. Blood for enumeration and characterization of CTCs was drawn before the start of systemic treatment. Clinical data were collected from patient charts. All patients with a CTC count ≥5/7.5 mL blood who were included in the clinical trial between February 2008 and February 2012 were selected for the current study. Patients were recruited from six hospitals in the Rotterdam region. The Erasmus MC and local Institutional Review Boards approved the study (METC 06-248). All patients provided written informed consent.

Sample processing

Enumeration and characterization of CTCs using the CellSearch System and the generation of cDNA, linear preamplification, and reverse transcription quantitative polymerase chain reaction (RT-qPCR; using Taqman Gene Expression Assays; Applied Biosystems, Carlsbad, CA) were performed as described in detail before [10,12].

Archived formalin-fixed paraffin embedded (FFPE) primary tumors and axillary lymph node metastases were collected from pathology laboratories. Only paraffin blocks with ≥30% tumor cells on hematoxylin and eosin slides were selected. Isolation of RNA from FFPE samples was done using the High-Pure RNA Paraffin Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Quantity and quality checks of isolated RNA were performed using the Nanodrop 1000-v.3.7 (Thermo Scientific, Wilmington, USA), the MultiNA Microchip Electrophoresis system (Shimadzu, Kyoto, Japan), and multiplexed RT-qPCR for reference genes.

In all CTC and FFPE tumor samples, we measured our previously described panel of 55 epithelial tumor- and CTC-specific genes. These genes had been selected based on literature for involvement in tumorigenesis and/or mutagenesis along with absent or low expression by leukocytes. Consequently, our panel consists of clinically relevant genes that are reliably measurable in ≥5 CTCs by RT-qPCR [10,12]. To confirm similarly good assay performance on CTC and FFPE tumor samples, we compared expression levels between nine paired fresh frozen and FFPE primary tumor samples and only continued with the 20 genes that significantly correlated (Pearson correlation $P>0.05$; Table 1).

Normalization and statistical analysis

Expression levels of individual genes in CTC and tumor samples were quantified relative to the average C_q of three reference genes (*GUSB*, *HMBS*, and *HPRT1*) using the ΔC_q method [13]. Samples with an average reference gene $C_q>26$ were considered to be of insufficient RNA quality and excluded from further analysis. To correct for the leukocyte background in the CTC samples, the median ΔC_q of each gene transcript in 31 CellSearch enriched healthy blood donor samples was used as cut-off. All ΔC_q values below this cut-off were considered undetectable. A compare batches (ComBat) normalization was conducted to enable comparison of corresponding profiles and limit technical variations [14-16].

We used a Pearson correlation analysis to compare the overall expression levels of 35 genes in primary tumors to corresponding CTCs. To enable further statistical testing two groups were formed of concordant and discordant profiles, based on all Pearson correlation coefficients of 62 primary tumors x 62 CTC samples. Among these 3,844 correlations were 62 corresponding primary tumor/CTC pairs of the same patient and

TABLE 1 (RIGHT PAGE). Selection of genes from our previously described [10] CTC-specific panel of 55 tumor-associated gene transcripts. Based on Pearson's correlation, genes that significantly correlated ($P<0.05$) between nine paired fresh frozen and FFPE primary tumor samples were selected for further comparison between FFPE primary tumor tissues and the fresh frozen CTC samples. Twenty assays performed poorly in the comparison between fresh frozen and FFPE samples, leaving 35 genes suitable for comparison of paired primary tumor tissues and CTC samples.

Gene	Pearson r	*P* value	In 35 gene panel?
FOXA1	0.99	<0.05	Yes
ITGA6	0.99	<0.05	Yes
KRT19	0.99	<0.05	Yes
IL17BR	0.98	<0.05	Yes
PKP3	0.98	<0.05	Yes
CXCL14	0.97	<0.05	Yes
KRT17	0.97	<0.05	Yes
EEF1A2	0.96	<0.05	Yes
IGFBP2	0.96	<0.05	Yes
EPCAM	0.96	<0.05	Yes
TFF1	0.96	<0.05	Yes
CEP55	0.96	<0.05	Yes
ESR1	0.96	<0.05	Yes
PLAU	0.95	<0.05	Yes
SPDEF	0.95	<0.05	Yes
DUSP4	0.94	<0.05	Yes
KRT7	0.93	<0.05	Yes
AGR2	0.93	<0.05	Yes
SCGB1D2	0.93	<0.05	Yes
FGFR4	0.92	<0.05	Yes
TFF3	0.91	<0.05	Yes
ERBB4	0.91	<0.05	Yes
PTRF	0.91	<0.05	Yes
CRABP2	0.90	<0.05	Yes
LAD1	0.90	<0.05	Yes
FKBP10	0.87	<0.05	Yes
CCND1	0.85	<0.05	Yes
PIP	0.84	<0.05	Yes
TSPAN13	0.84	<0.05	Yes
DTX3	0.83	<0.05	Yes
MUC1	0.83	<0.05	Yes
S100A7	0.83	<0.05	Yes
ACTA1	0.82	<0.05	Yes
IGFBP4	0.82	<0.05	Yes
MELK	0.82	<0.05	Yes
TOX3	0.81	>0.05	No
CEACAM5	0.77	>0.05	No
MUCL1	0.76	>0.05	No
PLOD2	0.73	>0.1	No
TIMP3	0.73	>0.1	No
DTL	0.69	>0.1	No
CLDN3	0.69	>0.1	No
KRT18	0.69	>0.1	No
SCGB2A2	0.67	>0.1	No
ERBB3	0.66	>0.1	No
IGFBP5	0.64	>0.1	No
CD24	0.63	>0.1	No
KIF11	0.62	>0.1	No
SEPP1	0.59	>0.1	No
FEN1	0.57	>0.1	No
KPNA2	0.39	>0.1	No
CTTN	0.35	>0.1	No
FGFR3	0.35	>0.1	No
S100A16	0.35	>0.1	No
MKI67	0.26	>0.1	No

3,782 non-corresponding pairs of different patients. The mean correlation coefficient from corresponding samples from one patient was 0.72, which was significantly higher than the 0.54 from non-corresponding pairs from different patients (*P*<0.0001; Figure 2A). The top 10% strongest correlations among all 3,844 pairs were arbitrarily chosen as concordant pairs, leading to a cut-off of r=0.74.

To determine the ER-status of CTCs, we first established an mRNA cut-off value for ER-positivity by comparing *ESR1* expression levels in primary tumors with known ER-status from routine pathological reports. ER-positivity was defined as immunohistochemical staining in >10% of tumor cells. Expression levels of *ESR1* in 61 primary tumors (one tumor's ER-status was unknown) correlated with ER-status from the pathology reports and led to a reliable *ESR1* cut-off in our patient cohort (Figure 3). All subsequent analyses were based on the *ESR1* expression levels both in the primary tumors and CTCs.

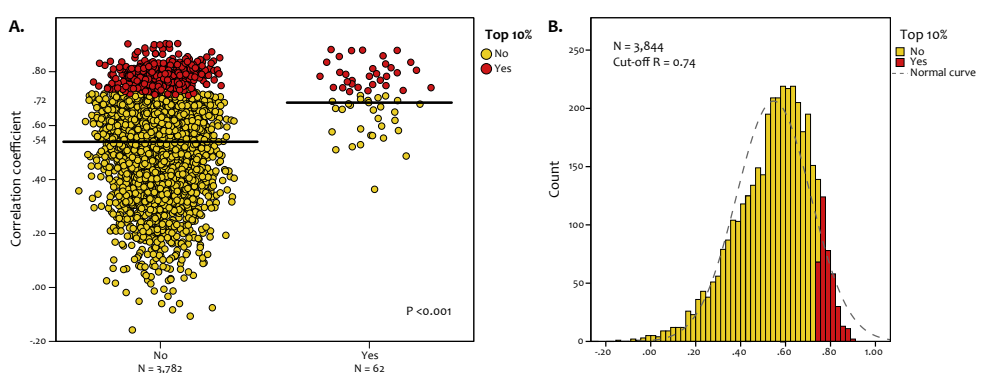

FIGURE 2.A. Scatterplot showing Pearson correlation coefficients of 3,782 non-matching primary tumor – CTC pairs of different patients (left side) and 62 matching primary tumor – CTC pairs (right side). The matching pairs correlated significantly better. The top 10% highest correlation coefficients are shown in light blue. Horizontal lines represent the means. The reported *P* value is from an independent samples t test. B. Histogram showing the distribution of all 3,844 matching and non-matching correlation coefficients. The top 10% with cut-off r=0.74 was chosen to define patients with concordant, highly correlating primary tumor versus CTC gene expression profiles. This cut-off was subsequently used to define two patient groups of concordant and discordant profiles among the 62 study patients.

The Datan Framework GenEx Pro package version 5.4.1 software (MultiD Analyses AB, Göteborg, Sweden), SPSS 20.0 (IBM Corporation, Armonk, NY), and R version 3.0.1 (http://www.R-project.org/) were used to analyze gene expression levels. ComBat normalization was done using the Surrogate Variable Analysis package within R. Standard statistical testing was done using SPSS 20.0 (IBM Corporation, Armonk, NY). Differences in continuous variables were tested using Student's t test or non-parametric Mann-Whitney U, depending on the distribution. Categorical variables were tested by chi-square tests. Correlations were tested either by Pearson (gene expression data) or Spearman correlation (CTC count). Clinical outcome was expressed as time-to-treatment switch (TTS: the interval between start of first-line and second-line treatment or death, whichever comes first) and overall survival (OS: the interval between start of first-line treatment and death or last known to be alive). Associations with clinical outcome were visualized in Kaplan-Meier plots and tested by log-rank tests. All statistical tests were two-sided and *P*<0.05 was considered statistically significant.

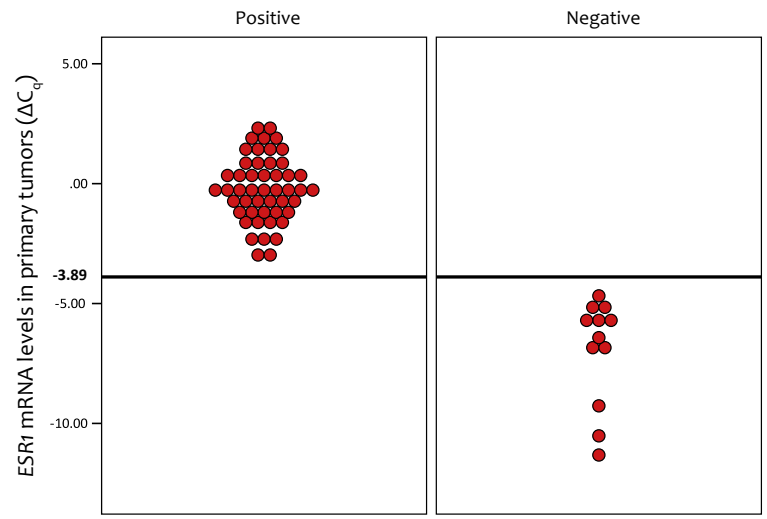

FIGURE 3. Determination of an mRNA cut-off value to assess ER positivity in CTC samples. This cut-off was based on *ESR1* expression levels in 61 primary tumors with known ER-status as assessed by routine pathology. Tumors were scored as ER-positive by the pathologist when >10% of tumor cells showed nuclear staining by immunohistochemistry. The optimal cut-off for *ESR1* expression was found with a sensitivity and specificity of 100%.

Table 2. Clinicopathological characteristics of all patients and tumors.

	All patients		Concordant profiles		Discordant profiles		*P* value
N	62	100%	32	100%	30	100%	
Age at MBC (mean ± sd)	59.8 ± 12.6		62.1 ± 11.4		57.4 ± 13.4		0.14
Tumor classification							0.46
T1 - 2	47	76%	23	72%	24	80%	
T3-4	15	24%	9	28%	6	20%	
Lymph node metastases							0.14
No	16	26%	11	34%	5	17%	
N+	43	69%	19	59%	24	80%	
Nx	3	5%	2	6%	1	3%	
Bloom and Richardson grade							0.63
1	6	10%	4	13%	2	7%	
2	38	61%	18	56%	20	67%	
3	18	29%	10	31%	8	27%	
Tumor histology							0.98
Ductal	37	60%	19	59%	18	60%	
Lobular	12	19%	7	22%	5	17%	
Mixed ductal/lobular	6	10%	3	9%	3	10%	
Other	7	11%	3	9%	4	13%	
Hormone receptor expression							
ER positive[a]	49	79%	26	81%	23	77%	1.00
PR positive[b]	34	55%	17	53%	17	57%	0.79
HER2 positive[c]	15	24%	5	16%	10	33%	0.07
Triple negative	10	16%	5	16%	5	17%	1.00
Menopausal status at primary diagnosis							0.19
Premenopausal	24	39%	10	31%	14	47%	
Postmenopausal	35	56%	21	66%	14	47%	
Unknown	3	5%	1	3%	2	7%	
(Neo)adjuvant treatment[d]							
None	25	40%	21	66%	16	53%	0.44
Chemotherapy	29	47%	16	50%	13	43%	0.62
Anthracyclines	27	44%	15	47%	12	40%	
Taxanes	7	11%	2	6%	5	17%	
Other	2	3%	1	3%	1	3%	
Hormonal therapy	28	45%	15	47%	13	43%	0.80
Tamoxifen	25	40%	14	44%	11	37%	
Aromatase inhibitors	8	13%	5	16%	3	10%	
Trastuzumab	2	3%	-		2	7%	0.23
Interval between primary tumor and metastases						0.28	
< 1 year or synchronous	17	27%	6	19%	11	37%	
1 – 5 years	22	36%	13	41%	9	30%	
≥ 5 years	23	37%	13	41%	10	33%	
Location of metastasese							0.58
Bone	47	76%	23	72%	24	80%	
Visceral	46	74%	25	78%	21	70%	
Secondary breast tumor/local relapse	5	8%	2	6%	3	10%	0.67
First-line systemic treatment for MBC[d]							
Chemotherapy	42	68%	20	63%	22	73%	0.42
Anthracyclines	7	11%	3	9%	4	13%	
Taxanes	27	44%	14	44%	13	43%	
Other	17	27%	9	28%	8	27%	
Hormonal therapy	29	47%	16	50%	13	43%	0.62
Tamoxifen	8	13%	4	13%	4	13%	
Aromatase inhibitors	21	34%	12	38%	9	30%	
Targeted therapy	25	40%	11	34%	14	47%	0.26
Trastuzumab	15	24%	5	16%	10	33%	
Bevacizumab	7	11%	4	13%	3	10%	
Other	5	8%	3	9%	2	7%	
Baseline CTC count (median, IQR)	29.5 (11 - 98.5)		48.5 (12.0 - 154.3)		18 (9 - 68.8)		0.07
Primary tumor tissue							
Median % invasive cells (IQR)	60 (44 - 75)		60 (41 - 79)		60 (44 - 75)		0.75
Median % infiltrate (IQR)	15 (7 - 29)		16 (5 - 30)		15 (10 - 25)		0.98
Median % normal tissue (IQR)	15 (10 - 25)		10 (10 - 20)		16 (10 - 25)		0.18

RESULTS

Gene expression profiles of CTCs, primary tumor, and lymph node metastasis

We selected 62 patients for the current study from the 262 evaluated for eligibility (Figure 1). Clinicopathological characteristics of these patients are shown in Table 2. The median interval between resection of the primary tumor and start of first-line palliative treatment was 33 months (interquartile range (IQR) 0 – 88 months). Thirty-seven patients (60%) had received (neo)adjuvant treatment in the form of chemotherapy (N=29), hormonal treatment (N=28), and/or trastuzumab (N=2). The median follow-up time of the 19 patients still alive at the time of analysis was 31.2 months (range 19 – 59 months). In Supplementary Table S1, characteristics and prior treatments are specified per patient.

The main objective of this study was to assess the proportion of patients with discrepancies in overall molecular characteristics between CTCs taken before the start of first-line therapy for metastatic disease and corresponding primary tumors. Based on the expression of the 35 selected genes, we found discordant profiles in 30 patients (48%). No differences were found in clinicopathological characteristics between patients with concordant and discordant profiles (Table 2). More patients in the discordant group had synchronous metastases or an interval between primary tumor surgery and CTC draw <1 year compared to patients in the concordant group, but this difference did not reach statistical significance (37% versus 19%; *P*=0.28). Twenty-one patients with concordant (66%) versus 16 patients with discordant profiles (53%) had received (neo) adjuvant treatment (*P*=0.44). However, patients with discordant profiles tended to have lower CTC counts (median 18 versus 48.5, *P*=0.07), and more often HER2-positive primary tumors (33% versus 16%, *P*=0.07).

We tested whether differences in sample input may have confounded the analyses. In the primary tumors, the median percentage of tumor cells, infiltrate and normal tissue was comparable between patients with concordant or discordant profiles (Table 2). Although CTC counts in the group of patients with concordant profiles were higher, there was

FIGURE 4. Correlation plot of baseline CTC counts versus Pearson correlation coefficients of the CTCs versus the primary tumor gene expression profiles in all 62 patients. The four quadrants are based on the median CTC count of 29.5 (vertical line) and the cut-off of r = 0.74 of concordant or discordant CTC versus primary tumor profiles (horizontal line). Only a weak influence of CTC counts on observed discordances was found. In patients with a CTC count higher than the median of 29.5 we still observed a discordance rate of 36%. The reported r and *P* values are from Spearman correlation.

only a weak correlation between CTC counts and the correlation coefficients of primary tumor versus CTC profiles (Spearman r = 0.25, *P* = 0.05). Furthermore, in patients with CTC counts greater than the overall median of 29.5 we still found discordant profiles in 36% of patients (Figure 4). Therefore, we concluded that CTC counts were no major contributor to the observed discrepancies in gene expression profiles.

Additional gene expression profiles from axillary lymph node metastases taken at the time of primary tumor resection were available from seven patients (Figure 5). These profiles closely matched the primary tumor profiles in all seven patients. In two patients (nrs. 4 and 5) the CTC profile was discordant from the lymph node metastasis. Interestingly, in two other patients (nrs. 1 and 7) the CTC profiles better correlated with the lymph node metastasis profiles than with the primary tumor profiles.

Discrepancies in ER expression

Being an important treatment target, we had special interest for discrepancies in ER-status between CTCs and primary tumors. Applying the established cut-off value for *ESR1*, we found ER-positive CTCs in 48 patients (77%). Compared to the corresponding primary tumors, we observed discrepancies in ER-status in 15 of 62 patients (24%; Table 3). Expression of ER was gained in 7 out of the 13 patients (54%) with originally ER-negative primary tumors, whereas it was lost in 8 out of the 49 patients (16%) with ER-positive primary tumors. The length of the interval until first-line treatment for metastatic disease had no influence on the occurrence of ER-switches, although a non-significant association toward more switches in patients with ER-positive primary tumors and longer intervals was observed (table 4). Of the 49 patients with ER-positive primary tumors, 27 had

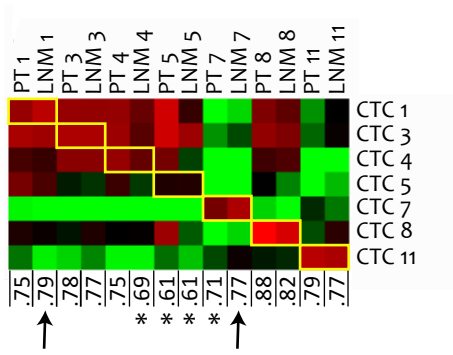

FIGURE 5. Correlation matrix based on the expression of 35 tumor-associated, CTC-specific genes in seven FFPE primary tumors, lymph node metastases, and corresponding CTC samples taken at the time of metastatic disease. Numbers below the matrix are the Pearson correlation coefficients of the vertical FFPE primary tumor or lymph node metastasis with the corresponding CTC sample (visualized in yellow boxes). Asterisks indicate discordant profiles based on the top 10% correlation coefficients with cut-off r=0.74 (Figure 2). In patients 5 and 7 the CTC profiles were discordant from the primary tumor profiles; in patients 4 and 5 the CTC profiles were discordant from the lymph node profiles. In patients 1 and 7 the CTCs correlated better with the lymph node metastases than with the primary tumors, as can be concluded from the more intense red color (arrows). Red color represents correlation coefficients higher than the median, black corresponds to the median, and green stands for correlation coefficients lower than the median. CTC = circulating tumor cells; LNM = lymph node metastasis; PT = primary tumor.

received adjuvant hormonal therapy. Six patients thereafter switched from an ER-positive primary tumor to ER-negative CTCs (22%) compared to two of the 22 patients that had not received endocrine therapy before (9%; *P*=0.20).

ER-status		CTCs: negative	CTCs: positive	
primary tumor	negative	6	7	13
	positive	8	41	49
		14	48	62

TABLE 3. Discrepancies in ER/*ESR1* expression between primary tumors and corresponding CTCs.

Associations with clinical outcome

In all 62 patients, the median TTS was 8.7 (95% CI 7.2 – 10.2) months with a median OS of 23.0 (95% CI 13.7 – 32.4) months. We found no difference in TTS or OS between patients with concordant or discordant CTC versus primary tumor profiles (log-rank *P*=0.95 and *P*=0.50, respectively, Figure 6A and B).

In our exploratory analyses, a statistically significant difference in median TTS by ER-status of CTCs versus primary tumors was observed (*P*=0.001; Figure 6C). Patients with ER-negative tumors and discordant ER-positive CTCs (*N*=7) had improved TTS (median 8.5 months (95% CI 0.0 – 22.7) over those with concordant ER-negative CTCs (*N*=6; median 2.1 months (95% CI 0.0 – 8.8); *P*=0.05). Based on the ER-status of the primary tumor, 20 patients received palliative hormonal treatment only (not preceded by chemotherapy).

		ER-status Primary Tumor: ER-negative: No switch	ER-negative: switch	ER-negative: Total	ER-positive: No switch	ER-positive: switch	ER-positive: Total
Interval	Synchronous or <1 year	1 (33%)	2 (67%)	3 (100%)	13 (93%)	1 (7%)	14 (100%)
	1 – 5 years	4 (50%)	4 (50%)	8 (100%)	12 (86%)	2 (14%)	14 100%)
	>5 years	1 (50%)	1 (50%)	2 (100%)	16 (76%)	5 (24%)	21 (100%)
Linear-by-linear association		*P* = 0.69			*P* = 0.19		

TABLE 4. Discrepancies in ER/*ESR1* expression between primary tumors and corresponding CTCs according to the interval between primary tumor surgery and start of first-line treatment for metastatic disease.

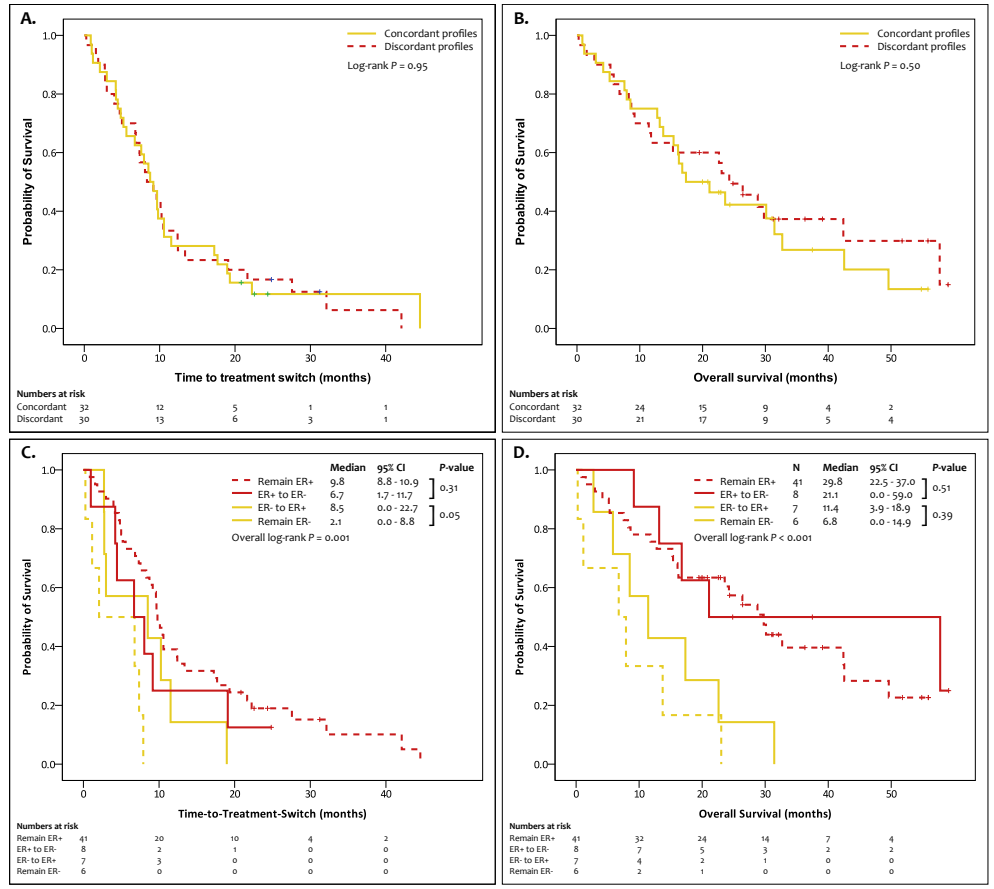

FIGURE 6. ABOVE: Time-to-treatment-switch (TTS; A.) and overall survival (OS; B.) of all 62 patients according to concordant or discordant primary tumor versus corresponding CTC gene expression profiles. No significant differences were observed in median TTS and OS. BELOW: Time-to-treatment-switch (C.) and overall survival (D.) as a function of the ER-status of CTCs versus the primary tumors. Patients at risk at various time points are indicated below the plots. Reported *P* values between two groups are from Log-rank tests and between four groups from Log-rank tests for trend.

Out of these, the three patients with ER-negative CTCs had shorter TTS of 1.0, 4.4, and 6.7 months compared to the 17 patients with concordant ER-positive CTCs, for whom median TTS was 12.4 months. However, formal statistical tests are not meaningful due to the small patient numbers.

Differences in OS were mainly driven by the ER-status of the primary tumor and no differences between the concordant and discordant groups were observed within the group of patients with ER-positive or ER-negative primary tumors (*P*=0.51 and *P*=0.39, respectively, Figure 6D).

Discussion

In this study, we compared gene expression profiles of CTCs taken at the time of metastatic disease to the corresponding primary breast tumors. We assessed the degree of molecular discordance and found that gene expression profiles of 35 epithelial tumor-associated, CTC-specific genes in CTCs differed from the corresponding primary tumor in 48% of MBC patients. Patients with concordant and discordant profiles did not differ in clinicopathological characteristics.

Differences in sample input can largely influence the results obtained and confound the discrepancies in gene expression profiles observed. To ascertain reliable CTC-driven gene expression profiles, only patients with a CTC count ≥5 were included in this study. Importantly, even after CellSearch enrichment, CTCs are left in a background of leukocytes that may influence expression levels of certain genes [12]. To circumvent this, we selected genes based on literature for their involvement in tumori- and/or mutagenesis and absent or low level expression in leukocytes. Although limiting the choice of genes, this assured reliable CTC-driven profiles in patients with ≥5 CTCs [10]. A normalization step using healthy blood donors further limited leukocyte contribution to the CTC gene expression profiles. The assays used on the CTC samples were also applied to the FFPE samples and a normalization step was applied to eliminate technical variations. We cannot exclude influence of other cell types – such as stromal tissue – on the profiles obtained from primary tumors since these tissues were not macrodissected. However, only epithelial tumor cell-associated gene transcripts were measured, limiting the contribution of stromal cells. The median percentage of tumor cells was 60% in patients with concordant and discordant profiles. Although patients with concordant profiles had higher CTC counts, we only found a weak correlation between CTC counts and profile correlation coefficients maximally explaining 6% of the variance in observed discrepancies. Besides, profiles were still discordant in 36% of the patients with CTC counts greater than the overall median. Altogether, differences in sample input did not seem to cause the observed discrepancies in molecular characteristics between primary tumors and CTCs.

The high proportion of patients with discordant profiles underscores the importance of considering tumor heterogeneity in the clinics. Reassessment of ER and HER2-status of metastatic disease is recommended in clinical guidelines [7], but still frequently omitted due to the invasive nature of tissue biopsies. Furthermore, a biopsy from one metastatic site can lead to false conclusions since spatial and temporal heterogeneity is disregarded. A "liquid biopsy" using CTC characteristics constitutes an easily accessible and patient-friendly way to repetitively monitor metastatic tumor cell characteristics – probably of multiple metastatic sites – throughout the course of treatment. Although no distant metastatic tissue was available from the patients included in our study, an axillary lymph node metastasis taken at the time of primary tumor resection was available from seven patients. In all seven patients, this profile correlated well with the primary tumor; in two patients there was a discordant profile with CTCs. Interestingly, in two other patients, CTCs better resembled the lymph node metastasis than the primary tumor. Future studies should focus on the comparison between primary tumors, distant metastases, and CTCs to establish whether we can use CTCs as direct derivatives of distant metastatic lesions.

Several studies investigated differences in characteristics between primary breast tumors and metastatic sites, especially for the treatment targets ER and HER2. Conversion rates for ER and HER2 generally lie around 15% [4,5,17-21]. Discordances between primary tumors and CTCs have been investigated less extensively. Reported discrepancy rates for ER vary between 21 and 79% of patients, with losses of ER more frequently being encountered than gained ER expression [reviewed in [9]]. Based on the expression of *ESR1* in CTCs, we observed discrepancies in ER-status compared to the primary tumor

in 24% of cases. Unexpectedly, an upregulation of *ESR1* was more frequently observed than a downregulation. However, given the small number of patients with ER-negative primary tumors these numbers have to be interpreted with caution. In patients with ER-positive disease, an association was observed of longer disease-free intervals with a higher chance of switch in ER-status. Furthermore, ER-negative CTCs were found in 22% of patients who had received adjuvant endocrine treatment compared to 9% of patients who had not. However, both observed differences did not reach statistical significance. In the overall group of 62 patients, discordances in ER-status were of prognostic significance for TTS, but not for OS. This is likely due to the diluting effects of subsequent chemotherapeutic treatments on OS. Unfortunately, too few patients received palliative endocrine therapy not preceded by chemotherapy to allow for statistical testing. The results observed in this study might indicate a predictive and prognostic value of ER-switches, especially in ER-positive patients, worthwhile of exploring further.

Discordant overall molecular profiles between CTCs and primary tumors had no prognostic significance in our study. This lack of prognostic value might be influenced by the choice of genes in our panel. Although selected from literature for involvement in tumor development and progression, the predictive and/or prognostic value of most genes remains largely unknown. Alternatively, changes in gene expression can be induced by factors present in the circulation, but not in the tumor. These changes might then reflect a difference in environment rather than true tumor evolution and this way might bear no prognostic information. Notwithstanding, our patient group is too small to investigate the prognostic significance of 35 individual genes and therefore we decided to only use the composite molecular profile of all 35 genes. Biological changes in individual genes and pathways therefore remain to be investigated, preferentially also by comparison with metastatic tissue.

Discrepancies in HER2-status between CTCs and primary tumors have been reported in 19-90% of patients [9]. Unfortunately, we were not able to reliably measure *ERBB2* mRNA expression levels in CTCs since *ERBB2* is also expressed at low levels by leukocytes. This

again points to the technical issues surrounding CTC isolation and characterization, which greatly hamper research in this field.

Several limitations apply to our results. Although the use of a validated and FDA-approved system for clinical use is a strength, the EpCAM-dependency of the used CellSearch System is a weakness. Previously, we showed that the CellSearch system does not detect mesenchymal transitioned breast cancer cell lines with only low or no expression of EpCAM [22]. We therefore only compared characteristics of epithelial CTCs to the primary tumors. The complex mechanisms of molecular changes in CTCs, including epithelial-to-mesenchymal transition, remain to be unraveled further. Since single cell information was lost during RT-qPCR, we were unable to explore the extent of heterogeneity between single CTCs. Also, the expression of ER was investigated on the mRNA instead of the usual protein level. In our group of patients we found a perfect correlation between protein and mRNA expression for ER in primary tumor tissues. However, whether this applies to the CTCs remains unknown. Furthermore, sampling bias in tissue biopsies and technical factors, such as the limited sensitivity of currently available assays, need to be considered when comparing the results obtained from different studies [23].

Conversions in receptor status between primary tumors and metastatic sites are of prognostic significance. Patients who lose expression of a receptor have shorter median OS compared to patients with sustained expression [1,4,5]. Amir et al. [21] investigated the impact of discordance in ER and HER2 status on patient management by reporting the results of metastatic tissue biopsies to the treating physicians. Changes in therapy were reported in 14% of patients and mostly concerned addition of trastuzumab in patients with a gain of HER2 and a switch from endocrine treatment to chemotherapy in patients with a loss of ER. To fully appreciate the clinical relevance of ER and HER2 expression in CTCs of MBC patients, well-designed prospective clinical trials are needed to investigate whether treatment decisions in MBC should be based on CTC characteristics. The first trials assessing this have already been initiated [24-26].

In conclusion, overall CTC characteristics and the expression of ER in CTCs differ from the primary tumor in a significant proportion of MBC patients. Conversions in ER-status between primary tumors and CTCs might be of prognostic significance and may impact treatment decision-making. To fully appreciate the value of CTC characterization, technical challenges have to be overcome first. An urgent need for validated characterization assays exists to open the path to larger prospective trials investigating the clinical value of CTC characteristics. Potentially, CTCs might become an invaluable tool for a personalized cancer treatment approach and thereby improve the prognosis of MBC patients.

Acknowledgments

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SUPPLEMENTARY TABLE S1. Patient and tumor characteristics specified per patient.

			CTC characteristics		Primary tumor characteristics													Clinical characteristics											
Pt ID	PT-CTC (r)	C/D	CTC count	ER	Year PT	Histology	T	N	M	ER	PR	HER2	MAI	BR	Tumor	Infiltrate	Normal tissue	Menopause (PT)	Second BC	Neoadjuvant tx	Adjuvant tx	Age MBC	PT-MBC (yrs)	Visceral M	Location M	Palliative tx	TTS	2nd-line tx	OS	†
1	.75	C	6	+	1992	D	2	1	x	+	+	-	0	2	70%	20%	10%	Post	Yes	-	1. CMF + tamoxifen 2. FEC + exemestane	68.5	18.0	Yes	B, LN, O	Paclitaxel	11	Yes	16	D
3	.78	C	14	+	2011	M	2	2	1	+	+	-	1	2	80%	10%	10%	Pre	-	-	-	50.7	0	-	B	Tamoxifen	23	No	23	A
4	.75	C	173	+	2002	O	2	1	x	+	-	-	10	2	45%	45%	10%	Pre	-	-	AC + tamoxifen	51.8	8.7	Yes	B, LN, O	Paclitaxel + bevacizumab	6	Yes	20	A
5	.61	D	5	+	2011	D	2	1	1	+	+	-	7	2	60%	20%	15%	Pre	-	-	-	47.6	0	Yes	B, L	FEC	4	Yes	19	A
7	.71	D	563	-	2011	O	4	3	0	-	-	-	10	3	75%	20%	5%	Post	-	-	CMF	79.7	0.8	Yes	P, L, O	Paclitaxel	0	Yes	0	D
8	.88	C	162	+	2004	D	2	1	0	+	-	+	15	3	60%	30%	10%	Post	-	-	AC + tamoxifen + letrozol	64.8	7.7	Yes	B, L	FAC + docetaxel + trastuzumab	18	Yes	23	A
11	.79	C	1101	-	2010	D	2	2	0	-	-	-	20	3	55%	35%	5%	Post	-	-	FEC + docetaxel	67.5	1.8	Yes	B, P	Paclitaxel	2	Yes	14	D
12	.68	D	29	+	2008	D	1	0	x	+	-	-	14	2	65%	15%	20%	Post	-	-	-	64.5	2.7	-	B	Anastrozole	7	Yes	8	D
13	.73	D	11	+	2006	D	1	1	x	+	-	+	16	3	85%	10%	5%	Post	-	-	AC + paclitaxel + trastuzumab + tamoxifen	59.4	2.9	Yes	B, P	Vinorelbine + trastuzumab	8	Yes	15	D
14	.36	D	6	-	2002	O	1	1	x	+	+	uk	8	2	60%	20%	20%	Pre	-	-	Tamoxifen	52.4	9.1	-	B, LN	Docetaxel + paclitaxel + trastuzumab + anastrozole	25	No	25	A
15	.90	C	140	+	2009	D	4	3	1	+	-	-	1	2	85%	5%	10%	Uk	-	-	-	56.9	0	Yes	LR, B, O	Tamoxifen	11	Yes	50	D
16	.71	D	7	+	2007	D	2	2	0	-	-	-	11	3	45%	5%	40%	Post	-	-	-	84.8	2.0	Yes	B, P	Pegylated liposomal doxorubicin	3	Yes	3	D
17	.88	C	178	+	2009	L	3	2	0	+	+	-	1	1	40%	20%	20%	Post	-	-	Tamoxifen	75.3	1.0	Yes	O	Exemestane	5	Yes	5	D
18	.77	C	12	+	1991	D	2	0	x	+	+	-	5	2	40%	20%	40%	Pre	-	-	-	66.7	19.7	Yes	B, LN, L	FAC + anastrozole	8	Yes	8	D
19	.76	C	64	+	2006	D	2	1	x	+	+	-	13	3	80%	5%	5%	Pre	-	-	TAC + tamoxifen	41.1	2.6	Yes	B, LN, L	Paclitaxel + capecitabine + trastuzumab	10	Yes	24	A
20	.70	D	124	+	2009	D	3	1	1	-	-	-	5	2	80%	5%	15%	Post	-	-	-	53.5	0	Yes	B, LN, L	Paclitaxel + trastuzumab	3	Yes	6	D
21	.58	D	20	+	2011	L	4	1	1	+	+	+	1	1	75%	5%	20%	Post	-	-	-	59.0	0	-	B	Docetaxel + trastuzumab + anastrozole	14	Yes	26	A
23	.79	C	159	+	2007	D	1	0	x	+	+	+	22	3	35%	20%	40%	Pre	-	-	-	50.5	4.2	Yes	B, LN, L	Docetaxel + trastuzumab	5	Yes	16	D

AC = adriamycin, cyclophosphamide; BR = Bloom & Richardson; CMF = cyclophosphamide, methotrexate, fluorouracil; ER = estrogen receptor; FEC = fluorouracil, epirubicin, cyclophosphamide; HER2 = human epidermal growth factor receptor 2; MAI = mitotic activity index; MBC = metastatic breast cancer; OS = overall survival; PR = progesterone receptor; TAC = doxetaxel, adriamycine, cyclophosphamide; TTS = time-to-treatment-switch; Uk = unknown

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SUPPLEMENTARY TABLE S1 (CONTINUED).

			CTC characteristics		Primary tumor characteristics													Clinical characteristics											
Pt ID	PT-CTC (r)	C/D	CTC count	ER	Year PT	Histology	T	N	M	ER	PR	HER2	MAI	BR	Tumor	Infiltrate	Normal tissue	Menopause (PT)	Second BC	Neoadjuvant tx	Adjuvant tx	Age MBC	PT-MBC (yrs)	Visceral M	Location M	Palliative tx	TTS	2nd-line tx	OS	†
25	.50	D	25	+	2003	D	1	0	x	+	-	+	5	2	60%	15%	25%	Pre	-	-	AC + tamoxifen	36.0	5.8	Yes	B, L	CMF + trastuzumab	10	Yes	30	D
26	.81	C	886	-	2004	D	2	0	x	-	-	-	26	3	60%	30%	10%	Post	Yes (LR)	-	FEC + docetaxel	66.5	3.8	Yes	LN, L	Cisplatin + fluorouracil	1	Yes	1	D
27	.74	D	9	-	2002	D	1	1	x	+	+	+	12	2	75%	10%	15%	Pre	-	-	AC + docetaxel + tamoxifen	43.5	6.1	Yes	B, L	Docetaxel + trastuzumab + sunitinib	19	Yes	59	A
28	.76	C	11	+	2007	D	1	0	x	-	-	-	17	3	80%	5%	15%	Post	-	-	FEC	63.7	2.7	Yes	B, LN, L	Carboplatin + docetaxel + capecitabine	19	Yes	31	D
29	.69	D	16	+	2010	D	2	x	1	+	+	+	3	2	65%	10%	25%	Post	-	-	-	61.3	0	-	B	Tamoxifen	12	Yes	39	A
30	.76	C	67	-	2001	L	3	1	0	+	+	-	0	1	55%	30%	15%	Post	-	-	FEC + tamoxifen	67.0	9.4	Yes	B, LN, O	Exemestane	7	Yes	37	A
31	.72	D	95	+	2011	D	4	1	1	+	+	-	5	2	40%	10%	25%	Pre	-	-	-	34.0	0	Yes	B, L	FEC	22	Yes	32	A
32	.83	C	12	+	2004	D	2	2	0	-	-	-	11	3	30%	60%	5%	Post	-	-	FEC	54.5	3.8	Yes	LN, L	Paclitaxel + bevacizumab	9	Yes	9	D
33	.67	D	52	+	2003	D	1	2	0	+	+	-	19	3	70%	20%	10%	Pre	-	-	AC + tamoxifen	45.0	5.7	Yes	B, P	Docetaxel + bevacizumab	7	Yes	42	D
34	.76	C	1572	+	2000	O	3	1	x	+	uk	+	4	2	60%	30%	10%	Post	-	-	Tamoxifen	69.1	10.6	Yes	B, P, L	Docetaxel + trastuzumab + anastrozole	9	Yes	30	D
35	.81	C	11	+	2008	D	1	0	x	+	-	-	14	3	50%	40%	10%	Post	-	-	-	74.1	2.8	Yes	LN, P, L, O	CMF	1	Yes	1	D
36	.82	C	173	+	2009	D	4	3	1	+	+	-	4	2	30%	10%	10%	Post	-	-	-	71.4	0	Yes	B, LN, P, L, O	Exemestane	10	Yes	15	D
37	.59	D	30	+	2011	L	2	0	1	+	+	+	0	2	55%	25%	10%	Pre	-	-	-	52.4	0	-	B	TDM1 + trastuzumab + pertuzumab	31	No	31	A
38	.82	C	35	+	2004	D	2	0	0	+	+	-	1	2	80%	10%	10%	Post	-	-	Tamoxifen	81.6	7.6	Yes	B, L	Paclitaxel + capecitabine	24	No	24	A
39	.52	D	9	+	2003	D	1	0	x	-	-	uk	8	2	50%	40%	10%	Post	Yes	-	-	79.3	6.2	Yes	LN, P, L	Paclitaxel + tamoxifen	10	Yes	23	D
40	.85	C	50	-	2002	L	4	0	0	+	+	-	0	1	60%	10%	30%	Post	-	-	FEC + anastrozole	72.9	8.1	-	B	Exemestane + everolimus	4	Yes	17	D
41	.75	C	138	+	2003	D	2	0	x	+	+	+	13	3	70%	5%	15%	Pre	-	-	-	47.8	5.9	Yes	B, L	Docetaxel + trastuzumab + tamoxifen	45	Yes	56	A
42	.65	D	95	+	2005	L	2	3	0	+	+	-	3	2	60%	10%	30%	Pre	-	-	AC + tamoxifen	56.8	5.9	Yes	O	Carboplatin + paclitaxel	5	Yes	5	D
43	.55	D	109	+	2004	L	2	2	x	+	+	-	0	2	60%	10%	30%	Pre	-	FEC	Tamoxifen + letrozole	53.1	7.1	Yes	B, L	CMF	10	Yes	24	D
44	.60	D	60	+	2006	D	2	1	0	+	+	-	4	2	35%	30%	35%	Pre	-	-	FEC + docetaxel + tamoxifen	36.6	3.9	Yes	B, L	Exemestane	28	Yes	36	A
45	.75	C	81	-	2009	L	1	x	1	+	-	-	0	2	45%	5%	50%	Post	-	-	-	70.1	0	Yes	B, O	Exemestane	1	Yes	21	D
46	.55	D	157	-	2008	D	2	1	x	+	-	+	5	2	35%	40%	10%	Post	-	-	Exemestane	68.4	1.8	Yes	B, L	CMF + trastuzumab	9	Yes	9	D
47	.84	C	7	+	2002	L	2	1	0	+	+	-	0	2	70%	5%	20%	Pre	-	-	FEC + tamoxifen	54.3	9.5	-	B	Letrozole	21	No	21	A
48	.77	C	19	+	2006	D	2	1	x	+	-	-	7	2	85%	10%	5%	Post	-	-	Tamoxifen + anastrozole	64.0	2.7	Yes	L	FAC + megestrol acetate	9	Yes	13	D
49	.64	D	41	+	2009	O	4	3	1	+	uk	-	22	3	85%	5%	10%	Post	-	-	-	76.7	0	Yes	O	Letrozole	5	Yes	12	D
50	.69	D	753	+	2009	M	1	1	1	+	+	-	19	3	80%	5%	15%	Post	-	-	-	54.0	0	Yes	B, L, O	Letrozole	12	Yes	29	D
51	.85	C	5	+	2006	D	2	0	x	+	+	-	2	2	75%	10%	15%	Pre	-	-	FEC + tamoxifen + letrozole	54.1	3.4	Yes	B, L	Capecitabine + docetaxel	10	Yes	33	D
52	.52	D	12	+	2010	D	4	1	1	+	+	-	1	2	50%	25%	25%	Post	-	-	-	64.3	0	Yes	B, O	Epirubicine + docetaxel	2	Yes	2	D
53	.75	C	35	-	2002	M	1	3	0	+	+	-	0	2	35%	30%	30%	Post	-	-	FEC + tamoxifen + letrozole	66.8	8.4	Yes	B, LN, O	Paclitaxel + intrathecal methotrexate	4	Yes	13	D
54	.81	C	16	+	2003	D	1	2	0	+	+	uk	7	2	75%	14%	10%	Pre	-	-	AC + tamoxifen	45.8	7.3	-	LN	Tamoxifen	22	Yes	31	A
55	.66	D	11	-	2005	M	1	0	x	+	-	+	5	2	35%	45%	20%	Post	-	-	-	59.9	3.7	-	B, LN	Paclitaxel + trastuzumab	8	Yes	58	D
56	.76	C	7	-	2008	O	2	0	x	-	-	-	49	3	55%	30%	15%	Pre	-	-	FAC	32.8	2.1	Yes	LN, P, L, O	Gemcitabine + carboplatin + PARPi	8	Yes	8	D
57	.77	C	47	+	2007	D	1	0	x	+	+	-	4	2	85%	5%	5%	Post	-	-	-	57.5	2.0	-	B	Anastrozole	19	Yes	55	A
58	.88	C	85	+	2008	L	3	x	1	+	+	-	0	1	60%	0%	40%	Post	-	-	-	79.8	0	-	B	Exemestane	4	Yes	4	D
59	.64	D	11	+	1993	M	2	1	x	+	+	-	4	2	70%	10%	10%	Uk	-	-	Tamoxifen	65.3	15.8	Yes	B, L	Exemestane + FGFR1 inhibitor	10	Yes	26	D
60	.80	C	67	+	1998	D	1	1	x	+	uk	-	0	2	80%	5%	10%	Post	-	-	Tamoxifen	62.7	10.6	-	B	Letrozole	17	Yes	43	D
61	.64	D	14	-	2007	D	2	2	x	-	-	-	28	3	65%	20%	10%	Post	-	-	AC + docetaxel	66.2	1.2	Yes	LN, P	Capecitabine	7	Yes	7	D
62	.57	D	6	+	1999	D	2	1	x	+	uk	uk	5	2	30%	60%	10%	Pre	-	-	AC + tamoxifen	53.3	9.7	-	LR, B	Letrozole	42	Yes	56	A
63	.67	D	28	+	2007	L	2	1	0	-	+	-	2	2	50%	5%	45%	Pre	-	-	FEC + tamoxifen	43.1	1.7	-	B	Letrozole	3	Yes	11	D
64	.73	D	5	+	2009	O	2	2	1	+	+	+	8	2	75%	15%	10%	Pre	-	-	-	38.9	0	-	B	Carboplatin + docetaxel + trastuzumab + tamoxifen	32	Yes	52	A
65	.50	D	7	+	2008	D	1	2	0	+	+	+	2	1	30%	10%	50%	Uk	Yes	Letrozol	1. FEC + docetaxel + trastuzumab + letrozole 2. FEC + docetaxel + tamoxifen	71.9	2.2	Yes	L	Paclitaxel + trastuzumab	2	Yes	9	D
66	.58	D	10	-	1998	D	1	1	x	uk	uk	uk	17	3	30%	55%	15%	Pre	Yes	-	AC	59.8	12.8	Yes	B, LN, P	Docetaxel	7	Yes	23	D
67	.79	C	31	+	2011	L	4	1	1	-	-	+	1	2	40%	40%	20%	Post	-	-	-	75.7	0	Yes	LN, O	Paclitaxel + trastuzumab	12	Yes	17	D
69	.84	C	6	+	2009	M	3	2	0	+	-	-	1	2	40%	20%	40%	Post	-	FEC	-	62.7	1.0	Yes	O	CMF	3	Yes	3	D

5

MOLECULAR CHARACTERISTICS OF CIRCULATING TUMOR CELLS RESEMBLE THE LIVER METASTASIS MORE CLOSELY THAN THE PRIMARY TUMOR IN METASTATIC COLORECTAL CANCER

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ABSTRACT

CTCs are a promising alternative for metastatic tissue biopsies for use in precision medicine approaches. We investigated to what extent the molecular characteristics of circulating tumor cells (CTCs) resemble the liver metastasis and/or the primary tumor from patients with metastatic colorectal cancer (MCRC). Methods: patients were retrospectively selected from a prospective study. Using the CellSearch System, CTCs were enumerated and isolated just prior to liver metastasectomy. A panel of 25 CTC-specific genes was measured by RT-qPCR in matching CTCs, primary tumors, and liver metastases. Spearman correlation coefficients were calculated and considered as continuous variables with r=1 representing absolute concordance and r=-1 representing absolute discordance. A cut-off of r>0.1 was applied in order to consider profiles to be concordant. Results: the CTC profiles were concordant with the liver metastasis in 17/23 patients (74%) and with the primary tumor in 13 patients (57%). The CTCs better resembled the liver metastasis in 13 patients (57%), and the primary tumorin five patients (22%). The correlations were not associated with clinical parameters. Nine genes (*CDH1, CDH17, CDX1, CEACAM5, FABP1, FCGBP, IGFBP3, IGFBP4,* and *MAPT*) displayed significant differential expressions, all of which were downregulated, in CTCs compared to the tissues in the 23 patients. Conclusions: in the majority of the patients, CTCs reflected the molecular characteristics of metastatic cells better than the primary tumors. Genes involved in cell adhesion and epithelial-to-mesenchymal transition were downregulated in the CTCs. Our results support the use of CTC characterization as a liquid biopsy for precision medicine.

Introduction

The treatment of metastatic colorectal cancer (MCRC) increasingly depends on the tumor's molecular characteristics. For example, inhibition of the Epidermal Growth Factor Receptor (EGFR) by cetuximab or panitumumab was shown to be futile in the 30-60% of MCRC patients with *KRAS* or *NRAS* mutated tumors, and as such, these treatments are now indicated only for patients with wild-type tumors [1,2]. Other tumor cell characteristics besides gene mutations may further affect patient outcome, as evidenced by a recent study showing the ability of a gene expression profile to predict outcome to chemotherapy in MCRC patients [3]. One may argue that treatment decisions are best based on the composite picture of several molecular features, including DNA mutations and transcription levels.

Blood sampling for circulating tumor cells (CTCs) has widely been proposed as a "liquid biopsy" to guide treatment decisions. In addition to the CTC count, which is strongly prognostic for survival in patients with MCRC as determined by the CellSearch System (Janssen Diagnostics, Raritan, NJ) [4], CTCs are generally thought to provide a real-time picture of different tumor characteristics, including the extent of heterogeneity at specific moments [5]. However, solid proof that CTCs can indeed function as surrogates for metastatic tissue is currently lacking, since research on the biology and predictive value of CTCs is hampered by technical difficulties. The characterization of CTCs is very challenging due to the rarity of CTCs in the circulation and the large background of leukocytes in which they are left even after CellSearch enrichment [6-9]. In this study, we used our previously described approach to reliably measure the expression of tumor-associated genes in CellSearch-enriched CTCs to compare the molecular characteristics of CTCs with the primary tumor and a liver metastasis from patients with MCRC. We investigated whether the characteristics of CTCs taken at the time of metastatic disease were closer to the liver metastasis or the primary tumor and, in this respect, whether or not we can use CTCs as surrogates for metastatic tissue biopsies.

Patients and Methods

Patients

Patients were retrospectively selected from a previously reported prospective clinical trial investigating the prognostic value of CTC enumeration for the one-year recurrence rate in patients with MCRC undergoing a liver metastasectomy [10]. The selection of patients for the current study is shown in Figure 1. The Erasmus MC Review Board approved the study (METC 06-089). All patients provided written informed consent.

Sample collection and processing

Archived formalin-fixed paraffin-embedded (FFPE) primary tumors and liver metastases were collected from pathology laboratories. The High-Pure RNA Paraffin Kit (Roche Applied Science, Penzberg, Germany) was used according to the manufacturer's instructions to isolate RNA from tumors with ≥30% tumor cells on haematoxylin and eosin (HE) staining. The details of blood sampling and processing for the CTC enumeration and characterization have been described before [10,11]. In brief, two samples of 30 mL blood in CellSave (Janssen Diagnostics, Raritan, NJ) and EDTA tubes were taken just prior to liver surgery and processed <24 h using the CellSearch System. The higher volume of blood used to enumerate CTCs from when compared to the usual 7.5 mL was part of the design of the original study and has been described before [11]. After a modified Ficoll density-gradient separation, mononuclear cells were collected and processed by the CellSearch System using the Epithelial Cell Kit for the CTC enumeration and the Profile Kit for the CTC isolation (both kits Janssen Diagnostics, Raritan, NJ). The isolation of mRNA from CTC samples was performed using the AllPrep DNA/RNA Micro Kit (Qiagen, Venlo, The Netherlands).

The gene expression profiles from all the CTC samples from all patients included in the prospective trial were determined in our previous study [9]. A panel of 34 CTC-specific genes was identified and proved to be reliably measurable in CTCs in the background of leukocytes. The genes had been selected based on literature for their association with MCRC development and progression. They were tested for absent or low-level

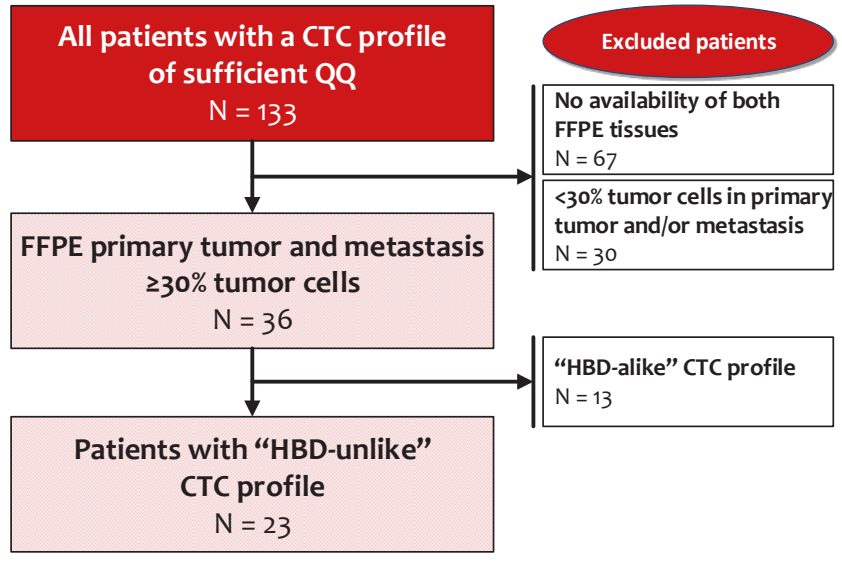


Figure 1. Study flowchart and the selection of patients for the analyses. The selection of patients available with a gene expression profile from the CTCs, the primary tumor, and the liver metastasis was based on the presence of sufficient epithelial signals in the CTC samples, as a measure for the presence of CTCs amongst the leukocytes. Of the 36 patients, 23 were designated as having an "HBD"-unlike and reliably CTC-driven profile. These patients were included in the analyses to compare the gene expression profiles of the CTCs to the primary tumors and the liver metastases.

expression in leukocytes, thereby rendering them measurable in the few CTCs present in the CellSearch-enriched samples. For the current study, we used the same panel of 34 genes for the selected primary tumor and liver metastasis tissues. The Taqman-based RT-qPCR assays used on the CTC samples were tested for performance on FFPE tumor tissue by comparing a separate group of 15 patient-matched fresh-frozen (FF) and FFPE tumor tissues. Only assays with significantly correlating expression levels (linear correlation $r>0.7$, $P<0.05$) were included in the final gene panel, which resulted in 25 of the 34 genes suitable for use in the comparison of the CTC, primary tumor, and metastasis profiles (Table 1).

Next, we selected patients with truly CTC-driven profiles from the total of 36 with available tissue profiles. Stochastic variations occurring in small numbers, such as CTC numbers from blood, limited the use of the CTC count to select patients with presumed circulating tumor content in the blood tube used for profiling. Instead, we constructed

Pt ID	Gene Name	Included in final panel?	CTC - PT		CTC - M		M - PT	
			Mean Δrank	*P*	Mean Δrank	*P*	Mean Δrank	*P*
1	*AGR2*	Yes	-2.39	0.43	-2.09	0.45	-0.3	0.9
2	*AKR1C3*	Yes	-2.3	0.45	-3.39	0.29	1.09	0.73
3	*CD44*	No*						
4	*CDH1*	Yes	-10.52	0.02	-11.26	0.001	0.74	0.85
5	*CDH17*	Yes	-8.17	0.03	-7.91	0.05	-0.26	0.89
6	*CDH5*	Yes	1.48	0.61	1.04	0.66	0.43	0.88
7	*CDX1*	Yes	-11.09	0.001	-11.13	0.004	0.04	0.98
8	*CEACAM5*	Yes	-11.09	0.002	-11.17	0.004	0.09	0.97
9	*COL4A1*	Yes	-3	0.21	-3.04	0.32	0.04	0.98
10	*CXCL1*	Yes	-4.43	0.12	0	1	-4.43	0.19
11	*EGFR*	No*						
12	*FABP1*	Yes	-7.35	0.02	-7.35	0.02	0	1
13	*FCGBP*	Yes	-11.26	0.02	1.13	0.68	-12.39	0.004
14	*GPX2*	Yes	-0.96	0.75	-1.78	0.48	0.83	0.76
15	*HOXB9*	No*						
16	*IGFBP3*	Yes	-11.09	0.003	-11.09	0.002	0	1
17	*IGFBP4*	Yes	-7.61	0.02	-6.43	0.08	-1.17	0.73
18	*IGFBP5*	Yes	-1	0.65	-1	0.63	0	1
19	*KRT19*	Yes	-1.09	0.7	-1.09	0.65	0	1
20	*KRT20*	Yes	-4.26	0.14	-3.61	0.23	-0.65	0.75
21	*KRT8*	No*						
22	*LAD1*	Yes	-2.48	0.38	-2.48	0.22	0	1
23	*MACROD1*	Yes	-1.3	0.72	-2.35	0.45	1.04	0.72
24	*MAPT*	Yes	-14.48	0.001	-12.52	0.003	-1.96	0.52
25	*NQO1*	No*						
26	*PRSS8*	Yes	-1.52	0.54	-1.52	0.51	0	1
27	*RARRES2*	Yes	-5	0.1	-5	0.11	0	1
28	*REG1A*	No*						
29	*S100A16*	No*						
30	*S100P*	Yes	1.17	0.68	3.65	0.17	-2.48	0.06
31	*SATB2*	No*						
32	*SLC6A8*	No*						
33	*TRIM2*	Yes	-5.7	0.08	-5.7	0.06	0	1
34	*TSPAN8*	Yes	-1.96	0.48	-1.3	0.57	-0.65	0.81

TABLE 1 (LEFT PAGE). List of the 34 genes that made up our CTC-specific gene panel that proved to be reliably measurable in CTCs in a background of leukocytes [9]. To allow for comparison between the FF CTC samples and the FFPE tumor samples, all Taqman assays were tested on matching FF and FFPE primary tumors from 15 patients. Only genes with correlating expression levels in the matching tissues (linear correlation r>0.7 and P<0.05) were included in the final gene panel. In total, 25 of the 34 genes were deemed reliably measurable in all samples and tissues and these genes were used to compare the characteristics of the CTCs to the corresponding FFPE primary tumor and liver metastasis. All individual gene expression levels were ranked over the 23 patients per sample and Δranks of one gene between two corresponding samples from a patient were calculated. The mean Δranks for the 25 genes across the 23 patients are shown in columns 4 (mean difference between the CTCs and the primary tumors), 6 (mean difference between the CTCs and the liver metastases), and 8 (mean difference between the primary tumors and the liver metastases). The mean Δranks were then tested by one-sample t tests with 1,000k bootstrapping against the 0 value; the resulting *P* values can be found in the columns 5, 7, and 9. Where there was no significant difference in the average expression of a gene between two samples, the mean Δrank would be close to and not statistically significantly different from 0. CTC= circulating tumor cells; M = metastasis; PT = primary tumor.

an epithelial score comprising the sum of the 34 epithelial genes' measured expression levels in a CellSearch-enriched sample multiplied by the *z*-value from non-parametric comparisons of the median C_q values between the 23 patients with ≥3 CTCs and 30 HBDs from the previous study [9].

$$\sum^{34genes} = -(-2.28*AGR2+2.61*AKR1C3-3.56*CD44+2.28*CDH1-2.53*CDH17-2.73*CDH5-2.68*CDX1-1.95*CEACAM5-2.38*COL4A1+3.09*CXCL1-1.64*EGFR-4.38*FABP1+2.39*FCGBP-3.98*GPX2-1.62*HOXB9+2.5*IGFBP3+2.62*IGFBP4-2.77*IGFBP5-3.1*KRT19-3.34*KRT20-3.69*KRT8-3.74*LAD1+1.08*MACROD1+2.84*MAPT+2.51*NQO1-3.25*PRSS8-1.89*RARRES2-2.21*REG1A-3.94*S100A16+1.94*S100P-2.7*SATB2+2.32*SLC6A8-2.7*TRIM2-3.27*TSPAN8)$$

The epithelial score had a strong correlation with the CTC count from the parallel enumeration blood tube (Spearman r=0.76, *P*<0.001, Figure 2A), indicating that the score did indeed reflect the epithelial input into the PCR. A cut-off score to identify patients with CTC-driven gene expression profiles was then determined from the Receiver Operating Characteristics (ROC) curve of the 23 patients with ≥3 CTCs versus 30 HBDs (Figure 2B). The optimal cut-off yielded a sensitivity of 91% and a specificity of 93% to discriminate patients from HBDs and was used to select patients with an "HBD-unlike" profile for the current study (Figure 2C and D).

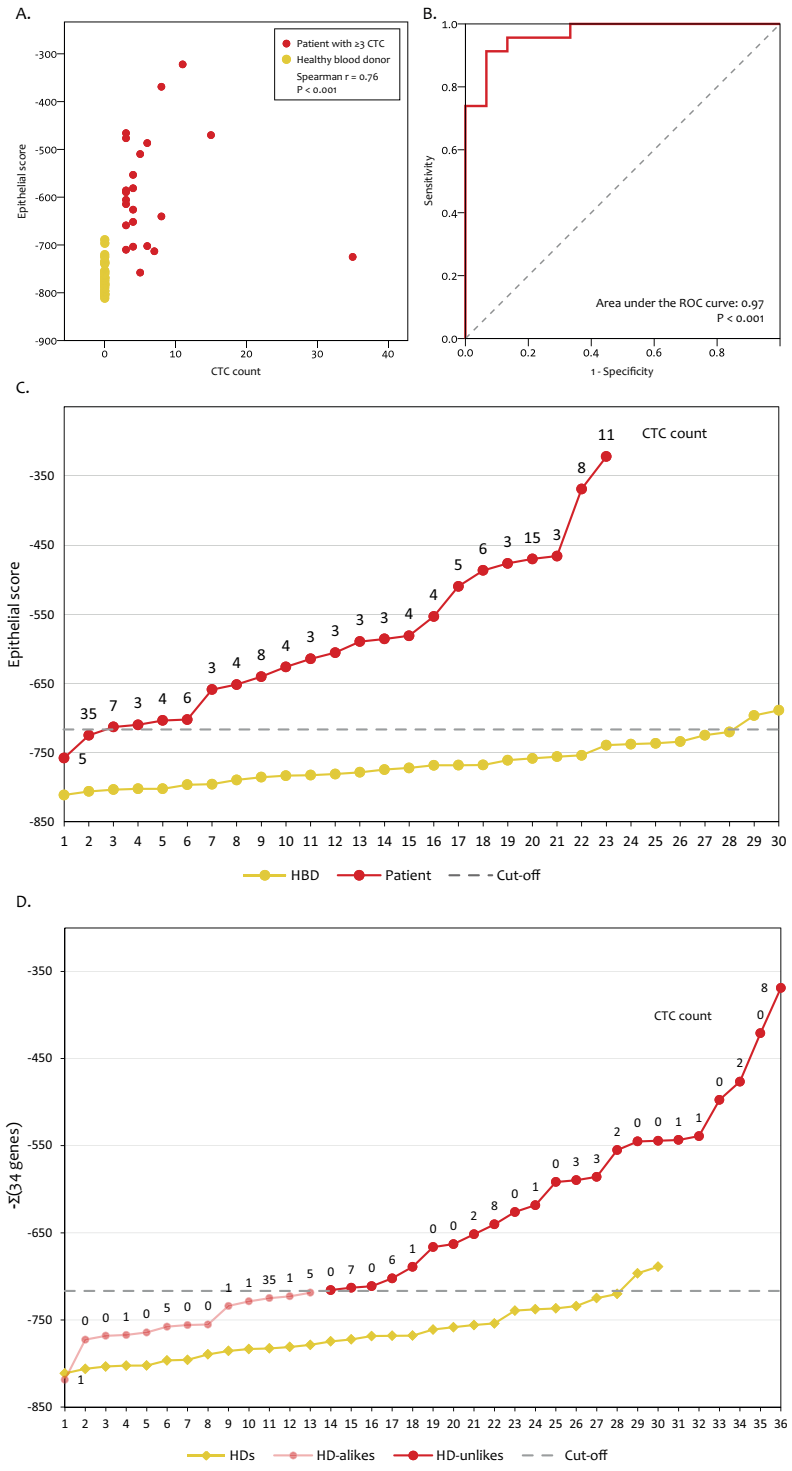


FIGURE 2 (LEFT PAGE). The selection of patients with CTC-driven profiles from the blood samples of the total 36 selected patients. Only patients with sufficient epithelial input were included in the analyses to compare gene expression profiles with CTCs, the primary tumor, and a liver metastasis. A. An epithelial score was calculated by adding the expression levels of the 34 CTC-specific genes multiplied by the z-value from the comparison between 23 patients with ≥3 CTCs and using the 30 HBDs from the prior study [9] as a weighing factor. The epithelial scores from the 23 patients with ≥3 CTCs and the 30 HBDs strongly correlated with the CTC count from the blood tube taken in parallel with the tubes for the characterization of CTCs (r=0.76, *P*<0.001). B. A Receiver Operating Characteristics (ROC) curve was constructed from the epithelial scores of the 23 patients with ≥3 CTCs and the 30 HBDs. The optimal cut-off value resulted in a sensitivity of 91% and a specificity of 93% to discriminate patients from HBDs. C. Line graph showing the epithelial scores of the 23 patients and the 30 HBDs. The dashed line shows the optimal cut-off value from the ROC curve. Two patients were assigned as HBDs, one of whom had a CTC count of 35. Most probably this is the result of a technical error in the enrichment of the CTCs or the gene profiling. Two HBDs had an epithelial score slightly above the cut-off value and were assigned as patients. D. The epithelial scores were calculated for the patients selected for the current study with FFPE primary tumors and liver metastases. Of the 36 patients, 23 had a score above the cut-off and were designated as having an "HBD"-unlike profile. These patients were included in the analyses to compare the gene expression profiles of the CTCs to the primary tumors and the liver metastases.

Normalization and statistical analysis

Three reference genes (*GUSB*, *HMBS*, *HPRT1*) were used as controls for sufficient overall mRNA quality (average reference gene C_q<26 in 92% of the samples in total). Following the ΔC_q method, expression levels were normalized relative to the average C_q of the reference genes [12]. The median ΔC_q of each gene transcript from the 30 HBDs was used as the cut-off to correct for the leukocyte background in the CTC samples, as previously described [7,9]. Different normalization approaches were tested in the first attempt to directly compare the gene expression levels of the CTC and FFPE samples. However, non-measurable levels in the CTC samples distorted these normalization procedures, forcing us to continue non-parametrically by separately ranking the C_q values of individual genes across the patients for the CTC, primary tumor, and liver metastasis samples separately. The three resulting ranks per gene per patient were visualized in heatmaps (Figure 3). Spearman correlation coefficients were calculated between the 25 gene profiles and considered as continuous variables with r=1 representing absolute concordance and r=-1 representing absolute discordance. A cut-off value to cite two profiles as concordant was chosen based on the mean of all correlation coefficients; the mean r was 0.1 and, consequently, all profiles with r>0.1 were considered concordant. Differences between categorical variables were tested by χ^2 or Fisher exact tests. The differences in gene

expression between two samples were tested by one-sample *t* tests. All statistical tests were two-sided and performed with 1,000k bootstrapping to correct for multiple testing; *P*<0.05 was considered statistically significant. The Datan Framework GenEx Pro package version 5.4.1 software (MultiD Analyses AB, Göteborg, Sweden) and SPSS 21.0 (IBM Corporation, Armonk, NY) were used for the analyses. The manuscript was written to conform with the reporting recommendations for tumor marker prognostic studies (REMARK; [13]).

Results

A total of 142 patients were included in the original prospective study investigating the prognostic value of the CTC count [10]. Archived FFPE primary tumor and liver metastasis tissues with ≥30% tumor cells on HE slides were available from 36 patients (Figure 1). However, the calculated epithelial score from the CTC sample was below the cut-off in 13 patients, leaving 23 patients with a reliable CTC-driven gene expression profile suitable for comparison with the primary tumor and liver metastasis (Figure 2D). The characteristics of these patients are shown in Table 2.

To compare the concordance of the three profiles per patient, heatmaps were constructed and Spearman correlation coefficients over the 25 ranks were calculated (Figure 3; Table 3). With a cut-off of r>0.1, the CTC profiles were concordant with the liver metastasis in 17 patients (74%) and with the primary tumor in 13 patients (57%). The primary tumor and metastasis profiles were concordant in 16 of the 23 patients (70%). Comparing the correlation coefficients from the correlation between the CTC versus primary tumor profiles and the CTC versus liver metastasis profiles with an error margin of Δr>0.1, the CTCs more closely resembled the metastasis in 13 patients (57%) and the primary tumor in five patients (22%; Table 3). In the remaining five patients, the Δr was ≤0.1 and/or both coefficients were ≤0.1. In patients 1 and 20, the CTCs neither resembled the primary tumor nor the liver metastasis. In patients 9, 14, and 17, both correlations seemed similar and the CTCs seemed to reflect both the characteristics from the primary tumor as well as the liver metastasis.

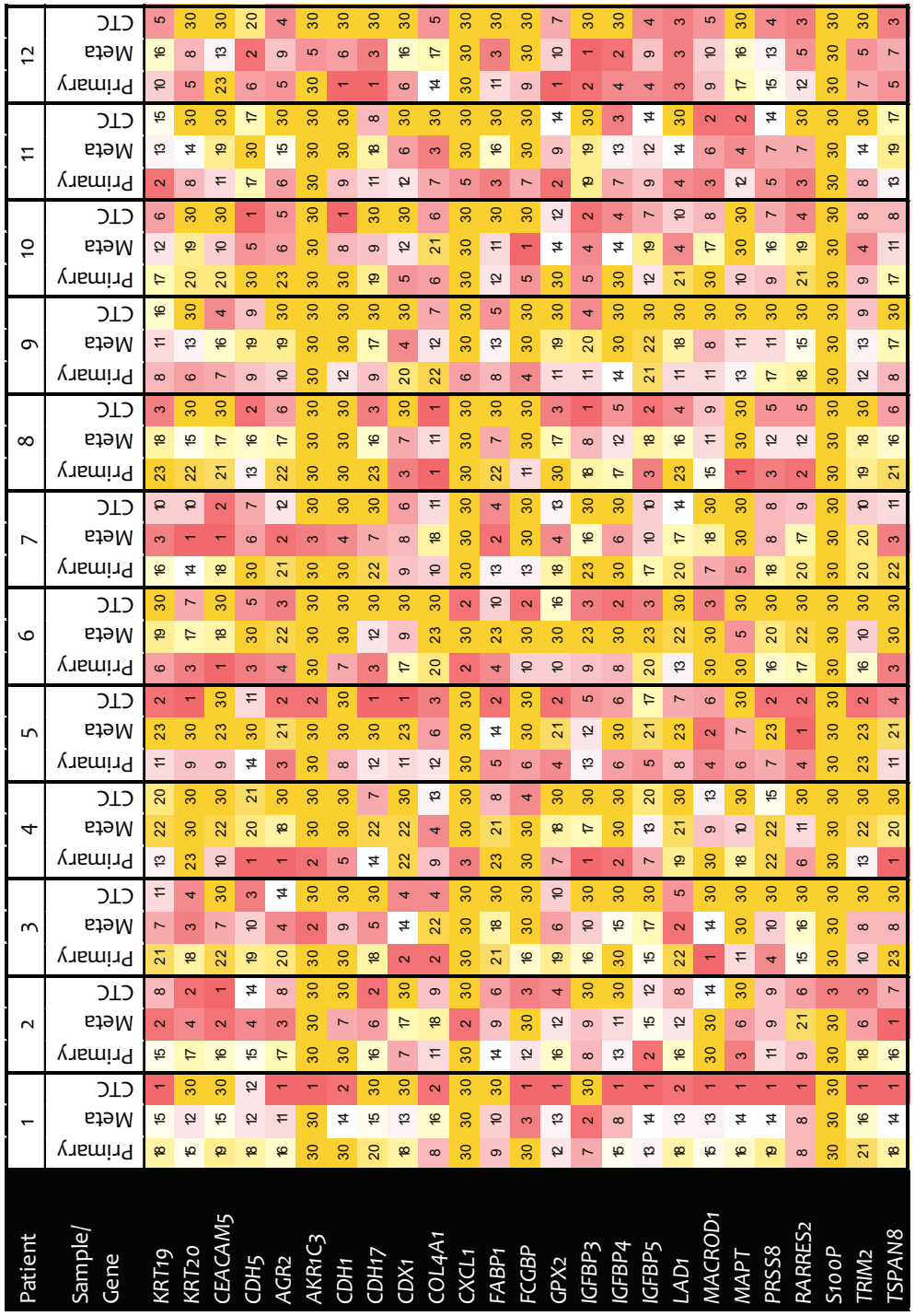
Table 2. Clinicopathological characteristics of the 23 patients with "HBD-unlike" profiles.

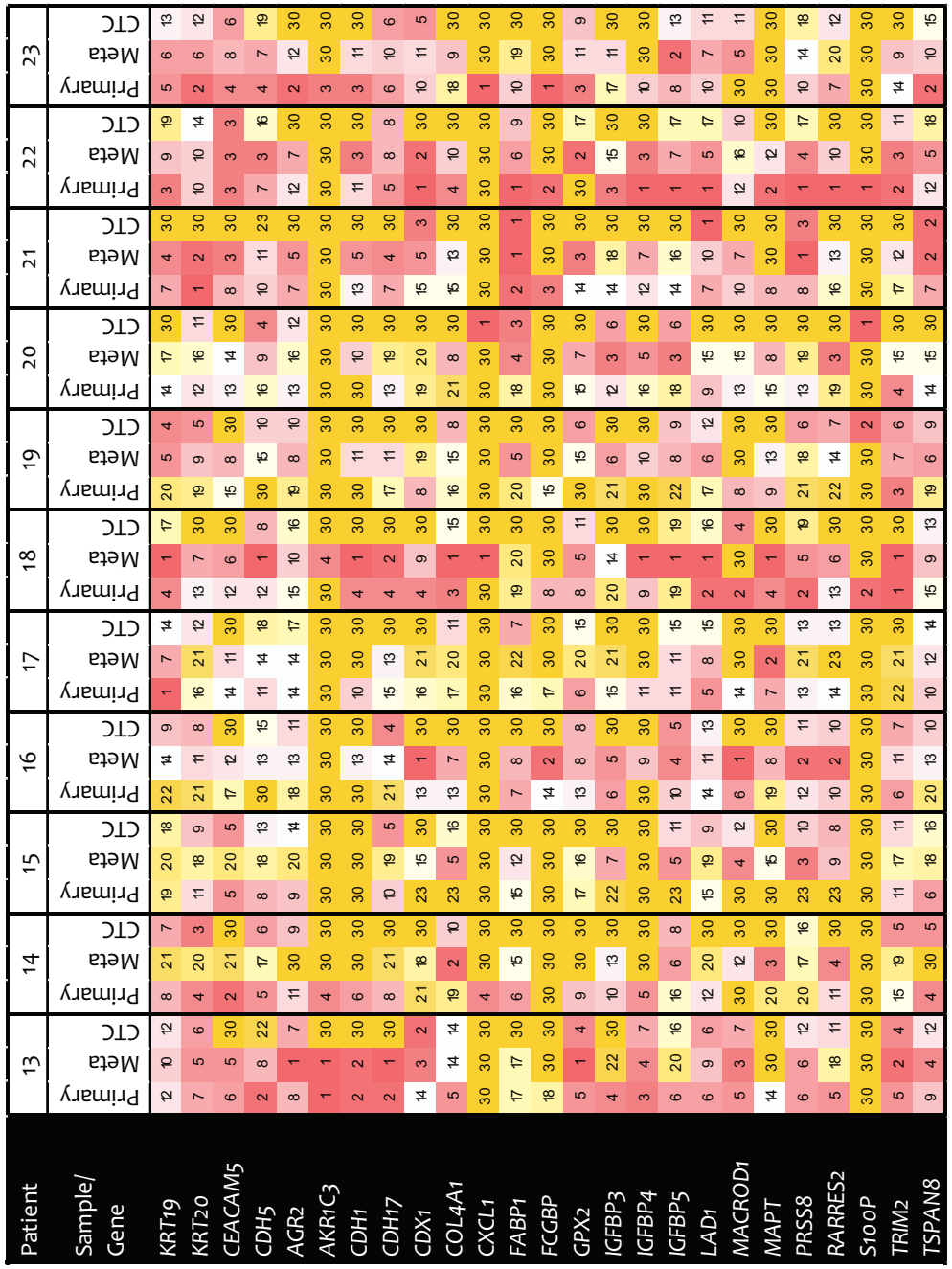
	N	%*
Total	23	100%
Age at inclusion (mean ± sd)	68 ± 10	
Sex (Male / female)	16 / 7	70% / 30%
Location primary tumor		
Right hemicolon	6	26%
Left hemicolon / sigmoid	12	52%
Rectum	5	22%
Staging		
T2	3	13%
T3	16	70%
T4	2	9%
Unknown	2	9%
N0	9	39%
N1-2	11	49%
Unknown	3	11%
Differentiation		
Well differentiated	1	4%
Moderately differentiated	15	65%
Poorly differentiated	1	4%
Unknown	6	26%
Presentation with metastases		
Synchronous	12	52%
Metachronous	11	48%
Median interval (IQR)	25 (17 – 39)	
Liver metastases only	21	91%
Dukes classification at first diagnosis		
A	1	4%
B	4	17%
C	5	22%
D	12	52%
Unknown	1	4%
Prior chemotherapy		
Neoadjuvant	1	4%
Adjuvant	3	11%
Induction	7	30%
Primary tumor in situ at CTC draw	4	17%
Number of CTCs before liver surgery (median, IQR)	1 (0-3)	
≥3 CTCs	6	26%

IQR = interquartile range; sd = standard deviation.
* Percentages do not always add up to 100% due to rounding.

We next examined whether clinicopathological parameters were associated with the strength of the correlations. The primary tumor was still *in situ* at the time of liver surgery and CTC sampling in five patients (Table 3). Here, the CTCs could be theoretically derived from both the primary tumor and the metastases. In two patients, the CTCs seemed to share characteristics with both the primary tumor and the liver metastasis, as defined by a positive correlation of r>0.1 with both the primary tumor and the liver metastasis. In

FIGURE 3. Heatmaps showing the ranks per gene, per sample, per patient. The expression levels for individual genes were ranked per sample over the 23 patients; undetectable expression levels were given a rank number of 30. Red represents higher than median gene expression levels, white represents the median gene expression, and yellow represents expression levels below the median or wholly undetectable.





patients 10 and 19, the CTCs correlated with the liver metastasis only, whereas in patient 18, the CTC characteristics correlated with the primary tumor only. No associations of the correlations' strength were observed regarding time or pattern of presentation with metastasis, the number of metastases, prior chemotherapy, or age (Table 4).

Lastly, we investigated the 25 individual genes for differences in expression levels between the three tumor compartments. For this, we calculated the difference between

Pt ID	Spearman r			CTCs closest to	Clinical parameters				
	PT-CTC	M-CTC	PT-M		CTC count	PT *in situ*	Prior chemo	Presentation with M	Number of M
1	0.08	0.08	0.55	Neither	0	N	N	Synchr	3
2	-0.18	0.12	-0.13	M	2	N	Y	Metachr	1
3	0.17	0.32	-0.21	M	0	N	Y	Synchr	1
4	-0.41	0.17	0.15	M	7	N	Y	Metachr	1
5	0.05	0.12	0.50	M	8	N	Y	Synchr	1
6	0.23	-0.45	0.01	PT	1	N	N	Synchr	1
7	0.33	0.43	-0.10	M	1	N	N	Metachr	1
8	0.24	0.37	0.42	M	0	Y	N	Synchr	2
9	0.20	0.21	-0.01	Both	0	N	N	Metachr	1
10	-0.11	0.28	0.26	M	0	Y	N	Synchr	1
11	0.13	0.42	0.43	M	0	Y	N	Synchr	2
12	0.13	0.03	0.54	PT	0	N	Y	Synchr	7
13	0.05	0.43	0.55	M	2	N	Y	Synchr	2
14	0.15	0.12	-0.38	Both	0	N	Y	Metachr	2
15	0.58	0.30	0.14	PT	8	N	N	Metachr	1
16	0.15	-0.09	0.77	PT	3	N	N	Metachr	1
17	0.25	0.31	0.59	Both	2	N	N	Synchr	2+
18	0.19	0.08	0.12	PT	0	Y	Y	Synchr	4+
19	-0.14	0.13	0.16	M	1	Y	N	Synchr	1
20	-0.15	0.04	0.16	Neither	0	N	N	Metachr	1
21	0.33	0.44	0.58	M	3	N	N	Metachr	2
22	-0.02	0.35	0.16	M	6	N	Y	Metachr	>10
23	0.06	0.56	-0.06	M	1	N	Y	Metachr	3

Table 3. Correlation coefficients from Spearman correlation analyses comparing the ranked 25 gene profiles from the CTCs, the primary tumor, and the liver metastasis per patient. The cut-off value of r>0.1 was used to consider two profiles concordant. To assess whether a CTC profile was closer to the liver metastasis than to the primary tumor, the difference between the correlation coefficients of the CTCs versus the primary tumor and the CTCs versus the liver metastasis had to be >0.1. The clinical parameters tested for the associations with the strength of correlation have been specified per patient. CTC= circulating tumor cells; M = metastasis; PT = primary tumor; Synchr = synchronous; Metachr = metachronous.

the ranks of two samples (Δrank) per gene per patient and the mean of the Δranks over the 23 patients. This resulted in three mean Δranks per gene (CTC-primary tumor, CTC-metastasis, metastasis-primary tumor; Table 1). In an instance where a gene was not differentially expressed between two tumor compartments, the mean Δrank would be close to and not statistically significantly different from zero. A one-sample *t* test against 0 was applied to determine whether genes were significantly over- or under-expressed (Table 1). The expression levels between the primary tumor and the liver metastases were overall similar; only *FCGBP* was downregulated in the liver metastases. In the CTCs, however, a larger number of genes was downregulated. In comparison to the primary tumor, the expression of *CDH1*, *CDH17*, *CDX1*, *CEACAM5*, *FABP1*, *FCGBP*, *IGFBP3*, *IGFBP4*, and *MAPT* were downregulated. Compared to the liver metastases, downregulations of the same genes were observed, with the exceptions of *FCGBP* and *IGFBP4*.

	N	CTC - PT		CTC - M		M - PT	
		Mean r	*P*	Mean r	*P*	Mean r	*P*
Mean all patients	23	0.10		0.21		0.23	
Synchronically metastasized	11	0.11	0.90	0.18	0.50	0.33	0.12
Metachronically metastasized	12	0.09		0.24		0.12	
Solitary metastasis	12	0.06	0.33	0.13	0.10	0.14	0.18
Multiple metastases	11	0.14		0.29		0.32	
Mean primary tumor in situ	5	0.06	0.60	0.26	0.47	0.28	0.47
Mean primary tumor resected	18	0.11		0.19		0.21	
Prior chemotherapy received	10	0.02	0.10	0.23	0.69	0.12	0.17
No chemotherapy received	13	0.16		0.19		0.30	
Linear correlations							
Age	23	0.27	0.22	-0.02	0.94	-0.15	0.49
Interval between surgery for PT and M	12	0.16	0.61	0.24	0.45	-0.44	0.15

Table 4. Associations between clinical parameters and the strength of the correlation between two tumor samples (CTCs versus primary tumor, CTCs versus liver metastasis, or liver metastasis versus primary tumor). For the categorical variables, the reported r values are the mean correlation coefficients from the Spearman rank correlation of the 25 gene profiles. The *P* values are from independent samples *t* tests. For the continuous variables of age and interval between the two surgeries, the reported r and *P* values are from linear correlations between the variables and correlation coefficients from the 25 gene profiles. CTC= circulating tumor cells; M = metastasis; PT = primary tumor.

Discussion

In this study, we observed that the molecular characteristics of CTCs obtained just prior to liver metastasectomy well reflected the characteristics of (one of) the liver metastasis and were generally closer to the metastasis than the primary tumor in patients with MCRC. Based on the expression of 25 CTC-specific and tumor-associated genes, we found the CTC profiles to correlate with the liver metastasis in 74% of the patients and with the primary tumor in 57% of the patients. No associations were observed between the strength of the correlations and clinicopathological characteristics.

To gain insight into the molecular changes occurring during tumor progression, we investigated the differences in the expression levels of the 25 individual genes between the three tumor compartments. Nine genes were downregulated in the CTCs, three of which (*CDH1*, *CDH17*, *CEACAM5*) are involved in cell adhesion. Downregulation of *CDH1*, encoding E-Cadherin, is a well-recognized event in the progression of epithelial cancers and the induction of epithelial-to-mesenchymal transition (EMT) [14,15]. The loss of epithelial markers, including E-cadherin, together with an overexpression of mesenchymal markers has been consistently observed in CTCs and is thought to reflect EMT as a means for CTCs to survive in the circulation [16-19]. Downregulation of insulin growth factor binding proteins 3 (*IGFBP3*) and 4 (*IGFBP4*), both proliferation-inhibiting and apoptosis-inducing factors, may help CTCs to survive [20]. Additionally, *IGFBP3/4* may play a role in EMT through interactions with the EMT-inducer transforming growth factor β (TGF-β) [20,21]. The significance of the downregulation of *CDX1*, *FABP1*, and *MAPT* in CTCs is unknown, although associations between the losses of these genes and the development and progression of colon cancer have been described [22-29]. Altogether, most of the downregulated genes in the CTCs seem to act as tumor suppressors, cell adhesion molecules, or have an involvement in EMT, a process that has well-acknowledged relevance for the survival and dissemination of CTCs [14,15]. The observed downregulations thus seem to have a functional role in CTC biology.

Several studies have compared the characteristics of CTCs to the primary tumors in different solid tumors, including MCRC. For example, mutations in the *KRAS* oncogene were found to be discordant between CTCs and primary tumors from MCRC patients in 6-55% of patients [30-34]. This discordance has been interpreted as tumor heterogeneity and a reflection of the characteristics of metastatic lesions instead of the primary tumor by the CTCs. However, solid proof that CTCs can indeed function as surrogates for metastatic tumor cells and thus prove to be a reliable alternative for tissue biopsies is lacking. Few studies have made direct comparisons between CTCs, the primary tumor, and distant metastatic tissue. In a study on metastatic breast cancer, the expression of the estrogen receptor was concordant between the CTCs and the primary tumor in 15 of the 22 (68%) patients and between the CTCs and the metastases in 10 of the 12 (83%) patients [35]. Notably, in the two patients where the metastasis was discordant from the primary tumor, the CTCs reflected the characteristics of the metastasis. In MCRC, the profiles from single CTCs – obtained with a micromanipulator after CellSearch-enrichment, followed by whole genome amplification, array comparative genomic hybridization and ultradeep sequencing – were compared to the primary tumors and distant metastatic sites of three patients [36]. In one patient, the copy number profile of a single CTC was 73% concordant with the liver metastasis, and 70% with the primary tumor. In the second patient, the CTCs were much closer to the primary tumor, while in the third patient all three profiles closely matched. These results seem comparable to the results from our study in that they support the hypothesis that CTCs are representative for metastatic tissue.

Still, our analyses should be considered exploratory since formal statistical analyses were restricted by the sample size and lack of preliminary data needed for upfront power calculations. Technical issues – mainly caused by the rarity of CTCs in the blood stream and the leukocyte contamination even after CellSearch enrichment – limited the number of genes that could be measured and compared. Nevertheless, we were able to build a CTC-specific gene panel through selection of MCRC-associated genes from literature and testing for absent or low-level expression in leukocytes. Tumor heterogeneity and

sampling bias could also be an influence on the results. Only one liver metastasis was profiled per patient, even from patients in whom multiple metastases were present. The number of CTCs that were detected was low and, due to stochastic variations, only a subset of CTCs from the total circulating CTC pool may have been interrogated. Furthermore, the biological behaviors of tumor subclones may differ, whereby smaller, but more aggressive clones may shed more CTCs than an abundant, but more indolent clone, which might be overrepresented in a tissue biopsy. To address the aforementioned issues, future studies should incorporate more extensive sampling of tumor tissues and compare the profiles to single CTC profiles, preferably though an RNA sequencing approach to gain better insight into oncogenic and mutagenic genes and pathways.

In conclusion, CTCs from the majority of patients with MCRC reflected the characteristics of the liver metastasis, supporting the use of CTCs as a surrogate for metastatic biopsies. The CTCs, overall, resembled the molecular characteristics of the liver metastasis better than the primary tumor. Several CTC-specific changes occurred and seemed to primarily represent EMT-related downregulations of cell-adhesion and tumor suppressor genes, which could have a biological function for CTC survival and migration. Our results support the hypothesis that CTCs may become a valuable tool for precision medicine by functioning as a liquid biopsy and providing real-time information on tumor characteristics.

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6

EFFICACY OF CABAZITAXEL IN CASTRATION-RESISTANT PROSTATE CANCER IS INDEPENDENT OF THE PRESENCE OF *AR-V7* IN CIRCULATING TUMOR CELLS

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ABSTRACT

The presence of the androgen receptor splice variant 7 (*AR-V7*) in circulating tumor cells (CTCs) from patients with metastatic castration-resistant prostate cancer (MCRPC) was recently demonstrated to be associated with resistance to abiraterone and enzalutamide. Cabazitaxel might, however, remain effective in *AR-V7*-positive patients. Objective: to investigate the association between *AR-V7* expression in CTCs and resistance to cabazitaxel. Design, setting, and participants: we selected patients with MCRPC from the multicenter, randomized, phase 2, open-label study in MCRPC on the pharmacodynamic effects of budesonide on cabazitaxel (Jevtana; CABARESC). Before the start of the first and third cabazitaxel cycle, CTCs were enumerated using the CellSearch System. In patients with ≥10 CTCs in 7.5 mL blood at baseline, the expression of AR-V7 was assessed by quantitative polymerase chain reaction. Outcome measures and statistical analysis: the primary endpoint was the assocation between the *AR-V7* status and the CTC response rate (decrease to fewer than five CTCs in 7.5 mL blood during treatment). Secondary endpoints were the prostate-specific antigen (PSA) response rate (RR) and overall survival (OS). Analyses were performed using chi-square and log-rank tests. Results and limitations: *AR-V7* was detected in 16 of 29 patients (55%) with ≥10 CTCs and was more frequently found in abiraterone pre-treated patients (5 of 5 (100%) treated versus 7 of 20 (35%) untreated; *P* = 0.009). We found no differences in CTC and PSA RR. The presence of *AR-V7* in CTCs was not associated with progression-free survival (hazard ratio (HR) 0.8; 95% confidence interval (CI) 0.4-1.8) or OS (HR 1.6; 95% CI 0.6-4.4). Conclusions: the response to cabazitaxel seems to be independent of the *AR-V7* status of CTCs from MCRPC patients. Consequently, cabazitaxel might be a valid treatment option for patients with *AR-V7*-positive CTCs.

Introduction

Several new treatment options have become available for patients with metastatic castration-resistant prostate cancer (MCRPC). Abiraterone and enzalutamide, both acting on androgen receptor (AR) signaling, improve overall survival (OS) both in the pre- and post-docetaxel setting [1-6]. Cabazitaxel, the next-generation taxane, has been developed to overcome docetaxel resistance and improves OS in MCRPC patients pretreated with docetaxel [3,7]. With the arrival of these treatments, the question of how to optimally sequence treatment lines for MCRPC patients has arisen. Preclinical and clinical data indicate cross-resistance between abiraterone, enzalutamide, and docetaxel [8-12]. However, patients pretreated with abiraterone, enzalutamide, and docetaxel still appear to benefit from cabazitaxel [7,13,14]. Reliable predictive factors reflecting tumor characteristics in real-time are thus urgently needed to guide treatment selection.

A circulating tumor cell (CTC) count from peripheral blood before and during treatment is an independent prognostic factor for progression-free survival (PFS) and OS in MCRPC, and it outperforms prostate-specific antigen (PSA) measurements as an early treatment response marker [15-19]. The presence of the AR splice variant 7 (*AR-V7*), coding for a truncated and constitutively active androgen receptor (AR), in CTCs has been found to be associated with resistance to enzalutamide and abiraterone but not to taxanes, mainly docetaxel [20,21]. We investigated the association of *AR-V7* in CTCs with the response to cabazitaxel in docetaxel pretreated MCRPC patients. We set up a highly specific reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay to measure messenger RNA (mRNA) expression levels of wild-type AR (*AR-WT*) and *AR-V7* in CTCs enriched by the CellSearch System (Janssen Diagnostics LLC, Raritan, NJ, USA). Extensive and robust data are available concerning the clinical relevance of CTCs enumerated by this relatively widely available US Food and Drug Administration (FDA)-cleared technique. Next, we explored associations between the presence of *AR-V7* in CTCs taken before the start of cabazitaxel and the outcome to cabazitaxel.

Patients and Methods

Patients

Patients with MCRPC were recruited from an ongoing multicenter, randomized phase 2 trial, investigating the effects of budesonide on cabazitaxel toxicity (CABARESC, Dutch Trial Registry no. NTR2991). All patients had progressive disease after docetaxel (three rising PSA measurements ≥2 weeks apart, PSA rise ≥2.0 µg/L, or radiologic progression). Full inclusion criteria are listed in Supplement 1. All patients received 25 mg/m^2 of cabazitaxel until progression, unacceptable toxicity, or the maximum of 10 cycles. The collection of CTC samples was a side study of the CABARESC trial. For this study, we selected patients who had been included between August 2012 and August 2014 with ≥10 CTCs in 7.5 mL blood before the start of cabazitaxel to ensure robust and CTC-specific downstream analysis. The Erasmus Medical Center and local institutional review boards approved the study (METC 11-324). All patients provided written informed consent.

Sample Processing

Before the start of the first and the third cycle of cabazitaxel, CTCs were enumerated from 7.5 mL blood drawn in a CellSave tube using the CellSearch System. Characterization of CTCs was done before the first cycle of cabazitaxel from 7.5 mL ethylenediaminetetraacetic acid (EDTA) blood, which was processed using the CellSearch Profile Kit. After RNA isolation, cDNA generation, and preamplification, expression levels of *AR-WT* and *AR-V7* were measured by RT-qPCR in an 11% aliquot of the original starting material using Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA) (Supplementary Table 1). Details on sample processing are available in Supplement 2.

The performance of the assays was tested through the analysis of 17 healthy blood donors (HBDs) and prostate (22RV1, LNCaP, PC3, and VCaP) and breast (CAMA1, MDA-MB-415, MDA-MB-453, MPE600, SUM185PE, and ZR75.1) cancer cell lines (Supplementary Table 1 and 2). A total of 100 cell-line cells were spiked in 7.5 mL HBD blood and CellSearch-enriched to serve as negative and positive controls: 22RV1 ($WT_{high}/V7_{high}$), CAMA1 ($WT_{low}/V7_{neg}$), LNCaP ($WT_{high}/V7_{low}$), MDA-MB-415 ($WT_{low}/V7_{neg}$), MDA-MB-453 ($WT_{low}/V7_{neg}$),

MPE600 ($WT_{low}/V7_{neg}$), PC3 ($WT_{neg}/V7_{neg}$), SUM185PE ($WT_{low}/V7_{low}$), VCaP ($WT_{high}/V7_{high}$), ZR75.1 ($WT_{low}/V7_{low}$). All samples were processed in a similar way to the patient blood samples.

Normalization and Statistical Analysis

Samples with an average cycle threshold for quantification (C_q) <26.5 for the three reference genes (gluceronidase, beta [*GUSB*]; hydroxymethylbilane [*HMBS*]; and hypoxanthine phosphoribosyltransferase 1 [*HPRT1*]) and an average C_q of the two epithelial genes <26.5 (epithelial cell adhesion molecule [*EPCAM*]; and keratin 19, type 1 [*KRT19*]) were considered evaluable. To correct for CTC count and epithelial tumor cell input, C_q values of *AR-V7* and *AR-WT* were normalized to the average C_q value of the epithelial genes (Spearman's r [r_s] with CTC count 0.7; *P*<0.01; Supplementary Figure 1A). Final epithelial tumor cell input in the aliquot of RNA used was calculated using the equation derived from the regression line of the correlation between the epithelial genes and the CTC count, thereby taking into account that only 11% of the originally isolated RNA from all CTCs in the sample was used for the characterization of *AR-V7* status (Supplementary Figure 1A). A cut-off value for positivity for *AR-V7* was determined based on the cell line and HBD experiments (Supplement 2).

The primary endpoint of this study was to compare the CTC response rate (CTC RR), defined as a decrease to fewer than five CTCs in 7.5 mL blood during treatment, between patients with *AR-V7*-positive and *AR-V7*-negative CTCs. Secondary objectives were the PSA RR (30% or 50% decline in PSA level from baseline to 12 weeks or earlier in case of treatment discontinuation), best PSA response during treatment, PFS (interval between registration and progression of disease or death), and OS (interval between registration and death). Associations between PFS or OS and the CTC response during treatment were analyzed after the second blood draw. Patients without events were censored at the last date recorded to be progression-free and/or alive. Reported end points were based on the Prostate Cancer Working Group 2 (PCWG2) guidelines [22].

The main hypothesis stated that there would be no difference in response to cabazitaxel by the presence or absence of *AR-V7*. Since limited data regarding the prevalence of *AR-V7* was available at the time of study design, no formal sample size calculations were performed. Therefore, our analyses were exploratory. Differences in the primary objective, CTC RR, and secondary objective, PSA-RR, were analyzed using the chi-square or Fisher exact tests. Survival was analyzed using Cox regression models and visualized in Kaplan-Meier plots. Other applied tests were the Student *t* test, the Mann-Whitney *U* test, and Pearson or the Spearman correlation, depending on the variable or distribution of a variable. All statistical tests were two-sided and performed using the SPSS 21.0 software package (IBM Corporation, Armonk, NY, USA). A *P* value <0.05 was considered statistically significant.

Results

AR-WT and *AR-V7* in CTCs

We first tested the sensitivity and specificity of our assays by comparing RNA fractions isolated from pure and spiked-in breast and prostate cancer cell-line cells before and after CellSearch enrichment (Supplementary Table 1 and 2). The *AR-V7* status could be reliably determined in three or more spiked-in epithelial cells. The cut-off was confirmed in our clinical samples, in which two patients with RNA from three CTCs in the used aliquot were positive for *AR-V7*; none of the patients with fewer than three CTCs were positive for *AR-V7* (Supplementary Table 2). The leukocyte background did not influence the outcomes of our analyses (Supplementary Figure 1B and 3C). Of the 17 HBDs tested, 16 were negative for the expression of *AR-WT* and *AR-V7* (Supplementary Table 2). One 67-year-old male HBD had detectable *AR-WT* in his peripheral blood. Since this donor was anonymous, no follow-up or further diagnostics were done.

We next selected patients with ≥10 CTCs at baseline to limit stochastic variations between the CTC enumeration and isolation tubes and to assure epithelial input. Twenty-nine patients with sufficient RNA quality and quantity and sufficient epithelial cell input in the CTC samples were identified (Figure 1). Table 1 shows all patient characteristics. Five

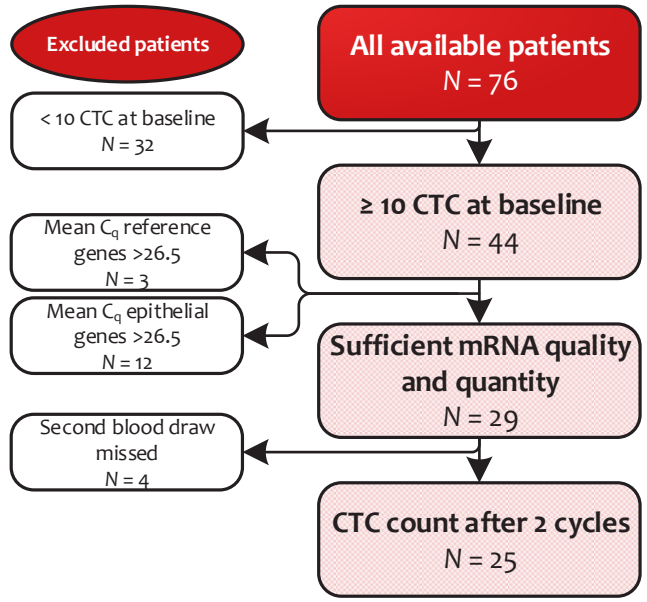

Figure 1. Study flow chart showing the selection of patients for the analyses. C_q = cycle threshold for quantification; CTC = circulating tumor cell.

patients had received abiraterone before enrollment. The expression of *AR-WT* in CTCs was detected in all patients, whereas *AR-V7* was detected in 16 patients (55%). All five patients who had previously been treated with abiraterone expressed *AR-V7* compared to seven of the 20 patients (35%) who had not received abiraterone (*P* = 0.01). We found no significant correlation between the expression levels of *AR-V7* and *AR-WT* in CTCs (r_s = 0.3, *P* = 0.12; Supplementary Figure 2A) and no difference in *AR-WT* expression levels between patients with and without *AR-V7* in the CTCs (*P* = 0.2; Supplementary Figure 2B).

AR-V7 and Response to Cabazitaxel

The primary endpoint of this study was the CTC RR, defined as a decrease to fewer than five CTCs per 7.5 mL blood after two cabazitaxel cycles, determined by the presence or absence of *AR-V7* in baseline CTCs. A secondary CTC sample was available from 25 patients. In three patients, the second draw was missed and one patient died after the second cycle because of a non-disease-related event. The overall CTC RR to cabazitaxel was 5 of 25 patients (20%). Fifteen patients had *AR-V7*-positive and ten had *AR-V7*-

TABLE 1. Patient characteristics in all patients, *AR-V7*-negative patients, and *AR-V7*-positive patients.

	All patients		AR-V7 in CTCs at baseline: Absent		Present		*P* value*
N	29	100%	13	100%	16	100%	
Age at registration (mean ± SD)	70 ± 7		68 ± 9		71 ± 6		0.3
WHO performance score							0.4
0	11	38%	6	46%	5	31%	
1	18	62%	7	54%	11	69%	
Type of castration							0.4
Surgical	4	14%	1	8%	3	19%	
LHRH agonist	25	86%	12	92%	13	81%	
Number of prior chemotherapy lines							0.9
One (docetaxel)	27	93%	12	92%	15	94%	
Two	2	7%	1	8%	1	6%	
Prior antiandrogens for MCRPC							
Abiraterone	5	17%	0	0%	5	31%	0.009
Orteronel	3	10%	3	23%	0	0%	0.09
Baseline chemistry**							
Lactate dehydrogenase (U/L , median (IQR))	453 (309 – 635)		431 (310 – 616)		456 (287 – 674)		0.9
Alkaline phosphatase (U/L , median (IQR))	163 (106 – 375)		160 (96 – 358)		228 (107 – 384)		0.7
Prostate specific antigen (µg/L, median (IQR))	321 (76 – 649)		107 (68 – 439)		475 (78 – 885)		0.08
Baseline CTC count (median (IQR))	100 (50 – 243)		94 (38 – 260)		110 (52 – 254)		0.6

ALP = alkaline phosphatase; *AR-V7* = androgen receptor splice variant 7; CTC = circulating tumor cell; IQR = interquartile range; LDH = lactate dehydrogenase; LHRH = luteinizing hormone-releasing hormone; MCRPC = metastatic castration-resistant prostate cancer; PSA = prostate-specific antigen; SD = standard deviation; WHO = World Health Organization.

* Reported *P* values are from independent samples Student *t* test (age), nonparametric Mann-Whitney *U* test (baseline chemistry and CTC count) and chi-square tests (categorical variables). IQR: interquartile range; sd: standard deviation.

** Upper limit of normal: LDH, 247 U/L; ALP 114 U/L; PSA 6.4 µg/L.

negative CTCs. The CTC RRs in both *AR-V7*-positive and *AR-V7*-negative patient groups were 20% (Table 2). Sequential PSA levels during cabazitaxel treatment for evaluation of the PSA-RR were available from 26 patients. Five (17%) and three patients (10%) achieved a ≥30% and ≥50% PSA response, respectively, after 12 weeks of treatment. At the end of treatment, the best PSA response was ≥30% in seven patients (24%) and ≥50% in three patients (10%). The 30% and 50% PSA RRs after 12 weeks and at the end of treatment in patients with and without *AR-V7* in CTCs were not statistically different (Table 2, Figure

2). We found no statistical difference in CTC RR and PSA RR between patients that had or had not received abiraterone before cabazitaxel.

AR-V7 and Survival

At the time of analysis, four patients were still receiving cabazitaxel treatment. The median follow-up time from the date of registration for the 12 patients still alive was 7 months (range 2–27 months). The median OS in all 29 patients was 10 months (95% confidence interval (CI) 5-14); median PFS was 5 months (95% CI 2-8). The five patients with a CTC response to cabazitaxel had significantly longer OS than the 20 patients without a CTC response (hazard ratio (HR): 0.1, 95% CI 0.01-0.9; *P* = 0.04), but had a comparable PFS (HR: 0.7, 95% CI 0.2-2.0; *P* = 0.5). The presence of *AR-V7* in CTCs at baseline was not associated with PFS (HR: 0.8; 95% CI 0.4-1.8; *P* = 0.6) or OS (HR: 1.6; 95% CI 0.6-4.4; *P* = 0.4; Figure 3). Treatment with abiraterone before or after cabazitaxel had no influence on OS (Supplementary Figure 3).

A. CTC response

AR-V7	No	Yes
No	8	2
Yes	12	3

Fisher's exact *P* = 1

B. PSA response at week 12

AR-V7	No	≥30%*	≥50%
No	9	3	2
Yes	12	2	1

χ^2 *P* = 0.7

C. Best PSA response

AR-V7	No	≥30%*	≥50%
No	7	4	2
Yes	12	3	1

χ^2 *P* = 0.6

TABLE 2. Presence of *AR-V7* in CTCs at baseline versus (A.) CTC response to cabazitaxel after two cycles, (B.) PSA response after 12 weeks of treatment, and (C.) best PSA response at the end of treatment.

CTC = circulating tumor cell; PSA = prostate-specific antigen.

*Numbers include patients with ≥50% PSA response. Sequential PSA values from three patients were missing (two *AR-V7*-positive and one negative). One *AR-V7*-positive patient discontinued treatment after two cycles of cabazitaxel and was not included in the analysis for PSA response after 12 weeks. The *AR-V7*-negative patient was still undergoing treatment and thus was included only in the analysis for PSA response after 12 weeks.

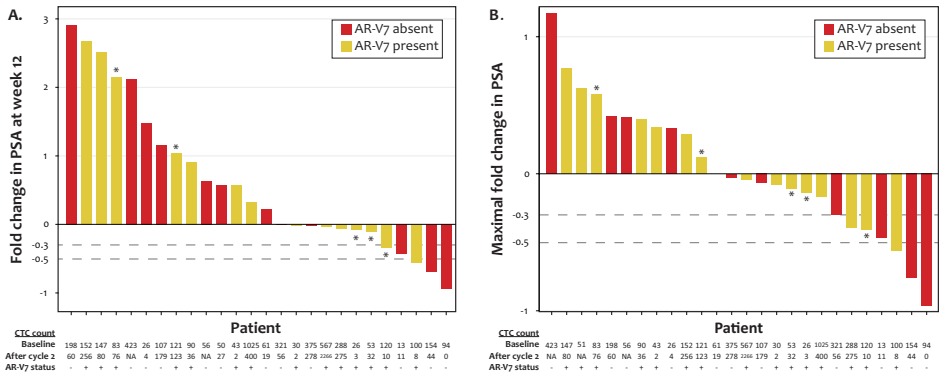

FIGURE 2. Waterfall plots of PSA responses to cabazitaxel treatment (A.) after 12 weeks and (B.) at the end of treatment. The dashed lines represent 30% and 50% decreases in PSA level relative to the baseline level. No differences in PSA responses were observed between *AR-V7*-positive and *AR-V7*-negative patients. Sequential PSA values from three patients were missing (two *AR-V7*-positive and one negative). One *AR-V7*-positive patient discontinued treatment after two cycles of cabazitaxel and was not included in the analysis for PSA response after 12 weeks. The *AR-V7*-negative patient was still undergoing treatment and thus was included only in the analysis for the PSA response after 12 weeks. CTC = circulating tumor cell; PSA = prostate-specific antigen.

* patients who had received treatment with abiraterone before cabazitaxel.

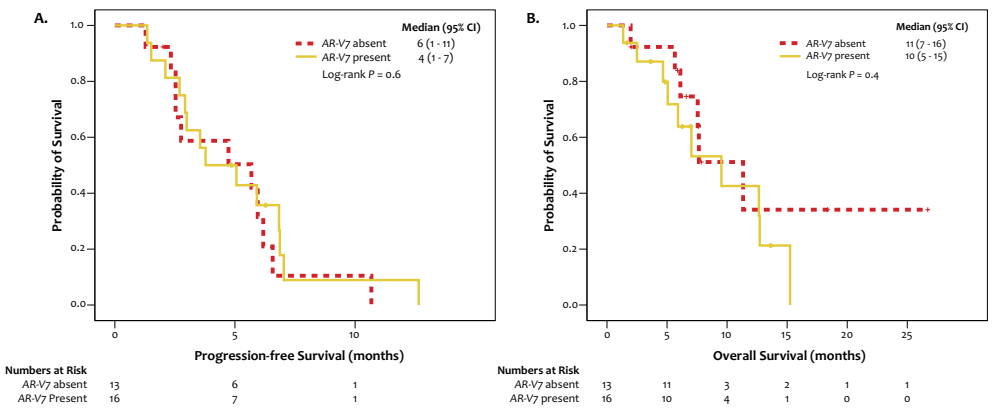

FIGURE 3. (A.) Progression-free and (B.) overall survival as a function of the presence of *AR-V7* in CTCs at baseline. The reported *P* value is from a log-rank test.

AR-V7 = androgen receptor splice variant 7; CI = confidence interval; HR = hazard ratio.

DISCUSSION

The presence of *AR-V7* in CTCs of MCRPC patients is associated with resistance to enzalutamide or abiraterone but not to taxanes [20,21]. In these studies, CTCs were enriched using an mRNA-based method; limited data exist about the method's clinical relevance in MCRPC. We explored the feasibility of the characterization of the presence of *AR-V7* in CTCs captured by the CellSearch System, which obtained FDA clearance for clinical use of the CTC count. We set up a robust RT-qPCR assay that reliably detects *AR-V7* in three or more CTCs and investigated the association between the *AR-V7* status of CTCs and outcome to cabazitaxel. In contrast to docetaxel, no cross-resistance seemed to emerge among cabazitaxel, abiraterone, and enzalutamide [8,14]. Consequently, we hypothesized that patients with *AR-V7*-positive CTCs would still benefit from cabazitaxel.

The prevalence *AR-V7* in our cohort of 29 docetaxel-pretreated MCRPC patients with ≥10 CTCs per 7.5 mL blood was 55%, which seems higher than the 29% in the previously reported enzalutamide/abiraterone cohort [20}, but comparable to the 46% in the prior taxane cohort [21]. We confirmed the higher prevalence of *AR-V7* in abiraterone-resistant patients. In line with our hypothesis, we found indications that the presence of *AR-V7* in CTCs taken prior to treatment might not be associated with the outcomes of cabazitaxel treatment in terms of CTC RR, PFS, and OS. The CTC RR, defined as a decrease to fewer than five CTCs in 7.5 mL during treatment, has been shown to be a robust surrogate end point for PFS and OS in several prior studies [15-19,23].

The lack of an association between *AR-V7* in CTCs and outcome is in agreement with the findings in 37 patients starting treatment with docetaxel (*N*=30) or cabazitaxel (*N*=7) [21], and contrasts with the results in patients treated with enzalutamide or abiraterone [20]. However, comparisons have to be made with caution because of the differences in methodology and patient selection. In the previous studies, CTCs were detected using the AdnaTest (AdnaGen, Langenhagen, Germany), whereas we used the CellSearch System. Both methods immunomagnetically enrich CTCs based on the expression of EpCAM, but there are important differences to consider; for example, the

AdnaTest also enriches CTCs expressing the human epidermal growth factor receptor 2 (HER2). Although frequently detected on breast cancer CTCs [24], the expression and clinical relevance to prostate cancer CTCs is unclear. After enrichment, the CellSearch identifies a CTC as an intact, nucleated cell with expression of cytokeratin as assessed by immunofluorescence. In the AdnaTest, all morphological information is lost after lysis of the enriched cells, so CTC enumeration is not possible. The presence of CTCs is assumed by the presence of the epithelial gene transcripts, thereby disregarding other characteristics such as the presence of a nucleus or intact cell membrane. Considering the differences in methodology, the AdnaTest and the CellSearch System might not detect comparable cell populations. Therefore, we have started a clinical trial to investigate the predictive value of the presence of *AR-V7* in CellSearch-enriched CTCs for outcome to cabazitaxel as well as to AR-targeted treatments.

The limitations of our study concern the CellSearch System's dependency on EpCAM expression on CTCs. In breast cancer, EpCAM-negative CTCs have been detected and have even been reported to be more strongly predictive of treatment resistance [25,26]. Whether this applies to prostate cancer CTCs remains to be investigated. Since only an aliquot of the total of isolated RNA could be used for the current study, patients with ≥10 CTCs – and thus a poor prognosis based on the high baseline CTC count – were selected. Patients with insufficient quality and quantity of mRNA were excluded from the analyses. Although potentially introducing a selection bias, this assured sufficient epithelial input to reliably measure the *AR-V7* status. Additionally, patients were recruited from a phase 2 study investigating cabazitaxel toxicity. As survival was not an end point of the main study and PFS was not defined in the study protocol, our PFS analyses were a composite of PSA, radiographic, and clinical progression, which were assessed at the discretion of the treating physician. This might explain the lack of prognostic value of CTCs for PFS. Last, our analyses were exploratory, because no formal power calculations were possible at the time of study design and only a small number of patients was included. We plan to validate our findings by extending the patient cohort, thereby including patients with <10 CTCs. Ultimately, prospective, randomized trials, taking into account all other baseline characteristics that might affect outcome, should offer insights into the exact role of cabazitaxel in the treatment of *AR-V7*-positive patients.

Conclusions

We demonstrated the feasibility of measuring the *AR-V7* status of MCRPC patients with ≥10 CTCs after CellSearch enrichment. We showed that the outcome of cabazitaxel treatment in these patients is not associated with the presence of this particular splice variant. Our results add important information to the existing evidence that CTCs are an invaluable tool for personalizing cancer treatments and improving the prognosis of MCRPC patients by allowing optimal treatment sequencing.

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Supplement 1.

In- and exclusion criteria for participation in CABARESC trial

Inclusion criteria:

- MCRPC with documented disease progression, defined as:
  - Rising PSA levels: at least two consecutive rises over a reference value and at least one week apart, or a PSA rise of ≥2.0 µg/L
    and/or
  - Appearance of new lesions or documented disease progression on a CT scan or bone scan.
- Previous treatment with docetaxel;
- Age ≥18 years;
- WHO performance status ≤1;
- Adequate renal function (serum creatinine ≤1.5 x upper limit of normal (ULN) and/or MDRD calculated creatinine clearance ≥50 mL/min) and hepatic function (total bilirubin ≤1.0 x ULN, alanine aminotransferase and aspartate aminotransferase ≤2.5 x ULN, or in case of liver metastases ≤5 x ULN, and alkaline phosphatase < 5 x ULN, or in case of bone metastases <10 x ULN), within 21 days before randomization;
- Adequate hematological blood counts (absolute neutrophil count ≥1.5 x 10^9/L and platelets ≥100 x 10^9/L) within 21 days before randomization;
- Castration, either surgically or by continued LHRH agonist therapy;
- Written informed consent according to ICH-GCP;

Exclusion criteria:

- Impossibility or unwillingness to take oral drugs;
- Serious illness or medical unstable conditions requiring treatment, symptomatic central nervous system metastases or history of a psychiatric disorder that would hinder the understanding and obtaining of informed consent;
- Use of medications or dietary supplements known to induce or inhibit CYP3A
- Use of hormonal agents other than GnRH agonists;

- Known hypersensitivity to corticosteroids;
- Any active systemic or local bacterial, viral, or fungal infection;
- Ulcerative colitis, Crohn's disease, or celiac disease (active or in medical history);
- Ostomy;
- Planned/active simultaneous yellow fever vaccine;
- Geographical, psychological, or other non-medical conditions interfering with follow-up.

Supplement 2.

Sample processing, normalization and analysis

Enumeration of CTCs was done from 7.5 mL of blood drawn into a CellSave Preservative tube (Janssen Diagnostics). Blood samples were processed within 96 hours using the Epithelial Cell Kit on the CellSearch System (both Janssen Diagnostics). In this system, epithelial cells are immunomagnetically enriched from whole blood using anti-EpCAM antibodies loaded with ferrofluid nanoparticles. Enriched cells are stained with the nuclear dye 4',6-diamidino-2-phenylindole (DAPI), anti-cytokeratin 8/18/19 labeled with phycoerythrin (PE), and anti-CD45 labeled with allophycocyanin (APC), followed by scanning using the CellTracks Analyzer (Janssen Diagnostics). All cells ≥4 µm, with round-to-oval morphology, positive for cytokeratin and DAPI, with at least 50% overlap in the DAPI and cytokeratin signal, and negative for CD45 were considered CTCs. All samples were analyzed by two independent, trained reviewers.

For molecular characterization of CTCs, 7.5 mL of blood from an EDTA tube was processed using the CellSearch Profile Kit (Janssen Diagnostics) within 24 hours to limit mRNA degradation. No staining step was performed after the immunomagnetical enrichment. Instead, buffer was aspirated after incubation in a hand magnet and enriched cells were lysed in buffer RLT+ (Qiagen, Valencia, CA), followed by storage at -80°C until subsequent RNA isolation using the AllPrep DNA/RNA Micro Kit (Qiagen). Of the resulting 12 µL with >200 bp RNA, 5 µL was used for the generation of 10.5 µL cDNA (RevertAid H Minus First Strand cDNA Synthesis Kit from Thermo Fisher Scientific,

Waltham, MA), followed by an RNAse H step (Ambion, Life Technologies) to degrade the remaining RNA. Next, 3 µL of the cDNA was used to specifically pre-amplify the transcripts generated by the nine Taqman assays depicted in Supplementary Table 1, which was done in 14 cycles according the protocol supplied by the manufacturer of the Taqman PreAmp Master Mix kit (Life Technologies, Carlsbad, CA). Following pre-amplification, the resulting 12 µL sample was 15-fold diluted prior to 35 cycles of RT-qPCR using an Mx3000P Real-Time PCR System (Agilent, Amsterdam, The Netherlands). For each sample, nine individual PCR reactions were performed in duplo in a final volume of 20 µL containing 5 µL diluted, pre-amplified cDNA, 30-50% (V/V) Taqman Universal Master Mix (4326614, Life Technologies), and 0.5-1 µL Taqman gene expression assay, which was done in 35 cycles according the protocol supplied by the manufacturer of the Taqman assays. Altogether, 1.5 µL of RNA from the original sample was used, which was further diluted for cDNA synthesis and pre-amplification, leaving an average aliquot of ~11% of the original starting material to measure the expression levels of *AR-WT* and *AR-V7* using Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA; Supplementary Table 1). The amount of epithelial cell input in the aliquot was calculated per patient using the average signal of *EPCAM* and *KRT19* from the aliquot, which correlated with the CTC count in 7.5 mL blood as assessed by the CellSearch System from the parallel CellSave tube (Spearman r=0.71; *P*<0.01; Supplementary Figure 1A). The final epithelial cell input per patient can be found in Supplementary Table 2. To correct for CTC count and epithelial tumor cell input, C_q values of *AR-V7* and *AR-WT* were normalized to the average C_q value of the epithelial genes *EPCAM* and *KRT19* measured in the same PCR plate as follows: ΔC_q *AR* = average C_q value of *EPCAM* and *KRT19* minus C_q value of *AR-V7* or *AR-WT*. Three reference genes (*GUSB*, *HMBS*, and *HPRT1*) served as internal control of isolated mRNA and cDNA quantity and quality. Samples with an average reference gene C_q value >26.5, indicative for low and/or poor RNA/cDNA quality, and/or an average epithelial gene C_q value >26.5, indicative for low/no epithelial CTC input in the final RNA/cDNA sample, were excluded from the analyses.

Assay performance was tested through analysis of healthy blood donors (HBD) and cell line experiments. All real time PCR assays were equally efficient both before and after pre-amplification (108%±4%, Supplementary Table 1). We measured the expression of *AR-WT* and *AR-V7* in pure cells of prostate (22RV1, LNCaP, PC3, and VCaP) and breast (CAMA1, MDA-MB-415, MDA-MB-453, MPE600, SUM185PE, and ZR75.1) cancer cell lines. These were used as negative and positive controls as follows: 22RV1 (WT_{high}/$V7_{high}$), CAMA1 (WT_{low}/$V7_{neg}$), LNCaP (WT_{high}/$V7_{low}$), MDA-MB-415 (WT_{low}/$V7_{neg}$), MDA-MB-453 (WT_{low}/$V7_{neg}$), MPE600 (WT_{low}/$V7_{neg}$), PC3 (WT_{neg}/$V7_{neg}$), SUM185PE (WT_{low}/$V7_{low}$), VCaP (WT_{high}/$V7_{high}$), ZR75.1 (WT_{low}/$V7_{low}$). Next, 100 cells of the same cell lines were spiked into 7.5 mL HBD blood, followed by CellSearch-enrichment, isolation of RNA, synthesis of cDNA, and PCR similar to the patient samples and as described above. In Supplementary Table 2 the results from these experiments are reported. To be able to assign patients as having *AR-V7*-positive or -negative CTCs, a cut-off value had to be established. As can be deduced from supplementary table 2, the ΔC_q value measured in the weakly positive breast cancer cell line ZR75.1 was -14.68. To keep a certain margin, we decided to set the cut-off for positivity, meaning any detectable AR-V7 signal, at a

Supplementary Table 1. Details of the assays used in the RT-qPCRs.

qPCR name	Approved Gene Symbol	Slope	R^2	Efficiency
		PreAmped PCR on serially diluted cDNA from VCAP cells		
AR-V7	*AR*	-3.02	0.98	107%
AR_WT	*AR*	-2.97	0.97	109%
AR-WT/fl	*AR*	-3.19	0.97	103%
EPCAM	*EPCAM*	-2.97	0.99	109%
KRT19	*KRT19*	-3.13	0.98	104%
GUSB	*GUSB*	-3.01	0.99	107%
HMBS	*HMBS*	-3.11	0.98	105%
HPRT1	*HPRT1*	-3.02	0.98	107%
		PreAmped PCR on cDNA from leukocytes of different HBDs		
CD45	*PTPRC*	-2.71	0.85	117%

Continued on next page

SUPPLEMENTARY TABLE 1 (CONTINUED).

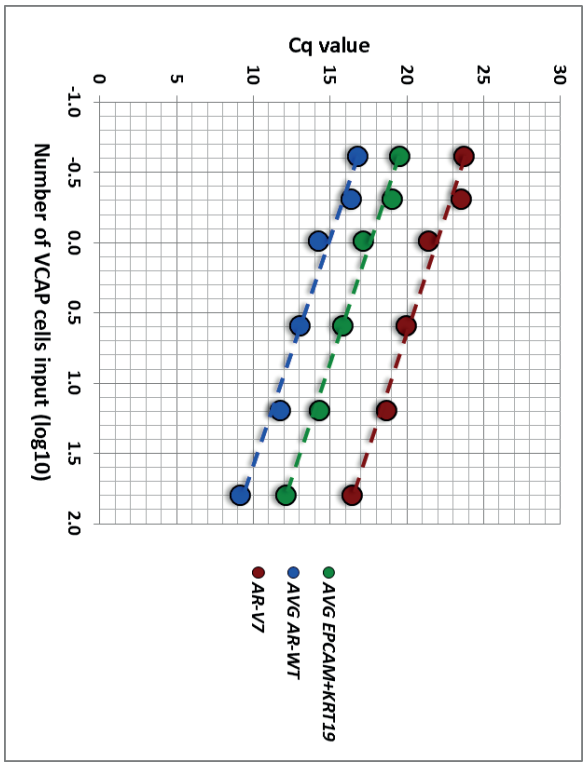
qPCR name	Approved Gene Symbol	Approved Gene Name	Location Sequence	Order info Applied Biosystems	F sequence	R sequence	probe sequence (FAM_MGB1_NFQ)	exon boundery	product size (bp)	Accession code qPCR
AR-V7	AR	androgen receptor	Xq12	AIPAEKK	GTCCATCTTGTCGTCTTC	GCAAGTCAGCCTTTCTTCA	GGGAGAAAAATTCCGGGTTGGC	3-> CE3	117	FJ235916
AR_WT	AR	androgen receptor	Xq12	Hs00171172_m1	*proprietary info ABI*	*proprietary info ABI*	AGGCCTTGCCTGGCTTCCGCAACTT	4 -> 5	72	NM_000044.3
AR-WT/fl	AR	androgen receptor	Xq12	AIRSAWo	CTGCTCAAGACGCTTCTA	ATCATTTCCGGAAAGTCCA	TCCGTGCAGCCTATTGCGAG	7 -> 8	132	NM_000044.3
EPCAM	EPCAM	epithelial cell adhesion molecule	2p21	In house design	AGTTTGCGGACTGCACTTCA	AATACTCGTGATAAATTTTGGATCCA	AAGGAGATCACAACGCGT	4/5 -> 5	72	NM_002354
KRT19	KRT19	keratin 19	17q21.2	Hs01051611_gH	*proprietary info ABI*	*proprietary info ABI*	ACAGCTGAGCATGAAAGCTGCCTTG	5 -> 6	66	NM_002276.4
GUSB	GUSB	glucuronidase, beta	7q11.21	Hs99999908_m1	*proprietary info ABI*	*proprietary info ABI*	TGAACAGTCACCGACGAGAGTGCTG	11 -> 12	81	NM_000181.3
HMBS	HMBS	hydroxymethylbilane synthase	11q23.3	Hs00609297_m1	*proprietary info ABI*	*proprietary info ABI*	ATGCGGCTGCAACGGCGGAAGAAAA	1 -> 2	64	NM_001258208.1
HPRT1	HPRT1	hypoxanthine phosphoribosyltransferase 1	Xq26.2	In house design	TTCCTTGGTCAGGCAGTATAATCC	GGTCCTTTTCACCAGCAAGCT	AGATGGTCAAGGTCGC	6 -> 7	64	NM_000194
CD45	PTPRC	protein tyrosine phosphatase, receptor type, C	1q31-q32	Hs00236304_m1	*proprietary info ABI*	*proprietary info ABI*	AGAGGCTGAATTCCAGAGACTTCCT	26 -> 27	81	NM_002838.4

Continued on next page

SUPPLEMENTARY TABLE 1 (CONTINUED).

ng cDNA input in PreAmped PCR	#VCAP cells input in Pre-Amped PCR*	#VCAP cells input in Pre-Amped PCR (log)	AVG EPCAM +KRT19 (Cq)	AVG AR-WT (Cq)	AR-V7 (Cq)
2.500	62.50	1.796	12.10	9.16	16.42
0.625	15.63	1.194	14.30	11.79	18.71
0.156	3.91	0.592	15.81	13.07	19.99
0.039	0.98	-0.010	17.18	14.26	21.40
0.020	0.49	-0.311	19.03	16.39	23.53
0.010	0.24	-0.612	19.54	16.83	23.71

* A typical mammalian cell contains 10-30 pg total RNA = 20-60 pg cDNA = ~40 pg cDNA
www.sabiosciences.com/newsletter/RNA.html



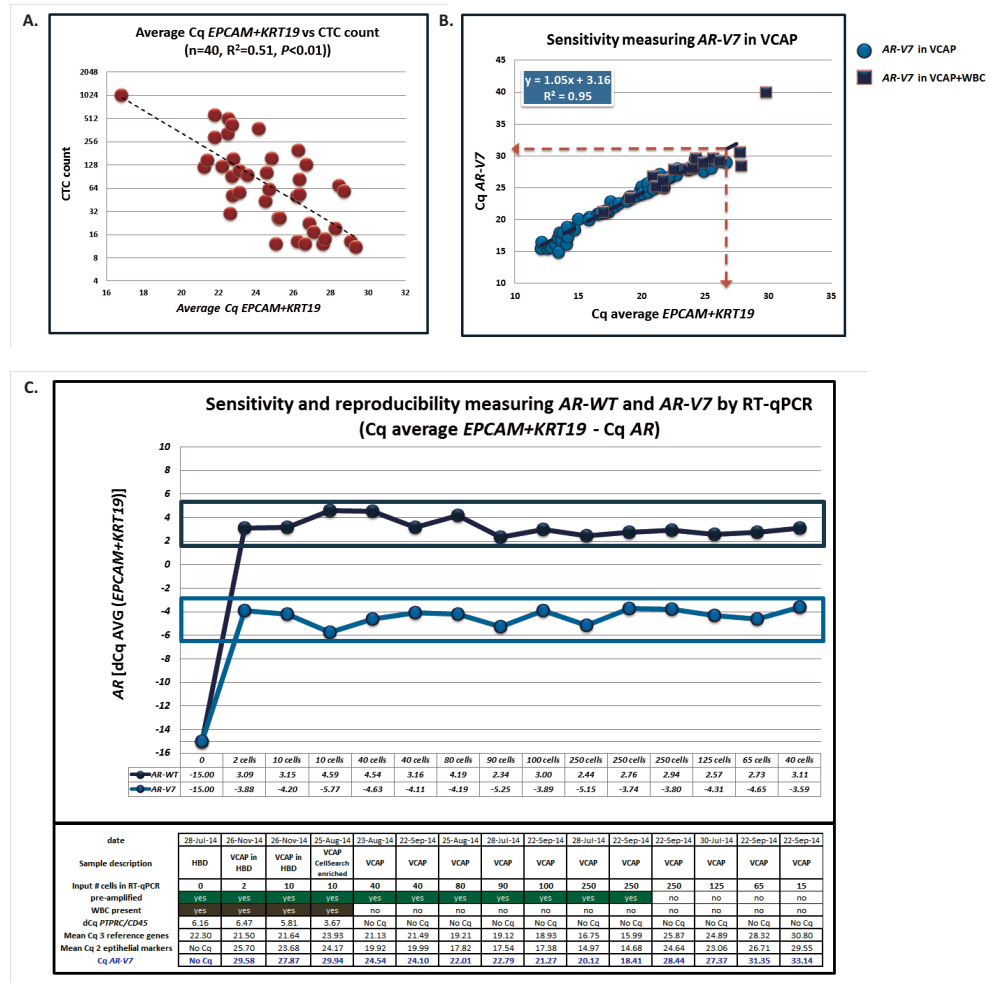
SUPPLEMENTARY TABLE 2 (LEGEND ON NEXT PAGE).

Sample description	Theoretical nr of cell line cells/ CTCs in PCR		Mean Cq reference genes*		Mean Cq epithelial genes**		Calculated nr of epithelial cells in PCR		ΔCq AR-WT*** (Cq epithelial-Cq AR-WT)		ΔCq AR-V7 (Cq epithelial - Cq AR-V7)	
CellSearch enrichment	before	after	before	after	before	after	before	after	before	after	before	after
Cell lines												
PC3	20	2	19.28	25.85	15.86	22.26	28	3	no Cq	no Cq	no Cq	no Cq
MPE600	66	7	20.41	23.86	15.61	23.35	103	7	-7.47	no Cq	no Cq	no Cq
ZR75.1	66	7	17.05	24.28	12.98	22.67	254	9	-3.55	no Cq	-14.68	no Cq
MDA-MB-415	66	7	18.94	23.21	14.62	20.52	145	19	-2.83	-2.55	no Cq	no Cq
CAMA1	66	7	17.03	24.35	13.86	20.97	188	16	-2.75	-2.11	no Cq	no Cq
MDA-MB-453	20	2	21.12	25.07	17.86	23.89	14	2	-1.48	-1.48	no Cq	no Cq
SUM185PE	66	7	18.33	22.49	14.07	20.19	175	21	-0.66	-0.68	-9.90	no Cq
LNCaP	66	7	15.99	23.04	17.92	24.84	46	4	4.14	4.30	-7.79	no Cq
LNCaP	20	2	17.40	24.30	19.58	25.68	8	1	4.06	3.14	-8.09	no Cq
VCaP	66	7	19.21	23.93	17.82	24.17	48	5	4.19	4.59	-4.19	-5.77
22RV1	20	2	19.97	23.55	18.83	23.32	10	2	-0.11	0.12	-4.97	-3.57
Healthy blood donors												
HBD-1		0		23.75		29.26		1		no Cq		no Cq
HBD-2		0		25.34		no Cq		0		no Cq		no Cq
HBD-3		0		28.89		no Cq		0		no Cq		no Cq
HBD-4		0		24.89		29.97		1		no Cq		no Cq
HBD-5		0		18.62		28.79		1		1.45		no Cq
HBD-6		0		22.80		27.44		2		no Cq		no Cq
HBD-7		0		24.03		27.03		2		2.68		no Cq
HBD-8		0		20.70		31.43		0		3.73		no Cq
HBD-9		0		20.57		27.43		2		no Cq		no Cq
HBD-10		0		21.39		26.98		2		-1.73		no Cq
HBD-11		0		17.73		25.19		4		2.11		no Cq
Patients												
CTC1155	68		19.89		16.82		68		0.84		-10.30	
CTC1172	8		23.60		21.24		15		2.31		-8.67	
CTC1167	10		18.20		21.41		14		1.79		-7.98	
CTC1184	38		22.47		21.82		12		0.50		-6.93	
CTC1170	19		22.45		21.82		12		3.32		-8.61	
CTC1143	7		21.93		22.20		11		3.31		-7.86	
CTC1175	21		21.22		22.52		10		-0.08		no Cq	
CTC1186	34		23.43		22.54		9		3.34		-7.79	
CTC1152	2		18.12		22.65		9		0.32		-10.00	
CTC1183	6		18.99		22.74		9		4.52		-8.01	
CTC1151	28		25.29		22.74		9		-2.42		no Cq	
CTC1147	3		17.72		22.77		9		-0.71		-7.24	
CTC1180	10		19.07		22.80		9		-0.12		-8.10	
CTC1159	4		24.73		23.12		8		-0.21		no Cq	
CTC1169	7		19.64		23.12		8		1.17		no Cq	
CTC1134	6		24.83		23.57		7		3.96		no Cq	
CTC1160	25		25.24		24.16		5		2.51		no Cq	
CTC1181	3		24.95		24.51		5		2.21		-6.31	
CTC1164	7		24.32		24.58		5		3.29		-6.41	
CTC1148	4		25.19		24.72		4		-0.50		no Cq	
CTC1179	10		24.57		24.84		4		0.59		no Cq	
CTC1166	1		20.54		25.08		4		1.14		no Cq	
CTC1156	2		21.56		25.20		4		3.11		no Cq	
CTC1157	2		21.94		25.27		4		3.38		-6.20	
CTC1176	3		22.57		26.21		3		3.89		no Cq	
CTC1174	1		24.65		26.23		3		0.48		no Cq	

Continued on next page

Sample description	Theoretical nr of cell line cells/ CTCs in PCR		Mean Cq reference genes*		Mean Cq epithelial genes**		ΔCq AR-WT*** (Cq epithelial-Cq AR-WT)		Calculated nr of epithelial cells in PCR		ΔCq AR-V7 (Cq epithelial-Cq AR-V7)	
CellSearch enrichment	before	after	before	after	before	after	before	after	before	after	before	after
CTC1168		13		26.11		26.28		1.56		3		no Cq
CTC1161		4		20.41		26.33		1.03		3		-4.53
CTC1171		5		23.79		26.36		4.62		3		-5.01
Patients not used in the final analysis due to a too low RNA and/or epithelial input (Cq > 26.5) in the final RT-qPCR												
CTC1145		3		26.28		28.72		2.54		1		no Cq
CTC1149		1		27.40		29.49		1.50		0		no Cq
CTC1165		3		27.46		26.69		3.35		0		no Cq
CTC1185		1		24.64		26.65		-8.30		2		no Cq
CTC1182		8		23.92		26.70		-0.15		2		no Cq
CTC1153		1		22.68		26.87		-0.89		2		no Cq
CTC1162		1		22.16		27.10		1.49		2		no Cq
CTC1158		8		27.70		27.14		-0.28		0		no Cq
CTC1163		1		19.85		27.61		1.46		2		no Cq
CTC1154		1		21.29		27.70		-0.22		2		no Cq
CTC1150		1		23.15		28.27		2.90		1		no Cq
CTC1177		4		22.12		28.48		2.17		1		no Cq
CTC1178		1		19.02		29.09		3.30		1		no Cq
CTC1173		1		20.29		29.37		3.49		1		no Cq
CTC1144		10		21.12		no Cq		4.00		0		no Cq

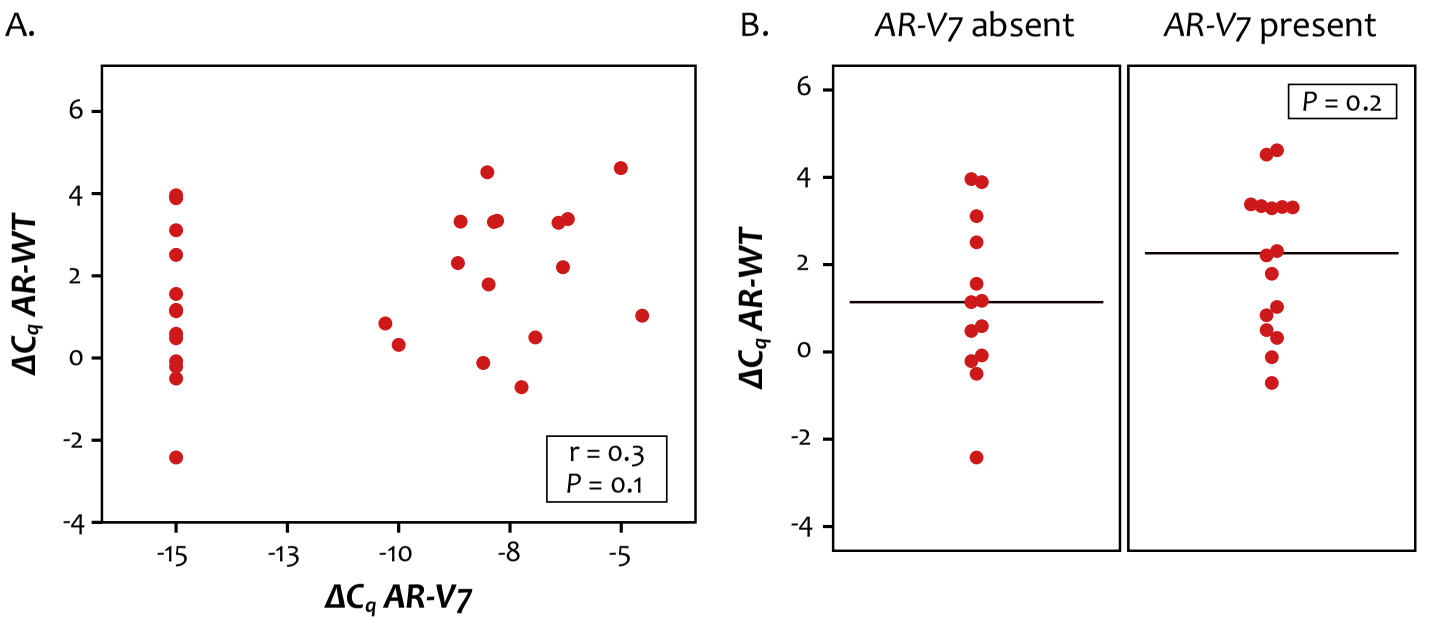
SUPPLEMENTARY TABLE 2 (CONTINUED). RT-qPCR data of all tested samples. Five healthy blood donors and 11 breast and prostate cancer cell lines – pure and after spiking in healthy blood donor blood and after CellSearch enrichment – served as negative and positive controls. In total, 44 patient samples were tested, of which 29 were of sufficient quality to be entered in the analyses. After CellSearch enrichment, an aliquot of 11% was used for the assessment of the AR-V7 status. The calculated final number of CTCs used for the PCR analysis was derived from the equation of the regression line of the CTC count from 7.5 mL blood – as measured by CellSearch enumeration from the CellSave tube – and the average C_q of *EPCAM* and *KRT19* (Supplementary Figure 1A), thereby taking into account that only 11% of the original sample was used.



date	28-Jul-14	26-Nov-14	26-Nov-14	25-Aug-14	23-Aug-14	22-Sep-14	25-Aug-14	28-Jul-14	22-Sep-14	28-Jul-14	22-Sep-14	22-Sep-14	30-Jul-14	22-Sep-14	22-Sep-14
Sample description	HBD	VCAP in HBD	VCAP in HBD	VCAP CellSearch enriched	VCAP	VCAP	VCAP	VCAP	VCAP	VCAP	VCAP	VCAP	VCAP	VCAP	VCAP
Input # cells in RT-qPCR	0	2	10	10	40	40	80	90	100	250	250	250	125	65	15
pre-amplified	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	no	no	no	no
WBC present	yes	yes	yes	yes	no	no	no	no	no	no	no	no	no	no	no
dCq *PTPRC/CD45*	6.16	6.47	5.81	3.67	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq	No Cq
Mean Cq 3 reference genes	22.30	21.50	21.64	23.93	21.13	21.49	19.21	19.12	18.93	16.75	15.99	25.87	24.89	28.32	30.80
Mean Cq 2 epithelial markers	No Cq	25.70	23.68	24.17	19.92	19.99	17.82	17.54	17.38	14.97	14.68	24.64	23.06	26.71	29.55
Cq *AR-V7*	No Cq	29.58	27.87	29.94	24.54	24.10	22.01	22.79	21.27	20.12	18.41	28.44	27.37	31.35	33.14

	0	2 cells	10 cells	10 cells	40 cells	40 cells	80 cells	90 cells	100 cells	250 cells	250 cells	250 cells	125 cells	65 cells	40 cells
AR-WT	-15.00	3.09	3.15	4.59	4.54	3.16	4.19	2.34	3.00	2.44	2.76	2.94	2.57	2.73	3.11
AR-V7	-15.00	-3.88	-4.20	-5.77	-4.63	-4.11	-4.19	-5.25	-3.89	-5.15	-3.74	-3.80	-4.31	-4.65	-3.59

SUPPLEMENTARY FIGURE 1. Sensitivity and specificity of the assays. A. Material from 40 MCRPC patients was used to evaluate the correlation between the CTC count after CellSearch enrichment and the average C_q value of *EPCAM* and *KRT19* in corresponding RNA samples (CTC count = $260466e^{-0.333*Cq\ average\ (EPCAM+KRT19)}$). B. Data from 62 individual experiments with input of RNA from 4 to 580 VCAP cells in the RT-qPCR were used to evaluate the linear correlation between the C_q value of *AR-V7* and the average C_q value of *EPCAM* +*KRT19*. Samples with an average C_q value of *EPCAM* + *KRT19* below 26.5 C_q were considered to contain a sufficient epithelial signal to allow measurement of *AR-V7* in these cells. Circles: unspiked VCAP cells; squares: VCAP cells spiked in HBD blood. C. Sensitivity and specificity measuring *AR-WT* and *AR-V7* by RT-qPCR in VCAP cells before and after spiking in HBD blood, before and after CellSearch enrichment and before and after pre-amplification. Data are expressed relative to the average expression of *EPCAM* + *KRT19* (ΔC_q) measured in these preparations. Within a window of ± 1.1 Cq, both transcripts can be reproducibly measured in material from as little as 2 VCAP cells. Dark blue circles: ΔC_q *AR-WT*; squares; light blue circles: ΔC_q *AR-V7*.



SUPPLEMENTARY FIGURE 2.A. Transcription levels of *AR-WT* versus *AR-V7*. No correlation was observed (r_s = 0.3, *P* = 0.1). B. Transcription levels of *AR-WT* in patients without *AR-V7* (left panel) and with *AR-V7* (right panel) in CTCs. The horizontal lines represent the medians. There was no difference in transcription levels (Mann Whitney *U*, *P* = 0.2).

SUPPLEMENTARY FIGURE 3. Overall survival in patients who had or had not received abiraterone before cabazitaxel (A.) and patients who received abiraterone or enzalutamide after cabazitaxel (B.). Clinical data concerning pre-treatments of five patients were still missing as the clinical trial was still ongoing and recruiting. From one patient no follow-up data were available yet.

7

THE USE OF CIRCULATING TUMOR CELLS IN GUIDING TREATMENT DECISIONS FOR PATIENTS WITH METASTATIC CASTRATION-RESISTANT PROSTATE CANCER

Wendy Onstenk, Willemijn de Klaver, Ronald de Wit, Martijn Lolkema, John Foekens and Stefan Sleijfer

Cancer Treatment Reviews, 2016; 46: p. 42-50

ABSTRACT

The therapeutic landscape of metastatic castration-resistant prostate cancer (MCRPC) has drastically changed over the past decade with the advent of several new anti-tumor agents. Oncologists increasingly face dilemmas concerning the best treatment sequence for individual patients since most of the novel compounds have been investigated and subsequently positioned either pre- or post-docetaxel. A currently unmet need exists for biomarkers able to guide treatment decisions and to capture treatment resistance at an early stage thereby allowing for an early change to an alternative strategy. Circulating tumor cells (CTCs) have in this context intensively been investigated over the last years. The CTC count, as determined by the CellSearch System (Janssen Diagnostics LLC, Raritan, NJ), is a strong, independent prognostic factor for overall survival in patients with MCRPC at various time points during treatment and, as an early response marker, outperforms traditional response evaluations using serum prostate-specific antigen (PSA) levels, scintigraphy as well as radiography. The focus of research is now shifting toward the predictive value of CTCs and the use of the characterization of CTCs to guide the selection of treatments with the highest chance of success for individual patients. Recently, the presence of the androgen receptor splice variant 7 (*AR-V7*) has been shown to be a promising predictive factor. In this review, we have explored the clinical value of the enumeration and characterization of CTCs for the treatment of MCRPC and have put the results obtained from recent studies investigating the prognostic and predictive value of CTCs into clinical perspective.

Introduction

Over the past decade, the advent of new drugs have led to a substantial improvement in the treatment of patients with metastatic castration-resistant prostate cancer (MCRPC). After the approval of docetaxel in 2004, six more agents have been registered, among which the next-generation taxane cabazitaxel, the androgen receptor (AR) antagonist enzalutamide, and the CYP17A1 inhibitor abiraterone [1,2]. In view of the preclinical and clinical evidence for the emergence of cross-resistance between docetaxel, abiraterone, and enzalutamide [2-6], the optimal treatment sequence yet remains to be determined. Importantly, optimal treatment sequencing may be patient-dependent, requiring deliberate (tailored) choices of specific agents for specific patients at specific times.

The options for a personalized treatment approach for patients with MCRPC are currently limited given the only few prognostic and predictive markers that are available for treatment selection and early evaluation of treatment efficacy. An initial Gleason score ≥7 and/or a short interval between the start of initial androgen deprivation therapy (ADT) and the development of MCRPC may select for patients who will likely benefit most from first-line docetaxel instead of AR-targeted treatment [7,8]. Monitoring of treatment response is mostly done through the dynamics of serum levels of prostate-specific antigen (PSA) and changes in bone scintigraphy and/or computed tomography (CT). However, these modalities are at most modestly useful and the read-out of efficacy needs at least three months after treatment start due to the long half-life and release from apoptotic cells of PSA, flare-up phenomena on bone scans, and slow changes in combination with inter-observer variability in tumor size on CT scans [2].

Circulating tumor cells (CTCs) are tumor cells present in the peripheral circulation of patients with different solid malignancies including MCRPC, which have detached from tumor sites. Although occurring at very low frequencies in the peripheral blood, CTC counts before and during treatment have proven to be an accurate early response marker with a strong independent prognostic value at all time-points during treatment [9,10]. Also, CTCs have generally been considered as surrogates for metastatic cells and the characterization of CTCs may in this respect function as a "*liquid biopsy*" to aid in

TABLE 1. Overview of the studies investigating the prognostic value of the CTC count as assessed by the CellSearch System before and during treatment in patients with MCRPC.

Reference	N	Treatment	Patients with ≥5 CTCs	Prognostic value
Danila et al. (2007) [68]	120	Any chemotherapy; first/second-line	57%	Baseline CTC count strongly associated with OS in univariate analysis (P<0.001)
De Bono et al. (2008) [10]	231	Any chemotherapy; first/second/third-line	57%	Baseline HR for OS: 3.3 (95% CI 2.2-5.1, P<0.0001); HR after 2-5 weeks: 4.5 (95% CI 3.0-6.7, P<0.0001); CTCs were more strongly prognostic than PSA at all time points
Goodman et al. (2009) [69]	100	Any chemotherapy; any line (1-7th)	Not reported	LDH and CTC both independent prognostic factors for OS; Baseline HR of the CTC count ≥4 for OS: 3.65, P<0.001
Olmos et al. (2009) [70]	119	Any chemotherapy; any line (1-5th)	50%	Baseline HR for OS: 3.25 (95% CI 1.4-7.4, P=0.005); Changes in CTC counts during treatment predict a change in prognosis, P<0.0001)
Scher et al. (2009) [65]	156	Docetaxel monotherapy or combination; first-line	54%	Baseline HR for OS: 1.58 (P<0.0001); Changes in CTC counts were strongly associated with OS at all times, whereas changes in PSA were only modestly associated with OS after 12 weeks
Danila et al. (2011) [56]	48	Abiraterone; second- or third-line	73%	Unfavorable CTC count after 4 weeks of abiraterone was association with worse OS (49 versus 122 weeks; P<0.001)
Scher et al. (2013) [71]	144	Cabozantinib; second-line or more	71%	A CTC conversion from unfavorable to favorable was associated with improved OS: HR 0.42 (95% CI 0.19-0.92; P=0.03)
Thalgott et al. (2013) [72]	55	First-line docetaxel or second-line treatment	57%	Unfavorable baseline CTC counts were associated with worse OS (P=0.003)
Vogelzang et al. (2013) [73]	208	Docetaxel ± lenalidomide; first-line	58%	Baseline HR for 2-year OS: 3.5 (P<0.05); HR for an increase in CTCs between baseline and cycle 4 for OS: 5.2 (P=0.03)
Goldkorn et al. (2014) [74]	263	Docetaxel ± atrasentan; first-line	51%	A decrease of CTCs to <5 during treatment was correlated to PSA response (63% versus 44%; P=0.01) and RECIST response (31% versus 14%; P=0.05); Baseline HR of the CTC conversion for 2-year OS: 2.74 (95% CI 1.72-4.37; P<0.001); HR of ≥50% decrease if baseline CTCs ≥5 for OS: 0.53 (95% CI 0.27-1.06; P=0.07); HR of the conversion favorable to unfavorable CTC count: 6.47 (95% CI 1.96-21.4; P=0.002)

Continued on next page

the tailoring of treatments [11,12]. In this review, we discuss the progress that has been made regarding the use of CTCs as a prognostic and predictive marker for patients with MCRPC, thereby focusing on the clinical relevance of CTCs and to what extent they may guide treatment decision-making and optimal treatment sequencing in MCRPC.

ENUMERATION OF CTCS

In 2008, a landmark paper was published showing the strong, independent prognostic value of a CTC count from peripheral blood in patients with MCRPC when taken before the start of a new treatment line [10]. The enumeration of CTCs was done from 7.5 mL of blood by the CellSearch System (Janssen Diagnostics, Raritan, NJ). This semi-automated system immunomagnetically enriches epithelial cells from peripheral blood using anti-epithelial cell adhesion molecule (EpCAM)-antibodies bound to ferrofluid nanoparticles. Enriched cells, consisting of CTCs and still a thousand-fold of contaminating leukocytes, are immunofluorescently stained and manually counted after digital microscopy; nucleated (4',6-diamidino-2-phenylindole (DAPI)^{pos}), cytokeratin (CK)^{pos}, and CD45^{neg} cells with a diameter ≥4x4 µm and a round to oval morphology are thereby considered CTCs. This way, patients can be stratified as having a favorable CTC count – defined as <5 CTC/7.5 mL – or an unfavorable CTC count of ≥5 CTC/7.5 mL. It was shown in 231 patients that having a favorable CTC count predicted for a significantly improved progression-free survival (PFS) and overall survival (OS) compared to an unfavorable count of ≥5 CTCs at all time-points before and during treatment [10]. Conversions of the CTC count, from unfavorable to favorable or *vice versa*, during treatment were shown to be associated with an improvement or deterioration of the prognosis, respectively, already 2-5 weeks after the start of treatment. By contrast, a 30% or 50% decline in PSA only started to be of prognostic significance after 6-8 weeks with maximum hazard ratio (HR) after 13-20 weeks. At all times, the HR of the favorable versus unfavorable CTC count for OS was greater than the HR for the PSA reduction. This study led to the Food and Drug Administration (FDA)-clearance of the CTC enumeration by the CellSearch System for clinical use in patients with MCRPC. Other studies have since confirmed the prognostic value of CTC counts as determined by the CellSearch System under different treatments (Table 1).

TABLE 1 (CONTINUED).

Reference	N	Treatment	Patients with ≥5 CTCs	Prognostic value
Okegawa et al. (2014) [75]	57	Docetaxel; first-line	58%	Baseline unfavorable CTC count predicted for worse OS (11 vs 25 months; P<0.001). Conversions in the CTC count during treatment were associated with OS.
Chang et al. (2015) [76]	70	Docetaxel or ketokonazol; first-line	43%	Baseline HR for OS: 2.73 (95% CI 1.21-6.13; P=0.02)
Fleisher et al. (2015) [77]	258	Enzalutamide; second- or third-line	49%	Conversions from unfavorable to favorable were observed in 48% of the patients and were associated with an OS benefit.
Lorente et al. (2015) [78]	439	Abiraterone or chemotherapy; first-/second-/third-line	Not reported	The baseline CTC count as continuous variable and a 30% decrease in CTC count after 4 and 12 weeks of treatment were both independently associated with OS (P=0.001)
Scher et al. (2015) [79]	711	Abiraterone or prednisone; second- or third-line	48%	CTC count after 12 weeks strongest prognostic factor for OS; biomarker panel of CTC counts and LDH after 12 weeks of treatment fulfilled all four Prentice criteria of individual patient-level surrogacy for OS.
Thalgott et al. (2015) [80]	33	Docetaxel; first-line	61%	CTCs were strongly prognostic for OS at all time points before and during treatment with HR 3.8-5.8; P<0.01; CTC conversions were more strongly predictive for OS than radiology and PSA evaluations

Given the strong, independent prognostic value at baseline and early moments during treatment, the CTC enumeration by the CellSearch System has been suggested and increasingly investigated as a surrogate end-point for OS in clinical trials. The combination of CTC and lactate dehydrogenase (LDH) dynamics during treatment fulfilled the criteria for individual patient-level surrogacy, supporting the use as a valid trial end-point instead of OS [13]. Already, CTC counts have been implemented as additional end-points in several phase I/II trials investigating the tolerability and efficacy of new treatments [14-19]. The use of CTCs as early outcome marker in the development track of novel drugs in MCRPC will likely improve the efficiency of early clinical trials through the shortening of the necessary follow-up time. Consequently, the time to registration for newly developed compounds can be expected to be reduced, as will the costs of development.

CIRCULATING TUMOR CELL CHARACTERISTICS

Besides a mere enumeration, the interrogation of CTCs for specific tumor characteristics has drawn major attention over the past few years. The genomic profiles of CTCs have been found to be largely comparable to primary tumors and/or metastatic tissue, suggesting that CTCs are able to reflect tumor characteristics including the extent of intra- and intertumoral heterogeneity [20-25]. However it remains to be established whether CTCs represent the characteristics of all the metastases or only of the most invasive clones and what influence factors present in the circulation have on the characteristics of CTCs. Moreover, CTCs have been shown to be tumorigenic and capable of forming new metastases [26-28]. The half-life of CTCs has been estimated to be in the order of hours rather than days [12,29-31], suggesting a real-time representation of tumor characteristics at the time of blood draw. Altogether, CTCs offer the opportunity to gain a snap-shot of tumor characteristics at a certain point in time and may therefore, as a liquid biopsy, harbor important characteristics of metastatic tumor cells in an individual patient. Until now, alterations in the expression, function, and localization of AR in CTCs and the clinical relevance thereof have mostly been investigated (Table 2).

Subcellular localization of AR

After activation by androgens, AR translocates from the cytoplasm to the nucleus in order to exert its function as a transcription factor for target genes. The presence of AR in the nucleus of tumor cells therefore indicates an active AR pathway. In this respect, evaluation of the subcellular localization has been suggested as marker for response or resistance to treatment, not only to abiraterone and enzalutamide, but also to docetaxel. For long, the working mechanism of docetaxel seemed to be the induction of mitotic arrest and apoptosis through the stabilization of microtubules. Recently, it was discovered that AR also interacts with microtubules for its nuclear transport and consequently at least part of the efficacy of docetaxel in MCRPC seems to result from an impairment of AR-signaling [3]. Darshan *et al.* [32] have shown that the absence of nuclear AR in patients treated with docetaxel correlates with clinical response, as assessed by confocal microscopy of CellSearch-enriched CTCs. In longitudinal samples

from 14 MCRPC patients during treatment with docetaxel, a shift was observed from nuclear AR before treatment to cytoplasmic AR during treatment to again nuclear AR at the time of progressive disease (PD). Overall, 71% of the patients who benefitted from taxane treatment had cytoplasmic AR whereas 72% of the CTC samples drawn at the time of PD showed a nuclear localization of AR.

To facilitate the characterization of CTCs for expression and localization of AR, a protocol has been developed to incorporate AR as an additional marker within the CellSearch System, enabling simultaneous characterization and enumeration of CTCs [33]. Comparing the intensity of AR staining between abiraterone/enzalutamide-naïve patients and patients who had progressed on abiraterone/enzalutamide, no difference in the staining intensity or subcellular localization was observed. However, an increase in the median intensity of nuclear AR expression compared to baseline was observed over sequential samples of five of the eight patients (63%) who progressed during abiraterone or enzalutamide [33]. This increase in AR staining intensity in patients progressing on abiraterone has also been described by Reyes *et al.* [34]. Applying a combined flowcytometric and microscopic method, the ImageStream X, to assess AR staining intensity and the subcellular localization of AR in the CTCs of 20 MCRPC patients, they found the median AR staining intensity to be three times higher in the ten patients progressing on abiraterone than in the ten patients who were abiraterone-naïve. No difference was observed in the subcellular localization of AR though. Interestingly, a correlation between high expression of AR, nuclear localization, and more intense staining of the proliferation marker Ki-67 in CTCs was found, suggesting active AR signaling in CTCs with high nuclear expression of AR.

Evidence for active AR signaling in CTCs has also been reported by Miyamoto *et al.* [35]. In this study, CTCs were captured using a CTC chip with anti-EpCAM-antibodies covered walls followed by characterization by automated fluorescence microscopy. Based on the expression of PSA and the membrane bound form of PSA (PSMA), three CTC categories with respect to AR-signaling were defined: AR-off ($PSA^{neg}/PSMA^{pos}$), AR-mixed ($PSA^{pos}/$

TABLE 2. Overview of studies investigating the predictive value of CTCs by characterizing CTCs for the expression and localization of AR and the presence of AR mutations, amplifications, and/or splice variants.

Reference	*N*	CTC Isolation; characterization	Parameter	Clinical relevance
Miyamoto et al. (2012) [35]	14	CTC-chip; IF	AR signaling phenotype of single CTCs	AR phenotype of CTCs was highly heterogeneous with an abundance of the "AR-off" phenotype. An increase of "AR-on" CTCs during abiraterone was associated with a decreased OS.
Darshan et al. (2011) [32]	14	CellSearch or density gradient separation; IF	AR subcellular localization	The presence of nuclear AR was predictive for resistance to taxanes.
Reyes et al. (2014) [34]	20	ImageStream X (combined FC and IF)	AR expression and subcellular localization	Higher intensity staining of AR in CTCs positively correlated with the intensity of the proliferation marker Ki-67. Nuclear localization of AR also correlated with the expression of Ki-67.
Dago et al. (2014) [22]	1	Micromanipulator; IF, WGA, NGS	AR subcellular localization *AR* amplifications	AR^{pos} CTCs decreased from 67% before to 11% during abiraterone to 96% at the time of PD. The localization shifted from cytoplasmic before and during abiraterone to nuclear at the time of PD. Amplifications of *AR* disappeared during treatment, but reappeared at the time of PD.
Crespo et al. (2015) [33]	48	CellSearch; IF	AR expression and subcellular localization	No difference in the staining intensity or localization of AR between patients naïve for versus progressing on abiraterone/enzalutamide. In 5/8 patients who had progressed on abiraterone/enzalutamide an increase of nuclear AR expression was observed compared to baseline.
Shaffer et al. (2007) [47]	9	CellSearch; FISH	*AR* amplifications	High copy number gains in 56% of the patients; clinical associations not reported.
Attard et al. (2009) [45]	33	CellSearch; FISH	*AR* amplifications	High copy number gains in 45% of the patients; clinical associations not reported.
Leversha et al. (2009) [46]	49	CellSearch; FISH	*AR* amplifications	High copy number gains in 35% of the patients; clinical associations not reported.
Magbanua et al. (2012) [25]	9	IM enrichment and FACS; WGA and aCGH	*AR* amplifications	High copy number gains in 78% of the patients. Overall genomic profiles of the CTCs were comparable to the corresponding archival primary tumors, except for the *AR* amplifications.
Jiang et al. (2010) [42]	35	CellSearch; NGS	*AR* mutations	In total, 27 mutations were identified in 20 (57%) patients; clinical associations not reported.

Continued on next page

Table 2 (continued).

Reference	N	CTC Isolation; characterization	Parameter	Clinical relevance
Miyamoto et al. (2015) [43]	13	CTC-iChip, micromanipulator; RNA sequencing	AR mutations AR splice variants *V1, V3, V4, V7, and V12*	The AR transcript was expressed in 60 of the 77 (78%) single CTCs sequenced; one AR mutation (T877A) was detected in 5/9 CTCs from 1/13 (8%) of the patients; 33 of 73 (43%) from 8/11 (73%) expressed ≥1 AR splice variant: *AR-V7* (73% of the patients), *Arv567es/AR-V12* (73% of the patients), or *AR-V1/AR-V3/AR-V4* (45% of the patients)
Steinestel et al. (2015) [44]	37	AdnaTest; PCR	AR mutations Presence of *AR-V7*	AR mutations in 5% and expression of *AR-V7* in 49% of the patients. The presence of *AR-V7* was positively correlated with the number of prior treatment lines; the presence of *AR-V7* was highly predictive for resistance to subsequent anti-AR treatment or chemotherapy (7% versus 71% PSA reduction of ≥50%; P<0.001)
Antonarakis et al. (2014) [50]	62	AdnaTest; PCR	Presence of *AR-V7*	*AR-V7* was detected in the CTCs of 29% of the patients. The presence of *AR-V7* was highly predictive for abiraterone and enzalutamide resistance and prognostic for PFS and OS.
Antonarakis et al. (2015) [52]	37	AdnaTest; PCR	Presence of *AR-V7*	*AR-V7* was detected in the CTCs of 46% of the patients. The presence of *AR-V7* was not predictive for resistance to taxanes and not prognostic for PFS and OS. *AR-V7*^{pos} patients treated with taxanes had a longer PFS compared to treatment with anti-AR treatment, no difference between the treatments was observed for *AR-V7*^{neg} patients.
Nakazawa et al. (2015) [51]	14	AdnaTest; PCR	Presence of *AR-V7*	Conversions in *AR-V7* status of CTCs frequently occurred; reversions only during taxane treatment. The clinical associations of these conversions and reversions were not reported.
Onstenk et al. (2015) [49]	29	CellSearch; PCR	Presence of *AR-V7*	*AR-V7* was detected in the CTCs of 55% of the patients. The presence of *AR-V7* in baseline CTCs was not associated with resistance to cabazitaxel and not prognostic for PFS and OS.

PSMA^{pos}), and AR-on (PSA^{pos}/PSMA^{neg}). In five patients with metastatic prostate cancer starting initial ADT, the AR-on phenotype was predominately present. The phenotype switched to AR-off during ADT, followed by the disappearance of CTCs after three months. By contrast, a wide variety of CTC phenotypes were observed in 14 MCRPC patients, with an abundance of the AR-off phenotype. In only 11% of the investigated CTCs, the AR-on phenotype was detected. Interestingly, an increase of CTCs with the AR-on phenotype during abiraterone treatment was found to be associated with a shorter median OS, suggesting that the occurrence of this AR-on phenotype may predict for resistance to anti-AR treatment.

AR mutations and amplifications

In addition to the presence and the localization of AR in CTCs, recent studies have focused on the specific aberrations of the *AR* gene as a cause of resistance to established treatments. Activating mutations in *AR*, leading to constitutive activity, have rarely been detected in hormone-sensitive tumors [36], but can be found in up to one-third of the patients with MCRPC [36-41]. Mutations have also been detected in CTCs from patients with MCRPC [42-44]. At the present time, only limited data from retrospective series of patients have been reported and prospective data reporting associations with clinical outcome remain to be awaited.

Amplifications of the *AR* gene resulting in AR protein overexpression and hypersensitization of prostate cancer cells to even castrate levels of androgens are a second possible mechanism for treatment resistance [38]. Amplifications have been detected in up to 50% of the castration-resistant prostate cancers [36-38] as well as in the CTCs from MCRPC patients [45-47]. Conveniently, a protocol has been developed to combine the CellSearch enumeration and characterization for gene amplifications by fluorescence *in situ* hybridization (FISH) in one CellSearch cartridge [48]. Applying this protocol, *AR* amplifications have been detected in all of the 33 evaluable patients with ≥4 CTCs starting treatment with abiraterone, with 15 patients (45%) having CTCs with >5 *AR* copies [45]. Observed copy number gains were remarkably heterogeneous between

single CTCs in one sample. Similar results, with amplification rates of 50-78%, have been reported from studies investigating *AR* amplifications by FISH on cytospun CTCs from CellSearch cartridges [46,47] or by array comparative genomic hybridization on immunomagnetically-enriched and fluorescence-activated cell sorted CTCs [25]. Again, marked heterogeneity in *AR* gene copy numbers between single CTCs was observed in most patients.

AR splice variants

To date, the potential predictive value of CTC characteristics has best been exemplified by recent reports on the association between the presence of the *AR* splice variant 7 (*AR-V7*) – coding for a truncated and constitutively active AR – and treatment outcome [44,49-52]. The presence of *AR-V7* in CTCs was shown to be highly predictive for resistance to anti-AR treatments [44,50]. From 31 patients starting abiraterone and 31 patients starting enzalutamide, CTCs were isolated and characterized for the presence of *AR*-V7 by the polymerase chain reaction (PCR)-based AdnaTest [50]. The overall prevalence of *AR-V7* at baseline was 29%. None of the patients with *AR-V7*^{pos} CTCs had a 50% PSA response rate (PSA-RR) compared to 53% and 68% of the patients with *AR-V7*^{neg} CTCs receiving enzalutamide or abiraterone, respectively (P=0.004 for both treatments). The presence of *AR-V7* in CTCs was an independent prognostic factor for OS with a HR of 6.9 (95% confidence interval (CI) 1.7-28.1, P=0.002) for the enzalutamide cohort and HR 12.7 (95% CI 1.3-125.3; P=0.006) for the abiraterone cohort.

In a second report by the same investigators applying the same methodology, the presence of *AR-V7* was detected in the CTCs of 46% of the 37 patients from a different cohort starting taxane treatment (docetaxel, N=30, or cabazitaxel, N=7) [52]. In this study, the PSA-RR was not significantly different between the *AR-V7*^{pos} and the *AR-V7*^{neg} patients (41% versus 65%, respectively; P=0.19) and the presence of *AR-V7* was not prognostic for PFS (HR 2.7; 95% CI 0.8-8.8; P=0.11) and OS (HR 0.7; 95% CI 0.1-3.8; P=0.66). A significant interaction between the presence of *AR-V7* and the type of treatment was observed; while the prognosis of the *AR-V7*^{neg} patients was comparable, the PFS of the

AR-V7^{pos} patients treated with taxanes seemed to be longer compared to the patients treated with abiraterone or enzalutamide from the first cohort. However, this indirect comparison of the different cohorts has to be interpreted with caution, amongst others since the patients treated with taxanes had more advanced disease.

From 21 of the 37 taxane treated patients, a secondary CTC sample during treatment was available. A conversion from *AR-V7*^{neg} to *AR-V7*^{pos} was observed in only 1/8 (11%) patients, but *vice versa* was the case in 7/12 (58%). Conversions have also been investigated over sequential treatment lines, where 70 CTC samples from 14 patients undergoing a total of 37 therapies were selected and analyzed using the AdnaTest [51]. Three patients remained *AR-V7*^{pos} over multiple treatment lines. In the other 11 patients, changes in the *AR*-V7 status of the CTC samples were observed. Interestingly, conversions from *AR-V7*^{pos} to *AR-V7*^{neg} only occurred during taxane and not anti-AR treatment. Although the results from this study suggest that the expression of *AR-V7* is influenced by the treatment given, the predictive value of the conversions in *AR-V7* status remains to be established. Also, it is unclear whether the observed changes in the *AR-V7* status are true conversions or the result of the disappearance of CTCs in the blood sample tested. In the work-up of the AdnaTest, cells are lysed and the enumeration of CTCs is not possible (See Table 3 for the characteristics of the AdnaTest versus the CellSearch System). The number of CTCs present in a sample was therefore not taken into account in the analyses and may be a confounder for the prognostic value of *AR-V7* in CTCs.

Given the constitutive activity of *AR-V7* as a result of the missing ligand-binding domain, treatments with AR-independent mechanisms of action such as cabazitaxel may remain effective. With this hypothesis, we measured the expression levels of *AR-V7* by RT-qPCR in 29 patients with PD after having been treated with at least docetaxel and starting cabazitaxel [49]. Data were collected as a part of a prospective phase II trial, for which the enumeration of CTCs formed part of the secondary objectives [49]. To ensure reliable CTC-derived signals as well as to avoid confounding by CTC count, we normalized the expression of *AR-V7* to the average of the epithelial genes *KRT19* and *EPCAM*, which

showed to correlate with the CTC count as derived from the parallel enumeration tube. The presence of *AR-V7* was detected in 16 patients (55%) at baseline and was more frequent in patients who had received prior abiraterone (100% versus 35%, *P*=0.009). No associations were found between the presence of *AR-V7* in baseline CTCs and response to cabazitaxel in terms of the CTC-RR or the PSA-RR. In the preliminary survival analyses in 29 patients, OS was not impacted by the presence of *AR-V7* (HR 1.6 (95% CI 0.6-4.4; *P*=0.45)).

	AdnaTest (Qiagen, Hannover, GE)	CellSearch System (Janssen Diagnostics LLC, Raritan, NJ)
Regulatory approval	CE certification, no clinical validation	FDA clearance for clinical use of the CTC enumeration
Input	5 mL whole blood	7.5 mL whole blood
Enrichment method	Immunomagnetical	Immunomagnetical
Enrichment markers	EpCAM and HER2	EpCAM
Detection method	PCR-based after lysis of enriched cells	Immunofluorescence staining of fixed and permeabilized enriched cells
Detection markers	PSMA, PSA, EGFR	CK8/18/19
Detection criteria	Concentration of ≥10 ng/µL for one or more of the detection markers in the presence of a sufficient actin signal	Intact cell of ≥4 µm with a round to oval morphology and a nucleus overlapping the cytokeratin for ≥50%; DAPI-pos, CK8/18/19pos, CD45neg
CTC quantification	Not possible	Count per 7.5 mL blood
Characterization possibilities	Limited to PCR for tumor-associated genes	Extensive; for example immunofluorescence staining of an additional marker, FISH of enriched cells, PCR for tumor-associated genes
Single CTC characterization possible	No, CTCs and contaminating leukocytes are lysed in a sample	Limited to the assessment of immunofluorescence staining of individual CTCs in the cartridge

TABLE 3. Comparison of the characteristics of the two enrichment methods that have been used in the studies investigating the prognostic and predictive value of the presence of *AR-V7* in CTCs: the AdnaTest and the CellSearch System. Many other detection, enrichment and characterization assays based on the different biological and physical properties of CTCs have been developed; these have been reviewed in [12].

Lastly, Miyamoto *et al.* [43] determined the AR splice variants 1, 3, 4, 7, and 12 in single CTCs by RNA sequencing after isolation on the CTC-iChip and picking by a micromanipulator. Heterogeneous expression levels of the different splice variants were observed both between and within patients. In 33 of the 73 (43%) single CTCs from 8 of the 11 patients (73%) at least one alternative splice variant was detected. This most frequently concerned *AR-V7* in 36% of the CTCs and 73% of the patients, followed by *ARv567es/AR-V12* in 25% and 73%, and *AR-V1/V3/V4* in 10% and 45% of the CTCs and the patients, respectively. Importantly, splice variants were not detected in corresponding primary prostate tumors, suggesting that alternative splicing occurs during disease progression. The prognostic value of all splice variants and the clinical relevance of the changes during treatment remain to be investigated and in this respect, several prospective clinical trials have been or will shortly be intiated (*e.g.* the CARVE (NCT02621190), the PRIMCAB (NCT02379390), and the ARMOR3 trial (NCT02438007).

Other predictive factors?

Besides AR, other factors regulating cancer-related pathways may contribute to disease progression and treatment resistance and may be clinically relevant to measure in CTCs (Table 4). One well-known example are the *TMPRSS2:ERG* rearrangements, resulting in fusion of an *ERG* oncogene with the AR-driven *TMPRSS2* promotor. Rearrangements have been detected in >50% of the hormone-sensitive prostate cancers and seem to be conserved during tumor progression and evolution [36,37,53]. The recent finding that patients with specific *ERG* rearrangements may be more sensitive to treatment with abiraterone makes the presence of this rearrangement a potential predictive factor [54]. Rearrangements have been detected in CTCs in frequencies ranging between 21-60% [29,45,55-59]. Overall, the rearrangement status of CTCs was homogeneous between different CTCs in one sample [45] and concordant with the primary tumor when assessed by FISH [45], while discordances have been described when comparing expression levels by RT-qPCR [29,56]. Whether technical issues or biological processes cause these discordances remains to be investigated. Two trials so far have investigated the predictive value of the presence of ERG rearrangements in CTCs for response to abiraterone in a

prospective-retrospective manner. Although both studies used the CellSearch System to enrich CTCs from whole blood, the subsequent characterization method differed, which may contribute to the conflicting results obtained. In the first study by Attard *et al.* [45], the CTCs from 49 patients with ≥4 CTCs at baseline and matching tumor tissue from 38 patients were characterized by FISH. Patients who were positive for *TMPRSS2:ERG* rearrangements responded better to abiraterone in terms of a ≥90% PSA response than the patients in whom no rearrangements were found (80% versus 32%, *P*=0.001). However, in the second study by Danila *et al.* [56] expression levels of *TMPRSS2:ERG* in CTCs were measured by RT-qPCR in the baseline CTCs from 41 MCPRC patients who had started abiraterone treatment. Herein, no associations with PSA response or OS were observed. To allow for prospective evaluation of the predictive value of *TMPRSS2:ERG* rearrangements for abiraterone sensitivity, the observed discordances between the results obtained by FISH and by PCR will have to be clarified first.

The expression of the proliferation marker Ki-67 has been detected in variable levels in CTCs and was shown to be positively correlated to the expression levels and nuclear localization of AR [34] as well as to more advanced stages of prostate cancer [29]. The presence of Ki-67-positive CTCs during treatment has been suggested as a marker of treatment resistance [29,34]. Similarly, the activity of telomerase – an enzyme that lengthens and protects the caps of the chromosomes and this way may protect tumor cells from apoptosis – has been investigated in CTCs. Telomerase activity was not only detected in CTCs, but was also shown to be an adverse prognostic factor in patients with a baseline CTC count of ≥5 as assessed by the CellSearch System [60]. Conversely, the presence of the apoptosis marker M30 – a neo-epitope of cytokeratin-18 emerging after cleavage of cytokeratin-18 by caspases – in CTCs during treatment has been suggested as a marker of treatment response [61]. The characterization of CTCs for the presence of other putative predictive factors, such as the loss of *PTEN* [45,59], expression of the enhancer of zeste homolog 2 (*EZH2*) [62], the epidermal growth factor receptor (*EGFR*) [47], or the insulin-like growth factor 1 receptor (*IGF1R*) [63], or the presence of transcripts for steroidogenic enzymes in CTCs [64] have been reported in several proof-of-principle

TABLE 4. Selection of studies investigating the characterization of CTCs for a selection of putative predictive markers beyond AR.

Reference	*N*	CTC isolation; characterization assay	Parameter	Clinical Relevance
Mao et al. (2008) [55]	10	Density gradient separation, IM enrichm; FISH, PCR	TMPRSS2:ERG rearrangements	Rearrangements in the CTCs of 60% of the patients; no TMPRSS2:ERG transcripts were detected by RT-PCR.
Attard et al. (2009) [45]	49	CellSearch; FISH	TMPRSS2:ERG rearrangements	Rearrangements in the CTCs of 47% of the patients; patients with ERG rearrangements had significantly more often a ≥90% PSA response to abiraterone (38 vs 7%, *P*=0.001)
Jost et al. (2010) [57]	14	IM enrichment, amplification, hybridization protection assay	TMPRSS2:ERG rearrangements	TMRPSS2:ERG transcripts detectable in 21% of the patients.
Stott et al. (2010) [29]	20	CTC-chip; PCR	TMPRSS2:ERG rearrangements	Rearrangements in the CTCs of 45% of the patients; concordance with primary tumor was 70% when assessed by FISH and 60% when assessed by RT-PCR.
Danila et al. (2011) [56]	41	CellSearch; PCR	TMPRSS2:ERG rearrangements	Rearrangements in 37% of the patients; concordance with the primary tumor (23 patients) 65% of the patients. No significant difference in the PSA response rates to abiraterone nor in OS according to the TMPRSS2:ERG fusion status.
Dijkstra et al. (2014) [58]	20	Buffy coat; PCR	TMPRSS2:ERG rearrangements	Rearrangements in the CTCs of 37% of the patients.
Punnoose et al. (2015) [59]	23	No enrichment; IF	TMPRSS2:ERG rearrangements	Rearrangements in the CTCs of 43% of the patients.
Stott et al. (2010) [29]	20	CTC-chip; IF	Ki-67 expression	Only 1-2% of the patients responding to ADT had Ki-67 positive CTCs compared to 27-73% of the patients with progressive mCRPC. In one mCRPC patient with a prolonged PSA stabilization to treatment, the Ki-67 was only 7%.
Reyes et al. (2014) [34]	20	ImageStream X (combined FC and IF)	Ki-67 expression	CTCs with high expression of AR also showed high expression of Ki-67. CTCs with nuclear localization of AR had higher expression of Ki-67 compared to CTCs with cytoplasmic AR localization.
Goldkorn et al. (2015) [60]	215	Microfiltration; Telomeric Repeat Amplification Protocol	Telomerase activity (TA)	Patients with high TA had more extensive disease (*P*=0.04); In patients with a baseline count of ≥5 higher TA was independently associated with worse OS (HR 1.14 (95% CI 1.06-1.23, *P*=0.001)

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Table 4 (continued).

Reference	*N*	CTC isolation; characterization assay	Parameter	Clinical Relevance
Larson et al. (2004) [61]	10	IM enrichment; IF, FC	M-30	The average fraction of M30-positive CTCs was 46.5% with a range of 11-91%.
Attard et al. (2009) [45]	33	CellSearch; FISH	PTEN loss	Loss of PTEN in 13 patients (26.5%)
Punnoose et al. (2015) [59]	23	No enrichment; FISH	PTEN loss	27% of the patients had homozygous PTEN loss in their CTCs and 15% had heterozygous PTEN loss; PTEN loss in CTCs was significantly associated with poor IS in univariate, but not multivariate analysis.
Shaffer et al. (2007) [47]	20	CellSearch; IF	EGFR	The average fraction of EGFR-positive CTCs was 56%, ranging from 0% to 100%.
De Bono et al. (2007) [63]	26	CellSearch; IF	IGF1R	88% of the patients had IGF1R-positive CTCs. This was associated with a higher chance of 50% PSA response to experimental IGF1R-inhibition.

studies. The clinical relevance of these factors for treatment decision-making and sequencing of currently available treatments remain to be established.

Discussion

The treatment landscape for MCRPC has become increasingly dense with the emergence of several new treatment options over the past years. The lack of reliable biomarkers precludes deliberate treatment choices to select the most appropriate therapy for individual patients. Efforts have been made to identify prognostic and predictive factors to guide clinical decisions and the enumeration and characterization of CTCs from peripheral blood by the CellSearch System have shown to be promising in this context. By the cut-off of ≥5 CTCs/7.5 mL of blood, patients with MCRPC can be subdivided into a favorable (<5 CTCs) or unfavorable prognostic group (≥5 CTCs) [10]. Given this strong, independent prognostic value, CTCs deserve to be incorporated in randomized clinical trials as surrogate end-point for OS and as baseline factor to ascertain that the treatment arms are well balanced. Additionally, CTC dynamics during treatment are a superior response evaluation marker over serum PSA levels and radiographic evaluations [10,65]. As such, the enumeration of CTCs has already increasingly been incorporated into clinical trials. Hopefully, the employment of CTCs will result in an acceleration in drug development and at the same time a diminution of the costs of development.

In parallel to the prognostic value, the predictive value of CTC characteristics in guiding up-front treatment decisions is being explored. Amongst others, the presence of mutations, amplifications or splice variants of AR have been assessed in CTCs with the aim to predict resistance to targeted treatments. Indeed, the presence of *AR-V7* in CTCs was shown to be able to predict resistance to abiraterone and enzalutamide [50], but not taxanes [49,66]. This way, baseline characterization of CTCs may support the choice of anti-AR-treatment or chemotherapy for an individual patient and save patients from ineffective treatments with accompanying unnecessary side-effects. Consecutive CTC enumerations and characterizations may help to keep track with the development of resistance during treatment, as a rising CTC count with shifts in the characteristics of the CTC pool may indicate outgrowth of a resistant clone and allow for early intervention through a change of treatment. Altogether, CTCs may help to increase treatment effectiveness and lower health-care costs.

Although encouraging results have been obtained over the past few years, there still is some way to go for CTCs to be implemented into standard clinical care. The detection rate of CTCs in patients with MCRPC lies around 80%, but not all patients with active disease have detectable CTCs in their blood, as would be expected. The CellSearch System relies on the expression of EpCAM and cytokeratin for the isolation and detection of CTCs, making that cells negative for EpCAM and/or cytokeratin will be missed. Indeed, cytokeratin-negative CTCs have been found and were shown to exhibit AR amplifications supporting their malignant origin [33]. To enable the detection of these cells, isolation and detection methods have been developed exploiting the physical and biological characteristics of CTCs, for example the size and deformability of CTCs compared to leukocytes or the expression of other cell surface markers besides EpCAM on CTCs [11].

However, each of the approaches brings its own intrinsic limitations. Knowledge about the biology and the behavior of tumor cells in the circulation has to be improved in order to allow for the development of more sensitive and specific assays to reliably capture CTCs preferably in a single cell manner.

Technical issues in the detection and isolation of CTCs also hinder the clinical applicability of the CTC characterization for use as a liquid biopsy to guide treatment decisions. Currently, characterization assays have to deal with the rarity of CTCs in the blood stream and have to be very sensitive and specific. For example, interrogation of CTCs for the presence of AR amplifications by FISH was only successful in 33 of 89 patients (37%) with ≥4 CTCs [45] and although single cell sequencing has proven to be feasible, sufficient quality DNA samples could only be retrieved from 12 of the 99 CTCs detected (12%) [67]. No validated characterization assays exist at this stage and consequently many different methods have been applied, limiting the possibilities for comparison of the results obtained from the different studies.

In conclusion, CTCs are a promising tool to help select the optimal treatment for individual patients with MCRPC. Whereas a CTC enumeration allows for early and reliable treatment response monitoring, CTC characterization may provide a comprehensive overview of tumor characteristics at real-time. A clear image of possible resistance mechanisms may be obtained through the evaluation of for example the AR mutation, amplification, and splice variant status. The expression and phosphorylation of other proteins beyond AR will in the future likely further improve the predictive value of CTCs and extend the possibilities for tailoring of treatment. While the clinical relevance of sequential CTC counts during treatment for use as an early response evaluation marker has clearly been shown, the value of a CTC characterization to guide treatment decisions in the clinic remains to be investigated. Future prospective clinical trials will have to prove whether CTCs can truly function as a liquid biopsy and shed light in the current dense treatment landscape for individual patients with MCRPC.

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DISCUSSION & FUTURE PERSPECTIVES

CTCs AS A TOOL FOR PRECISION MEDICINE IN ONCOLOGY

The work described in this thesis exemplifies the progress that has been made regarding the use of circulating tumor cells (CTCs) as a tool for precision medicine in oncology. Upon the commercial availability of the CellSearch System in 2004 and the subsequent US Food and Drug Administration (FDA) clearance for the clinical use of the CTC count for patients with metastatic breast cancer (MBC) in 2004 [1], for metastatic colorectal cancer (MCRC) patients in 2007 [2], and for metastatic castration-resistant prostate cancer (MCRPC) patients in 2008 [3], research on the clinical applicability of CTCs has gained momentum. While the initial focus was on the prognostic value of the CTC count to stratify patients into prognostic subgroups, over the years this has shifted to the predictive value of CTC characteristics to guide treatment decision-making by oncologists.

The need for tools to guide clinical decisions is urgent. Recent research on tumor biology has extended our knowledge of tumor progression and has identified several key oncogenic factors allowing for therapeutic interventions. For example, 80-85% of the gastro-intestinal tumors (GISTs) have been found to carry a *c-KIT* mutation, which results in a constitutive active protein product driving malignant behavior of this tumor type [4]. The advent of a specific inhibitor of this protein – imatinib mesylate – has improved the median survival of patients with advanced or metastatic GIST from <1 years to >5 years [5]. Attempts have been made to identify such factors in other tumor types, but, unfortunately, most oncogenic molecular aberrations have been detected in much lower frequencies. In fact, >90% of targetable alterations identified so far have been found in <5% of the patients with a specific tumor type [6], stressing the need to identify driver oncogenic factors for individual patients before selecting specific targeted treatments.

Furthermore, molecular aberrations have been shown not to be static, but rather follow a dynamic pattern, which constantly evolves during the course of the disease and under the pressure of treatments. The heterogeneity in molecular characteristics both between and within patients may have several important implications for the clinic: i) in order to select the most effective targeted treatment, molecular alterations in an individual tumor will have to be determined; ii) the actual molecular profile of a tumor

will have to be determined at the time of treatment decision-making; iii) changes in a tumor's molecular profile will have to be followed up in order to capture the emergence of treatment resistance early-on and to adjust treatment (Figure 1). As a result, minimally invasive methods to molecularly characterize tumor cells throughout a patient's treatment trajectory are highly desired. Representing the smallest comprehensive unit of a tumor, CTCs provide the opportunity to facilitate these molecular analyses.

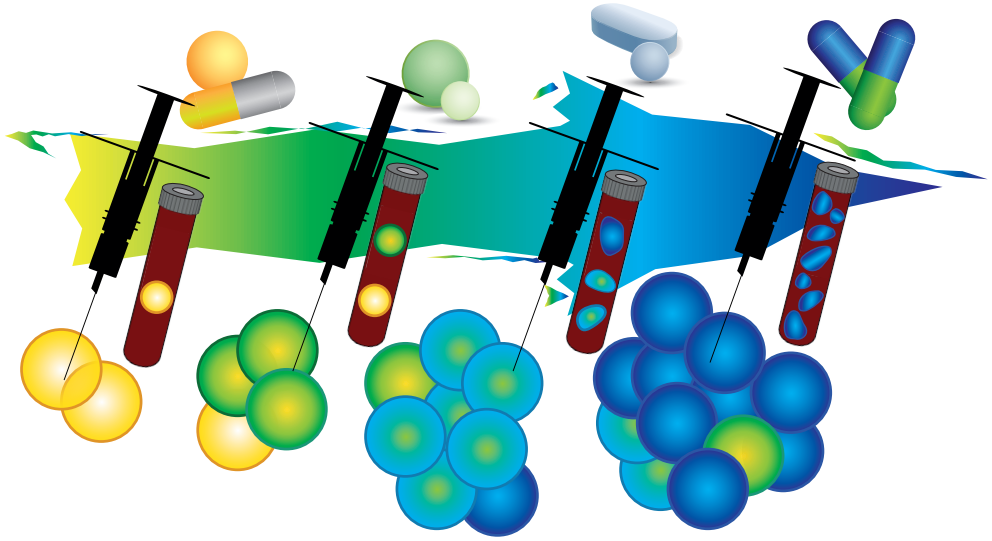

FIGURE 1. The concept of precision medicine in oncology. During a patient's treatment trajectory (represented by the arrow), the molecular make-up of tumor cells changes (represented by the different colors) due to spontaneous tumor evolution and/or under the pressure of administrered treatments. As a consequence of the molecular changes, tumors may become resistant to treatments they may have responded to before. To ascertain that the most effective treatment is given at any given point in time and to improve the prognosis of individual patients, well-informed treatment decisions based on a tumor's actual characteristics will have to be made and treatment may frequently have to be adapted based on the changes that have occurred (represented by the color matched pills). Assessment of tumor characteristics can be done on tumor tissue that has been obtained through for example a needle biopsy (represented by the black needles). However, minimally invasive tests are to be prefered to preserve the quality of life over repeated analyses. The characterization of CTCs from peripheral blood (represented by the blood tubes containing tumor cells) provides opportunities for use as a *liquid biopsy.*

Technical issues

Technical difficulties hamper research on the predictive value of CTCs and, consequently, their usage as a *liquid biopsy*. The low prevalence amongst hematological cells requires extremely sensitive and specific assays to detect, capture, and characterize CTCs from whole blood. Although the CellSearch System is able to detect 1 CTC amidst the billions of erythrocytes, leukocytes, and thrombocytes in 7.5 mL of blood, it only does so in 70-80% of the patients with MBC or MCRPC [7], 50% of the patients with MCRC [7], and even less in other carcinomas such as hepatocellular carcinoma (30%) [8], non-small cell lung carcinoma (24%) [9], pancreatic cancer (21%) [10], and ovarian cancer (14%) [11].

Sampling site

Differences in the biological behavior of tumors may be responsible for the observed differences in the prevalence of detectable CTCs by the CellSearch System. For example, ovarian cancer has the tendency to spread intra-abdominally and does not or only at late stages disseminate hematologically, which may explain the low occurrence of CTCs in the blood stream of patients with even high stage disease [12]. A large proportion of the CTCs from patients with MCRC may become trapped in the small capillaries of the liver and the lungs through which they travel before reaching the systemic peripheral circulation, as may be evidenced by the higher prevalence of CTCs in the portal vein compared to the hepatic vein and the occurrence of tumor micro-emboli in the pulmonary microcirculation [13, 14]. Also, CTC counts have been found to be higher in the central than in the peripheral circulation in patients with MBC [15], again suggesting filtration of CTCs from the circulation in the pulmonary microvasculature. Depending on the tumor type, we may thus have to consider different sources to capture CTCs.

Sampling volume

Increasing the sample volume to be tested for the presence of CTCs may results in improved CTC detection rates [16, 17]. Based on the CTC enumerations by the CellSearch System from the peripheral blood of 836 patients with MBC, MCRC, and MCRPC, it has been calculated that 99% of the patients would have ≥1 CTC in their circulation but that

up to 5 L of blood would have to be filtered to detect ≥1 CTC in all patients [16]. As such, alternative enrichment and detection methods have been developed, such as diagnostic leukapheresis [18] and an *in vivo* enrichment through a peripheral venous catheter-based medical wire (CellCollector, GILUPI, Potsdam, GE) [19]. However, these approaches are less patient-friendly compared to drawing a tube of blood and, importantly, will have to be investigated in large-scale clinical trials in a similar way as has been done for the CellSearch System before eventual clinical implementation.

New enrichment markers

Even when present, current isolation assays may lack the sensitivity to detect all or part of the CTCs in a sample. In MBC, our group has shown that a subset of CTCs does not express the epithelial cell adhesion molecule (EpCAM), which is the surface molecule necessary for the enrichment of CTCs by the CellSearch System (Introduction page 14, Figure 2) [20, 21]. Based on preliminary cell line data and a pilot study in MBC patients, melanoma cell adhesion molecule (MCAM, CD146) was identified as an alternative enrichment marker for EpCAM-negative CTCs [20]. The clinical relevance of the CTC enumeration by a combined EpCAM and MCAM enrichment approach has prospectively been tested in the clinical trial described in chapter 2. The aim for this study was to improve the sensitivity of the CellSearch System for the detection of CTCs in patients with locally advanced breast cancer (LABC) in order to improve the prognostic value and to facilitate downstream characterization of the isolated CTCs. Unfortunately, our primary objective to increase the capture rate of ≥1 CTC from 7.5 mL blood from the current 20% to 40% of the patients with LABC was not met. Still, a significant increase to 30% was observed. No correlation was found between the presence of EpCAM-postive CTCs and achievement of pathological complete response (pCR) to neoadjuvant chemotherapy (NAC). Remarkably, however, none of the patients with MCAM-positive CTCs reached a pCR to NAC compared with 23% of the patients without MCAM-positive CTCs. Although this difference was not statistically significant, survival data will have to be awaited to further conclude on the prognostic value of MCAM- and EpCAM-positive CTCs. Meanwhile, a study has been started to investigate the clinical

relevance of MCAM-positive CTCs in patients with MBC (IMPACT-MBC; NCT01957332). Besides the improvement in the detection of CTCs, a secondary aim of this study is to molecularly characterize the isolated MCAM-positive CTCs and to compare these to the EpCAM-positive CTCs. Since MCAM has been identified as an inducer of epithelial-to-mesenchymal-transition (EMT) [22, 23] – the process during which CTCs lose their epithelial phenotype to acquire a more mesenchymal phenotype as a means to survive in the circulation and to migrate to distant sites [22, 23] – it may well be that MCAM enriches for a more aggressive counterpart of CTCs. Recently, it has been shown that the overall pool of CTCs comprises a spectrum of phenotypes ranging from full epithelial to full mesenchymal CTCs and hybrid epithelial/mesenchymal phenotypes in between (Figure 2) [24]. Increases in mesenchymal CTCs during treatment have been found to be more strongly predictive of treatment resistance than increases in epithelial CTCs [24], which supports further investigation into the associations between the presence of MCAM-positive CTCs and the lack of pCR in patients with LABC as observed in our study.

Other enrichment and detection methods

In an attempt to overcome the limitations formed by the EpCAM-dependency of the CellSearch System a plethora of CTC enrichment and detection methods exploiting different phenotypical and physical properties of CTCs have been developed over the recent years [25]. However, no assay is currently able to directly isolate pure CTC fractions without contamination of leukocytes. To discriminate CTCs from the contaminating leukocytes, a secondary CTC detection step remains necessary. In the CellSearch System, CTCs are identified based on morphological and phenotypical criteria using fluorescence microscopy. A cell is considered a CTC when it has i) an intact, round or oval-shaped morphology; ii) a size of ≥4 x 4 µm; iii) positivity for 4'6-diamidino-2-phenylindole (DAPI), indicating the presence of double-stranded DNA in a cell nucleus, which should overlap ≥50% with the cytoplasm and be smaller than the cytoplasm, which is indicated by; iv) positive fluorescence staining for cytokeratin (CK); and v) negative staining for leukocyte marker CD45 (Introduction page 14, Figure 3). However, the selection based on positive staining for CK has been subject of recent debate, since CK-negative CTCs

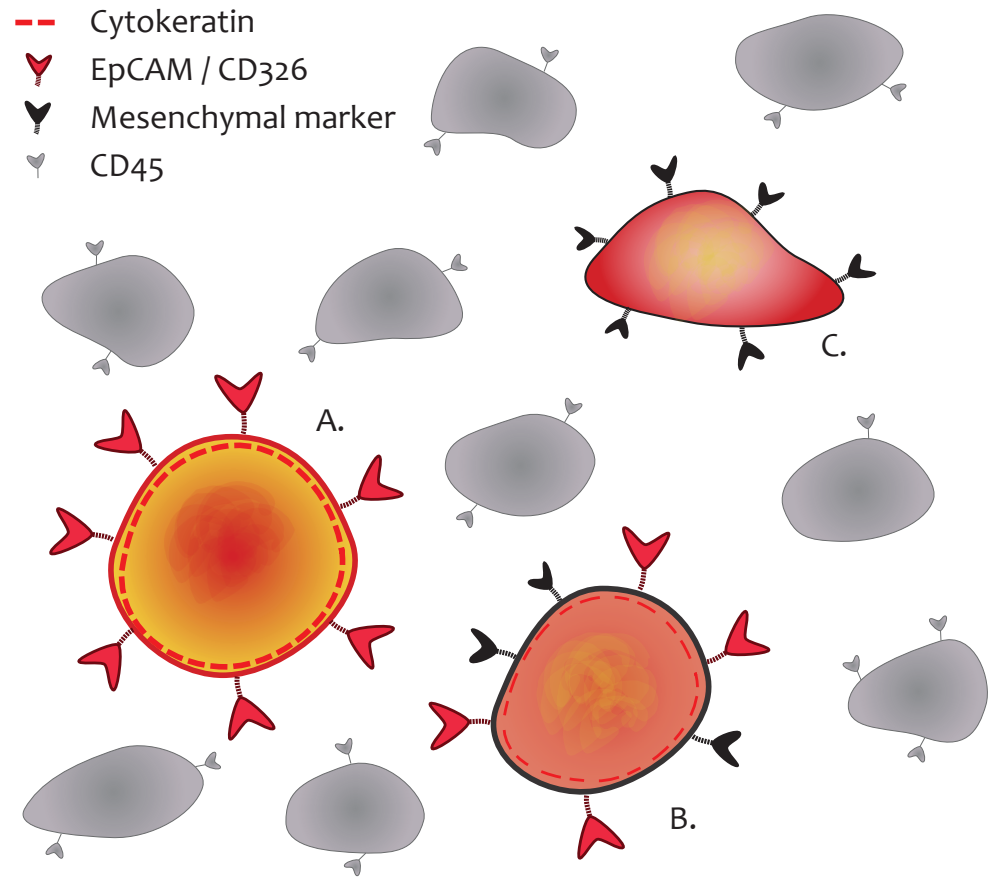

FIGURE 2. Epithelial-to-mesenchymal transition (EMT). After the detachment of CTCs from a solid tumor mass and under influence of factors in the blood circulation, the expression of epithelial markers such as EpCAM and CK may be downregulated or even lost and mesenchymal markers such as vimentin and N-cadherin may be upregulated. The total CTC pool in the blood hence comprises a spectrum of phenotypes ranging from fully epithelial (A.) to hybrid epithelial/mesenchymal (B.) to fully mesenchymal (C.). Mainly the loss of EpCAM (red markers) and CK (red dashed line) cause for a subset of CTCs to go undetected by most currently available detection methods.

have been shown to exist [24-26]. Like EpCAM, CK is downregulated during the process of EMT, meaning that even after enrichment these CTCs would remain undetectable if not recognized due to absent CK staining (Figure 2) [24, 26]. A proposed alternative marker from *in vitro* experiments to be used instead of CK is CD49f [27], although this marker remains to be tested on clinical samples. Alternative enrichment and detection

markers instead of or next to EpCAM and CK must be identified in order to detect, enumerate, and characterize the full spectrum of CTCs, including the subsets that are currenly frequently missed. The characterization of MCAM-positive CTCs will hopefully result in the identification of alternative enrichment and detection markers to improve the CTC detection rate.

Clinical relevance of the CTC receptor status in MBC

An improvement in the CTC detection rate will likely further boost research on the clinical relevance of the characterization of CTCs and the use of CTCs as a tool for tailored treatments based on the presence of predictive factors on/in CTCs. Already, promising results have been obtained concerning the expression of the estrogen receptor (ER) and the human epidermal growth factor receptor 2 (HER2) in CTCs from patients with MBC. As summarized in Chapter 3, different studies have investigated the discordances in receptor status of CTCs compared to the primary tumor. Although the results from the studies have to be compared with caution due to the differences in applied isolation and characterization assays, receptor status conversions have consistently been observed. Whereas the probability of a loss or gain of the expression of HER2 on CTCs in comparison to the primary tumor seems to be equal, the trend for ER is a loss in patients with initial ER-positive tumors. Although the frequencies of discordances in receptor status between CTCs and the primary tumor are difficult to estimate at this stage due to the methodological differences of the studies, it seems to affect a significant proportion of the patients. Given the potential direct therapeutic consequences – either inadequate treatment in patients in whom a negative conversion occurred or missing out on an effective treatment option in patients with a positive conversion – the clinical relevance of the receptor status of CTCs must be prospectively investigated.

As also described in chapter 3, the presence of HER2-positive CTCs has been found to be an adverse prognostic factor. Already, the first prospective clinical trials investigating the prognostic and predictive value of the expression of HER2 on CTCs have been reported [28-31]. Also, we have started a prospective, multicenter, multinational clinical trial to i)

test the efficacy of the HER2-targeted monoclonal antibody trastuzumab in MBC patients with an HER2-negative primary tumor and HER2-positive CTCs (CAREMORE-trastuzumab, NTR5115); and ii) test the impact of the expression of HER2 on CTCs on the efficacy of endocrine treatment in MBC patients with an initial ER-positive/HER2-negative primary tumor (CAREMORE-AI study; NTR5121). The results from these ongoing studies will have to be awaited.

Reflection of tumor characteristics by CTCs

While awaiting the results from prospective trials, studies are trying to shed light on the biology of CTCs. Although generally assumed, it has not been proven that CTCs derive from different metastatic sites and this way truly reflect the characteristics of the entire tumor load including the extent of heterogeneity between tumor clones. Mouse studies have shown that certain CTCs have acquired the capability of forming new metastases [32-34], suggesting that the characteristics of CTCs at least to some extent reflect the characteristics of the metastasis they have formed and are again derived from. However, the impact of the detachment from a solid mass and of factors present in the circulation on the characteristics of CTCs remain largely unknown.

In the study described in chapter 4, we have investigated to what extent the molecular profiles of the CTCs from 62 patients with MBC resembled the primary tumor, which was resected at median 33 months before the CTC blood draw. Using the gene panel of 35 CTC-specific genes that had been established in a prior study [35], we observed discordant overall profiles in 48% of the patients and in the expression of *ESR1* – the gene transcript coding for ER – in 24% of the patients. These discordances were not correlated with clinicopathological parameters. Only a gain of ER was of prognostic significance in our exploratory analyses; the discordances in overall gene expression profiles had no impact on survival. Unfortunately, tissue from distant metastatic sites was not available in this retrospective study and we were not able to investigate the resemblance with a metastasis to investigate whether CTCs best resemble the tumor that is present at the time of blood sampling. This would have been of particular interest for the patients

with discordant CTC versus primary tumor profiles, where the hypothesis is that CTCs no longer resemble the primary tumor due to changes that have occurred to the molecular tumor profile of the metastases and that they instead reflect the characteristics of the metastases.

In the prospective trial described in chapter 5, we applied a similar approach as in the study described above and compared the molecular profiles of CTCs from patients with MCRC to the primary tumor, and in this case also to a liver metastasis. Resection of liver metastases has become common practice for MCRC patients with metastases confined to the liver and hence metastatic tissue is readily available in this setting. From the 23 patients that were included in our study, blood was sampled at the time of liver surgery and tissue from the primary tumor and a liver metastasis were collected. In all samples, the expression of 25 MCRC-associated, CTC-specific genes were measured by RT-qPCR and the three resulting profiles were mutually compared. Interestingly, the profiles of the CTCs correlated with the liver metastasis in 74% of the patients, but with the primary tumor in only 57% of the patients. In another 57% of the patients, the correlation of the CTC profile with the liver metastasis profile was stronger than the correlation of the CTC profile with the primary tumor profile. Comparing the expression of the 25 individual genes between the three tumor compartments over the 23 patients revealed nine genes to be downregulated in the CTCs compared to the primary tumor and/or the liver metastasis. Most of these genes have been described as tumor-suppressors or to be involved in cell-adhesion or EMT, suggesting a functional reason for these genes to be downregulated. Altogether, our study has provided evidence that CTCs reflect the characteristics of the metastases better than the characteristics of the primary tumor and our data suggest that CTCs can indeed be used as surrogates for metastatic tissue.

The predictive value of CTC characteristics for patients with MCRPC

The use of CTCs as a minimally invasive means to investigate the changes in molecular characteristics occurring in a tumor has become of particular interest for patients with MCRPC. Over the last decade, several new compounds have been brought to the market,

among which the new generation taxane cabazitaxel, the CYP17A1-inhibitor abiraterone, and the androgen receptor (AR)-antagonist enzalutamide [36, 37]. Both abiraterone and enzalutamide have been found to be effective treatment options when placed before and after standard treatment with docetaxel chemotherapy [36, 37]. Recent reports on the emergence of cross-resistance – mainly between docetaxel, abiraterone, and enzalutamide [38, 39] – have further stressed the need to define the optimal treatment sequence and to keep track with the development of resistance mechanisms in tumor cells. The fact that a CTC represents the smallest yet integral unit of a tumor, which still contains information on many aspects of the tumor that may cause treatment resistance, such as chromosomal amplifications and translocations, DNA mutations, the upregulation of certain signaling pathways, and the expression of proteins, makes them an invaluable source in this context.

The promise of the characterization of CTCs to guide treatment decision-making has well been illustrated by a recent study showing a strong predictive value of the presence of the *AR* splice variant 7 (*AR-V7*) in CTCs for resistance to the AR-targeted treatments abiraterone and enzalutamide [40]. In this study, the presence of *AR-V7* transcripts in CTCs was measured using the clinically non-validated AdnaTest (Qiagen, Hannover, GE) in 62 patients with MCRPC. Both PFS and OS were shown to be significantly impacted by the presence of *AR-V7* in CTCs. Importantly, none of the 18 patients with *AR-V7*-positive CTCs responded to treatment compared to 27 of the 44 (61%) of the patients with *AR-V7*-negative CTCs (P=0.004). This led us to design the study that has been described in chapter 6. Herein, we have set up an assay to assess the presence of *AR-V7* in CTCs after CellSearch enrichment to test the predictive value of the presence of *AR-V7* in CTCs for response to cabazitaxel. Our hypothesis was that cabazitaxel would remain effective in patients with *AR-V7*-positive CTCs given its AR-independent mechanisms of action. The presence *AR-V7* was detected in the CTCs from 16 of the 29 patients included (55%). Indeed, the CTC response rates – defined as a decrease from ≥5 CTCs before the start of to <5 CTCs during treatment [3] – to cabazitaxel were 20% in both the patients with *AR-V7*-positive and *AR-V7*-negative CTCs at baseline, and survival was not impacted by

the presence of *AR-V7*. Although our study has shown that the *AR-V7* status of CTCs is no prognostic factor for patients that had received cabazitaxel – in contrast to abiraterone or enzalutamide – the true predictive value can only be established by prospective clinical trials, which have in the mean-time been initiated. Currently, we are testing the logistics and the feasibility to report the *AR-V7* status of CTCs back to the clinics within 10 days and before the start of a new treatment line (PRELUDE trial). These logistics will be used in the future multicenter, prospective CARVE trial, which will further investigate the predictive value of the presence of *AR-V7* in CellSearch-enriched CTCs for response to abiraterone/enzalutamide and cabazitaxel.

This thesis concludes with chapter 7, in which the clinical relevance of the CTC enumeration and characterization for the management of MCRPC was discussed. A liquid biopsy through CTC counts and characteristics may fill the gap caused by the lack of tools to enable the selection of the most optimal treatment for an individual patient at a specific point in time during his treatment trajectory. The enumeration of CTCs is able to indicate the aggressiveness of the disease before the start of treatment and CTC dynamics during treatment is a superior response evaluation marker over PSA and imaging [3]. The characteristics of CTCs mainly with regard to mutations and amplifications of *AR* and the presence of *AR* splice variants may predict which treatment would have the highest chance of success, thereby preventing ineffective treatments with unnecessary side-effects. This way, CTCs will help to improve the treatment of individual patients with MCRPC, ultimately improving the prognosis of the entire group of MCRPC patients and rendering the treatment for MCRPC more cost-effective.

Future perspectives

With the ever ongoing advancements in the biomedical technical field, further progress in the research on both the prognostic and the predictive value of CTCs can be foreseen. Already, genomic analysis of single cell CTCs has proven to be feasible [41, 42]. For the future, more efficient techniques to obtain pure CTC samples with the ability to analyze a multitude of genomic and proteomic factors in a single cell fashion can be anticipated. Other biomarkers, such as circulating tumor DNA (ctDNA) from peripheral blood and exosomes that have been released from tumor cells into the blood plasma, may complement the CTC analyses. However, given the comprehensive tumor picture a CTC as unit provides with information on the DNA, RNA, and protein level including heterogeneity between single CTCs, these assays will likely not replace CTCs.

Future studies will have to focus on the biology of CTCs, in addition to further unravel the predictive value of CTC characteristics. Pure CTCs samples without the contamination of leukocytes will have to shed light on the changes that CTCs undergo while circulating. The process of EMT and the reverse process of MET must be studied to identify detection and isolation markers allowing for the capture of all CTC subsets present in the peripheral blood, including the putative most aggressive subset of CTCs that have fully undergone EMT. Only then can the true prognostic and predictive power of CTCs be established. This may also provide new targets for treatment, for example by inhibiting initiators of EMT or blocking the reverse process of MET in order to prevent the formation of (new) metastases. Studies in patients with metastatic disease will further have to focus on the extent to which CTCs truly reflect the characteristics of the metastases and the information on tumor heterogeneity they carry. Preferably, multiple clones from multiple metastases would be sequenced and compared to single CTCs to establish whether CTCs derive from all the tumor clones present or from a subset of the most invasive clones.

The results obtained through yet to be initiated comprehensive large-scale trials investigating the characteristics of CTCs at the DNA, mRNA, and protein level will yield a wealth of information that will help us extract the best ways to treat tumors early-on. Already, large-scale sequencing efforts of metastatic tissues have been started, for example by the Dutch *Center for Personalized Cancer Treatment*. These efforts will help us to identify actionable genomic factors and tumor-specific signaling pathways, but will only to a limited extent give insight into the escape mechanisms, which may develop in tumors under treatment pressure. Also, genomic analyses will not be fully informative for the epigenetic changes, alternative mRNA splicing, or protein-related changes, such

as phosphorylation. Repeated analyses of CTCs could prove to be essential to keep up with all the changes occurring in a tumor over time, thereby considering all molecular features including but also beyond DNA aberrations to allow for early treatment interventions in order to block escape mechanisms. In this scenario, CTCs would be an inestimable tool for the oncologist to either prevent the formation of metastases in patients who present with early stage disease, or oppose the progression of disease in patients who present with late stage disease. Ultimately, cancer may turn into a chronic, manageable disease through repeated analyses of CTC characteristics and deliberate therapeutic adjustments.

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SUMMARY / SAMENVATTING

The studies that have been described in this thesis focus on improvement of the prognostic and predictive value of circulating tumor cells (CTCs) by optimization of the detection, capture, and characterization of CTCs from the peripheral blood of patients with different forms of cancer. In chapter 2 we investigated a new approach to increase the yield of CTCs in patients with locally advanced breast cancer (LABC) by combining the usual epithelial cell adhesion molecule (EpCAM)-based CTC enrichment with an experimental melanoma cell adhesion molecule (MCAM, CD146)-based enrichment on the CellSearch System. In a prior project, our group has shown that a subset of CTCs does not express EpCAM and identified MCAM as an alternative enrichment marker for EpCAM-negative CTCs. We prospectively tested the clinical relevance of the CTC enumeration by EpCAM and MCAM with the aim to improve the sensitivity of the CellSearch System for the detection of CTCs in patients with LABC in order to improve the prognostic value and to facilitate downstream characterization of the isolated CTCs. Unfortunately, our primary objective to increase the capture rate in LABC patients from the current 20% to 40% was not met. Still, a significant increase to 30% was observed. We investigated the correlation between the presence of EpCAM-positive and MCAM-positive CTCs and the response to neoadjuvant chemotherapy (NAC) and found that none of the patients with MCAM-positive CTCs reached a pathological complete response (pCR) to NAC compared with 23% of the patients without MCAM-positive CTCs. This difference was not statistically significant, though. The survival data have to be awaited in order to investigate the association of the presence of EpCAM-positive and MCAM-positive CTCs with disease-free survival (DFS) and overall survival (OS) data.

An improvement in the CTC detection rate will likely further boost research on the clinical relevance of the characterization of CTCs and the use of CTCs as a tool to tailor treatments based on the presence of predictive factors on/in CTCs. Chapter 3 contains a review article discussing the possibilities for and the clinical relevance of the characterization of CTCs from patients with metastatic breast cancer (MBC) mainly focusing on the expression of the human epidermal growth factor receptor 2 (HER2) and the estrogen receptor (ER). Receptor status conversions between primary tumors and CTCs have frequently been

observed, although the results obtained from different studies have to be compared with caution due to the differences in methodology mainly caused by the different CTC detection and characterization assays. Nonetheless, the probability of a loss or gain of the expression of HER2 on CTCs in comparison to the primary tumor seems to be equal, whereas the trend for ER is a loss in patients with initial ER-positive tumors. The presence of HER2-positive CTCs has been found to be an adverse prognostic factor for DFS, progression-free survival (PFS), and OS and is now subject of several prospective clinical trials investigating the predictive value of the expression of HER2 on CTCs, irrespective of the HER2 status of the primary tumor.

The main hypothesis regarding CTCs is that they are derived from different tumor sites that are present at the time of blood sampling and that CTCs this way reflect the characteristics of the entire tumor load, including the extent of heterogeneity between different tumor sites. In the study described in chapter 4, we have investigated to what extent the molecular profiles of the CTCs from 62 patients with MBC resembled the primary tumor, which was resected at median 33 months before the CTC blood draw. After comparison of the expression levels of 35 CTC-specific genes, we observed discordant overall profiles in 48% of the patients and in the expression of *ESR1* – the gene transcript coding for ER – in 24% of the patients. These discordances were not correlated with clinicopathological parameters. Only a gain of ER was of prognostic significance in our exploratory analyses; the discordances in overall gene expression profiles had no impact on survival.

In the prospective trial described in chapter 5, we applied a similar approach to compare the molecular profiles of CTCs from patients with MCRC to the primary tumor, and in this case also to a liver metastasis. Blood from 23 patients was sampled at the time of liver surgery and tissue from the primary tumor and a liver metastasis were collected. In all samples, the expression of 25 MCRC-associated, CTC-specific genes were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and the resulting profiles were mutually compared. The profiles of the CTCs correlated with the

liver metastasis in 74% of the patients, but with the primary tumor in only 57% of the patients. In 57% of the patients the correlation of the CTC profile with the liver metastasis profile was stronger than the correlation of the CTC profile with the primary tumor profile. Comparing the expression of the 25 individual genes between the three tumor compartments over the 23 patients revealed nine genes to be downregulated in the CTCs compared with the primary tumor and/or the liver metastasis. Most of these genes have been described as tumor-suppressor or to be involved in cell-adhesion or epithelial-to-mesenchymal-transition (EMT), suggesting a functional reason for these genes to be downregulated. Altogether, our study has provided evidence that CTCs reflect the characteristics of the metastases better than the characteristics of the primary tumor and our data suggest that CTCs can be used as surrogates for metastatic tissue.

In chapter 6 we investigated the prognostic and predictive value of the presence of androgen receptor splice variant 7 (*AR-V7*) transcripts in the CTCs from patients with metastatic castration-resistant prostate cancer (MCRPC). For this purpose, we set up an assay to measure the expression of *AR-V7* in CTCs after CellSearch enrichment and we investigated the association between the presence of *AR-V7* in CTCs and response to cabazitaxel. Our hypothesis was that cabazitaxel would remain effective in patients with *AR-V7*-positive CTCs given its androgen receptor (AR)-independent mechanisms of action in contrast to the AR-targeted treatments abiraterone and enzalutamide. The presence *AR-V7* was detected in the CTCs from 16 of the 29 patients included (55%) and the CTC response rates – defined as a decrease from ≥5 before the start of to <5 CTCs during treatment – to cabazitaxel were 20% in both the patients with *AR-V7*-positive and *AR-V7*-negative CTCs at baseline. Survival was also not impacted by the presence of *AR-V7*. Our study suggests that cabazitaxel would thus remain a valid treatment option for patients with *AR-V7*-positive CTCs.

This thesis concludes with chapter 7, in which the clinical relevance of the CTC enumeration and characterization for the management of MCRPC is discussed. Studies investigating the clinical relevance of the CTC enumeration and characterization for patients with

MCRPC are summarized and put into perspective. In short, the enumeration of CTCs may help to estimate the aggressiveness of the disease before the start of treatment and CTC dynamics during treatment can be used as a superior early response evaluation marker over PSA and imaging. The characteristics of CTCs mainly with regard to mutations and amplifications of *AR* and the presence of *AR* splice variants may predict which treatment would have the highest chance of success, thereby preventing the administration of ineffective treatments with unnecessary side-effects. This way, CTCs may improve the treatment of individual patients with MCRPC and ultimately the prognosis of the whole group of patients with MCRPC.

Circulerende tumorcellen

Het werk dat in dit proefschrift beschreven is, geeft de voortgang weer van het onderzoek naar het gebruik van circulerende tumorcellen (CTC's) als hulpmiddel voor een persoonsgerichte "therapie op maat" in de oncologie. Sinds het commercieel verkrijgbaar worden van het CellSearch Systeem (Janssen Diagnostics LLC, Raritan, NJ) in 2004 en vervolgens de goedkeuring door de Amerikaanse Food and Drug Administration (FDA) voor het klinisch gebruik van een CTC telling bij patiënten met uitgezaaide borstkanker in 2004 [1], uitgezaaide darmkanker in 2007 [2] en uitgezaaide prostaatkanker in 2008 [3] is het onderzoek naar CTC's in een stroomversnelling geraakt. Hoewel de focus in eerste instantie met name op de prognostische waarde van de CTC telling lag, is dit de laatste jaren verschoven naar de predictieve waarde van CTC's en het gebruik van CTC's ter ondersteuning van behandelbeslissingen door de oncoloog.

CTC's als hulpmiddel in de kliniek

Hulpmiddelen om klinische beslissingen te ondersteunen zijn dringend nodig. Recent onderzoek naar de biologie van tumoren heeft onze kennis over de groei van tumoren vergroot en heeft ertoe geleid dat we verschillende factoren hebben kunnen identificeren die benodigd zijn bij deze groei en die kunnen dienen als aangrijpingspunt voor gerichte therapie. Bijvoorbeeld, 80-85% van de gastro-intestinale stromatumoren (GIST's) draagt een mutatie in het *cKIT* oncogen, wat resulteert in een abnormaal en continu actief eiwitproduct met groei van de tumor als gevolg [4]. Behandeling met een remmer van dit abnormale eiwit – imatinib mesylaat – heeft de mediane overleving van patiënten met een GIST verbeterd van <1 jaar naar ≥5 jaar [5]. Ook in andere tumoren is en wordt gezocht naar moleculaire afwijkingen die op een gelijke manier tumorgroei veroorzaken. Echter, over het algemeen wordt de aanwezigheid van groeibevorderende factoren in veel lagere frequenties per tumorsoort gedetecteerd: 90% van de bekende oncogene mutaties worden gedetecteerd in <5% van de patiënten met een specifiek tumortype [6]. Er lijken dus bij veel tumorsoorten niet één maar vele verschillende factoren betrokken te zijn bij de groei van de tumor. Het bepalen van een moleculair profiel om de aanwezigheid van oncogene factoren in de tumor van individuele patiënten te

onderzoeken zal daarom noodzakelijk zijn om de meest effectieve behandeling op een bepaald moment te kunnen selecteren.

Naast de verschillen tussen patiënten blijkt het moleculaire tumor profiel van een individuele patiënt over de tijd niet constant te zijn; tumoren zijn onderhevig aan veranderingen en evolutie gedurende het ziekteproces en onder de toegediende behandelingen. Hierdoor ontstaan verschillen in de eigenschappen van een tumor, wat ook wel heterogeniteit wordt genoemd (pagina 11, figuur 1). Deze heterogeniteit is zichtbaar te maken in moleculaire profielen en kan zowel tussen patiënten als in een individuele patiënt bestaan. Heterogeniteit heeft verschillende implicaties voor de kliniek: i) de selectie van de meest effectieve behandeling dient te gebeuren op geleide van het moleculaire profiel van de tumor; ii) het moleculaire profiel dient actueel te zijn en zal daarom bepaald moeten worden direct vóór de te starten behandeling; en iii) veranderingen in het moleculaire profiel van een tumor zullen vervolgd moeten worden om het ontstaan van resistentie tegen de ingestelde behandeling op een zo vroeg mogelijk moment te kunnen ondervangen en de behandeling aan te kunnen passen (pagina 179, figuur 1). Minimaal invasieve methoden om tumorcellen gedurende het behandeltraject van een patiënt herhaaldelijk moleculair te kunnen onderzoeken zijn hierbij van groot belang. Aangezien CTC's eenvoudig te verkrijgen zijn middels een simpele bloedafname en een CTC de kleinste integrale eenheid van een tumor vertegenwoordigt, heeft een bloedafname voor de telling en karakterisatie van CTC's de potentie om een belangrijk hulpmiddel te worden voor de moleculaire tumor analyses.

Technische hindernissen

Het onderzoek naar de klinische waarde van CTC's en het gebruik als zogenaamd "*vloeibaar biopt*" wordt echter bemoeilijkt door technische obstakels. De zeer lage prevalentie van CTC's tussen de vele hematologische cellen vereist extreem sensitieve en specifieke methoden om de cellen te detecteren en te vangen uit volbloed om ze vervolgens te karakteriseren voor de moleculaire eigenschappen. Hoewel het CellSearch Systeem in staat is om 1 CTC te detecteren temidden van de miljarden rode bloedcellen,

witte bloedcellen en bloedplaatjes die aanwezig zijn in 7,5 mL bloed, gebeurt dit slechts in 70-80% van de patiënten met uitgezaaide borst- en prostaatkanker [7], 50% van de patiënten met uitgezaaide darmkanker [7] en zelfs nog minder frequent in andere tumortypes zoals leverkanker (30%) [8], niet-kleincellig longkanker (24%) [9], alvleesklierkanker (21%) [10] en eierstokkanker (14%) [11].

Plaats van afname

De verschillen die bestaan in het biologische gedrag van de tumoren kan een verklaring zijn voor de verschillende frequenties waarin CTC's door het CellSearch Systeem in het bloed worden gedetecteerd. Bijvoorbeeld, eierstokkanker is een ziekte die voornamelijk in de buikholte groeit en niet of slechts in vergevorderde stadia via het bloed uitzaait [12]. Dit zou mogelijk het lage percentage van patiënten met detecteerbare CTC's bij dit tumortype kunnen verklaren. Bij darmkanker gaan de CTC's eerst door de bloedvaten van de lever en de longen alvorens ze in de grote bloedsomloop komen. Filtratie van een groot deel van de CTC's in de lever en in de kleine vaatjes van de longen kan in dit geval zorgen voor een lager aantal CTC's bij patiënten met uitgezaaide darmkanker. Onderbouwing voor deze hypothese kan ook gevonden worden in het hogere aantal CTC's dat gedetecteerd werd in het bloed uit de poortader ten opzichte van bloed uit de leverader [13] alsmede het voorkomen van klompjes van tumorcellen in het vaatbed van de longen [14]. Daarnaast zijn verhoogde aantallen CTC's gevonden in bloedafnames uit de centrale, grote circulatie ten opzichte van de perifere circulatie bestaande uit kleinere bloedvaten [15], wat opnieuw aanwijzingen geeft voor het optreden van filtratie van CTC's. Mogelijk is de plaats van afname van de CTC's dus van belang en kan dit verschillen tussen de tumor typen.

Volume

Het vergroten van het volume dat onderzocht wordt voor de aanwezigheid van CTC's kan ook een manier zijn om de CTC detectie te verbeteren [16, 17]. Gebaseerd op de CTC tellingen bij 836 patiënten met uitgezaaide borst-, prostaat- en darmkanker is berekend dat 99% van de patiënten ≥1 CTC('s) in het bloed heeft, maar dat tot 5 L bloed onderzocht

moet worden om daadwerkelijk 1 CTC in al deze patiënten te detecteren. Om deze reden zijn alternatieve verrijkings- en detectiemethoden ontwikkeld, waaronder diagnostische leukaferese [18] en een *in vivo* verrijking middels een vergulde medische draad die via een infuus in de bloedbaan gebracht wordt (CellCollector, GILUPI, Potsdam, GE) [19]. Echter, deze methoden zijn minder patiënt-vriendelijk dan een reguliere bloedafname. Daarnaast zal het van groot belang zijn deze methoden eerst te onderzoeken in grootschalige klinische studies en de CTC telling en/of karakterisatie klinisch te valideren op eenzelfde manier als voor het CellSearch Systeem is gedaan alvorens over te gaan tot klinische implementatie.

Nieuwe detectie merkers

Een te lage sensitiviteit van de huidige detectiemethoden kan een andere verklaring zijn voor het lage aantal CTC's dat momenteel gedetecteerd wordt. Onze groep heeft in uitgezaaide borstkanker laten zien dat er een subgroep van CTC's bestaat welke geen of slechts zeer laag het eiwit EpCAM tot expressie brengt. Aangezien dit membraaneiwit door het CellSearch Systeem gebruikt wordt om CTC's uit volbloed te vangen, worden de EpCAM-negatieve CTC's met de huidige methode gemist (pagina 14, figuur 2). Gebaseerd op *in vitro* cellijn experimenten werd het eiwit MCAM (CD146) geïdentificeerd als een mogelijke alternatieve detectie merker voor EpCAM-negatieve CTC's [20, 21]. De klinische relevantie van een CTC telling middels zowel EpCAM als MCAM is prospectief getest in de klinische studie die beschreven is in hoofdstuk 2. Het doel van deze studie was om de sensitiviteit van het CellSearch Systeem te verbeteren voor de detectie van CTC's bij patiënten met lokaal gevorderde borstkanker. Uiteindelijk zou dit kunnen helpen de prognostische waarde van CTC's te versterken en de erop volgende karakterisatie te vergemakkelijken. Helaas werd het primaire doel van de studie – om de detectie van ≥1 CTC/7,5 mL bloed te verbeteren van de huidige 20% van de patiënten met lokaal gevorderde borstkanker naar een beoogde 40% – niet behaald. Desalniettemin werd een significante verbetering naar 30% van de patiënten in onze studie gevonden. Opvallend genoeg bereikte geen van de patiënten met MCAM-positieve CTC's een pathologisch complete respons op neo-adjuvante chemotherapie tegenover 23% van de patiënten

zonder MCAM-positieve CTC's. Hoewel dit verschil niet statistisch significant was, zullen de overlevingsdata afgewacht moeten worden om een definitieve conclusie te kunnen trekken over de prognostische waarde van MCAM- en EpCAM-positieve CTC's bij de patiënten in onze studie. In de tussentijd is een studie gestart waarin de klinische relevantie van MCAM-positieve CTC's bij patiënten met uitgezaaide borstkanker verder wordt onderzocht (IMPACT-MBC; NCT01957332). Naast de verbetering in de detectie van CTC's is een secundair doel van deze studie om geïsoleerde MCAM-positieve CTC's moleculair te karakteriseren en deze te vergelijken met de EpCAM-positieve CTC's. Aangezien MCAM geïdentificeerd is als een van de factoren die betrokken zijn bij epitheliale-naar-mesenchymale transitie (EMT; een proces waarin CTC's hun epitheliale fenotype kwijtraken en een meer mesenchymaal fenotype verkrijgen om op deze manier in de bloedstroom te kunnen overleven en naar weefsels op afstand te kunnen migreren) [22, 23] isoleert de MCAM verrijking mogelijk voor een agressievere subset van CTC's. Onlangs is aangetoond dat de gehele CTC fractie een spectrum aan fenotypes omvat dat reikt van volledig epitheliaal naar volledig mesenchymaal en gemengde epitheliaal-mesenchymale fenotypes daartussen (pagina 183, figuur 2) [23]. Een toename in het aantal mesenchymale CTC's gedurende de behandeling bleek sterker predictief te zijn voor resistentie tegen de ingestelde behandeling dan een toename van de epitheliale subset [24]. Deze resultaten steunen verder onderzoek naar de verbanden tussen de aanwezigheid van MCAM-positieve CTC's en het uitblijven van pathologisch complete respons op neo-adjuvante chemotherapie zoals gevonden werd bij de patiënten met lokaal gevorderde borstkanker in onze studie.

Andere detectiemethoden

Recente inspanningen om de beperkingen van het CellSearch Systeem, welke met name gevormd worden door de EpCAM-afhankelijkheid voor het detecteren en vangen van CTC's, te ondervangen hebben geleid tot een veelvoud aan CTC verrijkings- en detectiemethoden. Hoewel de verschillende methoden uitgaan van verschillende eigenschappen van CTC's – naast de aanwezigheid van membraaneiwitten onder andere ook de grootte en vervormbaarheid van de cellen ten opzichte van bloedcellen

– heeft iedere methode ook zijn eigen nadelen. Geen enkele methode is op dit moment in staat om pure CTC fracties te isoleren uit volbloed zonder "bijvangst" van witte bloedcellen. Om de CTC's van de witte bloedcellen te kunnen onderscheiden is altijd nog een vervolgstap nodig. Het CellSearch Systeem maakt hiervoor naast kenmerken in de vorm van de cellen gebruik van fluorescente antistoffen om de aan- of afwezigheid van bepaalde eiwitten te visualiseren. Een cel wordt beschouwd als een CTC als deze i) intact en rond of ovaal van vorm is; ii) een minimale grootte van 4 x 4 µm heeft; iii) positief is voor 4'6-diamidino-2-phenylindool (DAPI), wat de aanwezigheid van dubbelstrengs DNA in een celkern zichtbaar maakt, deze kleuring moet voor tenminste 50% binnen de cel liggen; iv) positieve fluorescente aankleuring van cytokeratine; en v) geen aankleuring voor de witte bloed cel merker CD45 laat zien (pagina 14, figuur 3). Recentelijk is de aankleuring van cytokeratine als criterium om een cel een CTC te noemen echter onderwerp van discussie geworden, nadat het bestaan van cytokeratine-negatieve CTC's werd aangetoond [24-26]. Net als EpCAM wordt de expressie van cytokeratine omlaag gebracht tijdens het proces van EMT, wat betekent dat deze cellen niet gedetecteerd kunnen worden wegens afwezige cytokeratine aankleuring zelfs al worden ze gevangen [24, 26]. Een mogelijke alternatieve merker voor cytokeratine zou CD49f kunnen zijn [27], hoewel deze merker alleen nog afkomstig is uit cellijn experimenten en getest dient te worden op patiënten materiaal. Al met al zullen alternatieve merkers voor de verrijking en detectie van CTC's in plaats van of naast EpCAM en cytokeratine geïdentificeerd moet worden om de detectie en telling van CTC's, inclusief de subset van CTC's die met de huidige methoden niet of moeilijk detecteerbaar is, uit volbloed te optimaliseren en de CTC karakterisatie te faciliteren. Het onderzoek naar het vóórkomen en de eigenschappen van MCAM-positieve CTC's zal hopelijk resulteren in nieuwe merkers die de CTC detectie kunnen verbeteren.

CTC's en de behandeling van patiënten met uitgezaaide borstkanker

Een verbetering in de CTC detectie kan een nieuwe impuls geven aan het onderzoek naar de klinische relevantie van de karakterisatie van CTC's en het gebruik van CTC's als hulpmiddel voor therapie op maat, gebaseerd op de eigenschappen van CTC's. Veelbelovende resultaten zijn al behaald waar het de expressie van de oestrogeen receptor (ER) en de humaan epidermale groeifactor receptor 2 (HER2) – beide belangrijke aangrijpingspunten voor de behandeling van patiënten met borstkanker – in CTC's bij patiënten met uitgezaaide borstkanker betreft. In hoofdstuk 3 is een overzicht gegeven van de frequentie van discordante expressie van ER en HER2 tussen de CTC's en de primaire borsttumor, welke onderzocht zijn in verschillende studies. Hoewel de resultaten op dit moment moeilijk vergelijkbaar zijn door de grote verschillen in de toegepaste CTC isolatie en karakterisatie methoden, zijn conversies in de receptor status van CTC's ten opzichte van de primaire tumor consistent geobserveerd. Waar de waarschijnlijkheid van het verliezen of verwerven van HER2 op CTC's in vergelijking met de primaire tumor vergelijkbaar lijkt te zijn, is de trend voor ER een verlies bij patiënten met een oorspronkelijk ER-positieve tumor. Hoewel exacte frequenties van discordante expressie op dit moment moeilijk aan te geven zijn door de grote verschillen tussen de studies, lijkt het om een significant deel van de patiënten te gaan. Gezien dat dit mogelijk directe therapeutische consequenties met zich meebrengt – ofwel het geven van een inadequate behandeling bij patiënten waar de receptor verloren is gegaan ofwel het niet overwegen van een effectieve behandeling bij patiënten waar een receptor in eerste instantie niet aanwezig was – is het van groot belang om de klinische relevantie van de receptor status van CTC's nader te onderzoeken in prospectieve klinische studies. Zoals ook beschreven in hoofdstuk 3 is de aanwezigheid van HER2 op CTC's prognostisch gebleken voor slechte overleving. De eerste prospectieve klinische studies die de prognostische en predictieve waarde van de HER2 status van CTC's hebben onderzocht zijn inmiddels ook gepubliceerd [28-31]. Op dit gebied heeft ook onze groep een prospectieve, multicenter, multinationale klinische studie gestart om i) de effectiviteit te testen van het anti-HER2 monoclonale antilichaam trastuzumab bij patiënten met uitgezaaide borstkanker met een HER2-negatieve primaire borsttumor en HER-positieve

CTC's (CAREMORE-trastuzumab, NTR5115); en ii) de impact te testen van de expressie van HER2 op CTC's op de effectiviteit van hormonale therapie bij patiënten met uitgezaaide borstkanker met een oorspronkelijk ER-positieve/HER2-negatieve primaire borsttumor (CAREMORE-AI study; NTR5121). De resultaten van deze nog lopende studies zullen afgewacht moeten worden.

CTC's versus de primaire tumor versus een uitzaaiing

In afwachting van de resultaten uit de lopende prospectieve klinische studies gaat het onderzoek naar de biologie van CTC's onverminderd door. Hoewel in het algemeen aangenomen, is nooit bewezen dat CTC's ook daadwerkelijk afkomstig zijn van de verschillende uitzaaiingen die aanwezig kunnen zijn bij een patiënt en dat CTC's op deze manier de eigenschappen van de gehele tumormassa weergeven, inclusief de mate van heterogeniteit. Studies in muizen hebben aangetoond dat bepaalde CTC's in staat zijn om nieuwe uitzaaiingen te vormen [32-34], wat suggereert dat CTC's tot op zekere hoogte de eigenschappen weergeven van de uitzaaiing die ze hebben gevormd en waar ze opnieuw van afkomstig zijn. De effecten van het loskomen van een tumor massa en van factoren uit de bloedbaan op de eigenschappen van CTC's blijven echter nog onbekend.

In de studie die beschreven is in hoofdstuk 4 is onderzocht in welke mate de moleculaire profielen van de CTC's van 62 patiënten met uitgezaaide borstkanker lijken op die van de primaire borsttumor, welke mediaan genomen 33 maanden eerder chirurgisch was verwijderd. Gebruik makend van het panel van 35 CTC-specifieke genen zoals gedefinieerd in een eerdere studie [35], vonden wij discordante profielen in 48% van de patiënten. De expressie van *ESR1* – het gen dat codeert voor ER – was discordant in 24% van de patiënten. De gevonden discordanties waren niet gecorreleerd aan klinische en pathologische parameters. Enkel het verwerven van ER had significante prognostische waarde in onze exploratieve analyses; discordantie over het gehele genpanel had geen gevolgen voor overleving. Helaas was weefsel van een uitzaaiing op afstand niet beschikbaar voor de patiënten uit deze retrospectieve studie waardoor we niet in staat waren om het CTC profiel te vergelijken met die van een uitzaaiing en te onderzoeken of

CTC's beter lijken op de tumor die op het moment van de bloedafname nog daadwerkelijk bij de patiënt was. Dit zou met name van toegevoegde waarde zijn voor de patiënten uit onze studie waar de CTC versus primaire tumor profielen discordant waren en waar de hypothese is dat de CTC's niet meer lijken op de primaire tumor door veranderingen die zijn ontstaan in de uitzaaiingen gedurende de tijd en dat de CTC's deze veranderingen weergeven.

In hoofdstuk 5 is een prospectieve studie beschreven waarin eenzelfde aanpak is gevolgd als in het onderzoek dat hierboven beschreven is. In dit geval is het moleculaire profiel van de CTC's van patiënten met uitgezaaide darmkanker vergeleken met die van de primaire darmtumor alsook met het profiel van een leveruitzaaiing. Het operatief verwijderen van leveruitzaaiingen is standaard zorg geworden voor patiënten waar de uitzaaiingen zich beperken tot de lever en hierdoor is weefsel van een uitzaaiing op afstand makkelijker verkrijgbaar geworden voor deze patiëntengroep. Van de 23 patiënten die geïncludeerd waren in de studie werd bloed afgenomen direct voorafgaand aan de leveroperatie en werd weefsel van een leveruitzaaiing en de primaire tumor verzameld. In zowel de CTC's uit het bloed als de tumorweefsels werd de expressie van een panel van 25 CTC-specifieke genen gemeten middels *reverse transcription quantitative polymerase chain reaction* (RT-qPCR) en deze werden onderling vergeleken. Interessant genoeg bleken het profiel van de CTC's overeen te komen met de leveruitzaaiing bij 74% van de patiënten, maar met de primaire darmtumor maar bij 57% van de patiënten. Bij 57% van de patiënten was de correlatie tussen het profiel van de CTC's en de leveruitzaaiing sterker dan die tussen de CTC's en de primaire darmtumor. Het vergelijken van de expressie van de 25 individuele genen over de 23 patiënten tussen de drie tumorcompartimenten resulteerde in negen genen die significant verlaagd tot expressie kwamen in de CTC's ten opzichte van de leveruitzaaiing en de primaire tumor. Over het algemeen zijn deze genen beschreven als zijnde tumor suppressor of betrokken bij celadhesie en/of EMT. Dit suggereert dat het verlagen van de expressie van deze genen door de tumorcellen een functionele reden heeft. Alles tezamen laat onze studie zien dat de eigenschappen van CTC's het beste

lijken op de uitzaaiing op afstand in plaats van op de primaire darmtumor en suggereren de data dat CTC's inderdaad gebruikt kunnen worden als surrogaat voor weefsel van uitzaaiingen.

CTC's en de behandeling van patiënten met uitgezaaide prostaatkanker

Het gebruik van CTC's als een minimaal invasieve manier om veranderingen in de moleculaire eigenschappen van een tumor te onderzoeken heeft recentelijk veel aandacht getrokken voor de behandeling van patiënten met uitgezaaide castratie-resistente prostaatkanker. De laatste jaren zijn er veel nieuwe behandelingen beschikbaar gekomen, waaronder de nieuwe generatie taxaan cabazitaxel, de CYP17A1-remmer abiraterone en de androgeen receptor (AR)-antagonist enzalutamide [36, 37]. Zowel abiraterone als enzalutamide zijn effectieve behandelopties gebleken voor en na behandeling met docetaxel chemotherapie, wat de optimale behandelvolgorde onduidelijk maakt [36, 37]. Het aantonen van het bestaan van kruisresistentie tegen behandelingen – met name tussen docetaxel, abiraterone en enzalutamide [38, 39] – onderstreept het belang van weloverwogen, geïnformeerde beslissingen over de meest optimale therapie op een specifiek moment. Het feit dat CTC's de kleinste volledige eenheid van een tumor vertegenwoordigen, waarbij informatie beschikbaar blijft aangaande velerlei aspecten van een tumor welke resistentie kunnen veroorzaken – zoals chromosomale amplificaties en translocaties, DNA mutaties, opregulatie van specifieke signaalpaden in de tumor en de aanwezigheid van eiwitten – maakt deze cellen van onschatbare waarde.

Dat de karakterisatie van CTC's ter ondersteuning van klinische behandelkeuzes veelbelovend is, werd recent geïllustreerd door een studie die aantoonde dat de aanwezigheid van de AR splice variant 7 (*AR-V7*) in CTC's van sterk ongunstige prognostische waarde is voor patiënten die behandeld werden met de anti-AR gerichte middelen abiraterone en enzalutamide [40]. In deze studie werden de CTC's van 62 patiënten met uitgezaaide prostaatkanker gedetecteerd en gekarakteriseerd voor de aanwezigheid van *AR-V7* middels de klinisch niet-gevalideerde AdnaTest (Qiagen, Hannover, GE). De *AR-V7* status van CTC's had een significant negatief effect op zowel

de progressie-vrije als totale overleving (respectievelijk PFS en OS). Opvallend was dat geen van de 18 patiënten met *AR-V7*-positieve CTC's respondeerde op de behandeling versus 27 van de 44 patiënten (61%) met *AR-V7*-negatieve CTC's (P=0.004). De resultaten uit deze studie leidden ons ertoe om de studie beschreven in hoofdstuk 6 op te zetten. In deze studie hebben wij een methode opgezet om de aanwezigheid van *AR-V7* te meten in CTC's die door de CellSearch gedetecteerd zijn. Het doel van de stdie was om de predictieve waarde van de aanwezigheid van *AR-V7*-positieve CTC's voor respons op cabazitaxel te onderzoeken. Onze hypothese was dat behandeling met cabazitaxel chemotherapie effectief zou blijven onafhankelijk van de aanwezigheid van *AR-V7* in CTC's gezien de merendeels AR-onafhankelijke werkingsmechanismen van cabazitaxel. Bij 16 van de 29 patiënten in onze studie (55%) werden *AR-V7*-positieve CTC's gedetecteerd. Zoals verwacht werd geen verschil waargenomen in de CTC respons – gedefinieerd als een afname van ≥5 CTC's voor start van de cabazitaxel naar <5 CTC's gedurende de behandeling [41] – op cabazitaxel; deze was 20% bij zowel de *AR-V7*-positieve als *AR-V7*-negatieve patiënten. Tevens werd geen effect van de aanwezigheid van *AR-V7* op overleving waargenomen. Hoewel onze studie laat zien dat de *AR-V7* status van CTC's geen prognostische waarde heeft onder behandeling met cabazitaxel – in tegenstelling tot abiraterone en enzalutamide – kan de werkelijke predictieve waarde alleen bevestigd worden middels prospectieve klinische studies, welke in de tussentijd zijn gestart. Zo hebben wij de PRELUDE studie gestart om de logistieke pijplijn te testen en de haalbaarheid te toetsen van het terug rapporteren van de *AR-V7* status van CTC's naar de kliniek binnen 10 dagen na bloedafname. Deze logistiek zal vervolgens ingezet worden voor de toekomstige multicentrische, prospectieve CARVE studie, welke de predictieve waarde zal onderzoeken van de aanwezigheid van *AR-V7* in door CellSearch gedetecteerde CTC's van patiënten met uitgezaaide castratie-resistente prostaatkanker die gaan starten met behandeling met ofwel abiraterone/enzalutamide ofwel cabazitaxel.

Dit proefschrift besluit met hoofdstuk 7, waarin de klinische relevantie van het tellen en karakteriseren van CTC's voor de behandeling van patiënten met uitgezaaide castratie-resistente prostaatkanker bediscussieerd wordt. Een vloeibaar biopt middels CTC telling

en eigenschappen kan mogelijk het gat opvullen dat gevormd wordt door het ontbreken van hulpmiddelen die de selectie mogelijk maken van de meest optimale behandeling voor een individuele patiënt op een specifiek tijdspunt gedurende zijn behandeltraject. Het tellen van CTC's informeert over de agressiviteit van de ziekte voor start van een behandeling; het veranderen van het aantal CTC's gedurende de behandeling kan iets zeggen over het wel of niet aanslaan van die behandeling [3]. De responsevaluatie middels herhaalde CTC tellingen is superieur gebleken aan de huidige evaluatiemethoden die bestaan uit het meten van het prostaat-specifieke antigeen (PSA) in het bloed en beeldvorming in de vorm van een CT-scan en/of een botscan. De eigenschappen van CTC's, met name met het oog op de aanwezigheid van *AR* mutaties en amplificaties en de aanwezigheid van *AR* splice varianten, kunnen mogelijk voorspellen welke behandeling de grootste kans van slagen heeft op een specifiek moment. Daarbij worden ineffectieve behandelingen met onnodige bijwerkingen voorkomen. Op deze manier kunnen CTC's bijdragen aan een verbeterde prognose voor de gehele groep van patiënten met uitgezaaide prostaatkanker en tevens de behandeling meer kosten-effectief maken.

Perspectief voor de toekomst

Met de immer doorgaande ontwikkelingen in het biomedisch technische vakgebied kan meer voortgang verwacht worden in het onderzoek naar zowel de prognostische als de predictieve waarde van CTC's. Het is nu al mogelijk gebleken om het DNA van individuele CTC's te analyseren [41, 42]. In de toekomst zullen naar verwachting meer efficiënte technieken ter beschikking komen om een veelvoud aan genetische en eiwit-gerelateerde factoren te meten in een enkele CTC. Andere biologische merkers, zoals het circulerend tumor DNA (ctDNA) uit bloed en exosomen – minipartikels uitgescheiden door cellen – in plasma, kunnen de CTC analyses mogelijk aanvullen. Echter, gezien het complete beeld dat CTC's van een tumor kunnen geven aangaande informatie vanuit DNA, RNA en eiwit niveau inclusief de mate van heterogeniteit in deze eigenschappen tussen verschillende CTC's is het niet aannemelijk dat deze merkers CTC's zullen vervangen.

Voor de toekomst zal de focus van studies moeten liggen op de biologie van CTC's als aanvulling op het onderzoek naar de predictieve waarde. Onderzoek in pure CTC fracties zonder bijvangst van bloedcellen zal moeten ophelderen welke veranderingen CTC's ondergaan wanneer ze circuleren in het bloed. Het proces van EMT en het tegengestelde proces van mesenchymale-naar-epitheliale-transitie (MET) moet worden bestudeerd om tot detectiemerkers te komen die het mogelijk maken om alle CTC's die aanwezig zijn in het bloed te vangen, inclusief de subset van CTC's die volledige EMT hebben ondergaan. Pas dan kan de echte prognostische en predictieve waarde van CTC's worden vastgesteld. Bovendien kan dit nieuwe aangrijpingspunten voor behandeling opleveren, bijvoorbeeld door de factoren die EMT in gang zetten te remmen of het tegengestelde proces van MET tegen te gaan zodat er geen (nieuwe) uitzaaiingen gevormd kunnen worden. Studies bij patiënten met uitgezaaide ziekte zullen moeten uitwijzen tot op welke hoogte de eigenschappen van CTC's overeenkomen met de uitzaaiingen en of ze werkelijk iets kunnen zeggen over de mate van heterogeniteit tussen verschillende uitzaaiingen. Hiervoor zou bij voorkeur het DNA uit verschillende tumorklonen van verschillende uitzaaiingen genetisch onderzocht moeten worden, waarna dit vergeleken zou moeten worden met het genetische profiel van verschillende losse CTC's om vast te kunnen stellen of CTC's afkomstig zijn van alle tumorklonen die aanwezig zijn of van een selectie van de meest agressieve tumorklonen.

Resultaten verkregen uit nog te starten, veelomvattende en grootschalige klinische studies naar de eigenschappen van CTC's op het niveau van het DNA, RNA en eiwit zullen een schat aan informatie opleveren, welke zal helpen om de beste manier te bepalen om tumoren vanaf een zo vroeg mogelijk stadium te behandelen. Inmiddels zijn zulke studies reeds gestart, waarbij weefsel afkomstig van een uitzaaiing wordt onderzocht, bijvoorbeeld het onderzoek zoals dat uitgevoerd wordt door het Nederlands Centrum voor Persoonsgerichte Behandeling van Kanker (*Center for Personalized Cancer Treatment, CPCT*). Deze inspanningen zullen ons verder op weg helpen om tumor-specifieke genetische factoren en signaalpaden te identificeren die als aangrijpingspunt voor therapie kunnen dienen. Echter, het onderzoeken van een uitzaaiing op een

specifiek tijdspunt zal weinig inzicht geven in het ontstaan van mechanismen die een tumor kan aanwenden om onder de druk van een behandeling uit te komen. Daarnaast geven puur genetische analyses niet alle informatie over de veranderingen die in een tumor kunnen optreden. Bijvoorbeeld epigenetische veranderingen, alternatieve splitsing van gen transcripten, of eiwit-gerelateerde veranderingen zoals fosforylatie worden niet meegenomen in de genetische analyses. Middels herhaalde bepalingen van de eigenschappen van CTC's kunnen veranderingen van een tumor gedurende de tijd en over verschillende behandelingen potentieel zichtbaar gemaakt worden. Doel hierbij is om alle moleculaire eigenschappen in aanvulling op de DNA afwijkingen in ogenschouw te nemen om zo vroeg mogelijk te kunnen acteren op het ontstaan van resistentie tegen de ingestelde behandeling. Uitgaande van dit scenario zullen CTC's een onmisbaar hulpmiddel voor de oncoloog kunnen gaan worden om ofwel het ontstaan van uitzaaiingen te voorkomen bij patiënten die zich presenteren met lokale ziekte ofwel de groei van de ziekte tegen te gaan bij patiënten die zich presenteren met reeds gevorderde ziekte. Uiteindelijk zal kanker op deze manier een chronische, goed te behandelen ziekte kunnen worden, gebaseerd op weloverwogen en goed geïnformeerde behandelkeuzes aan de hand van herhaalde analyses van de eigenschappen van CTC's.

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LIST OF ABBREVIATIONS

AC	Adriamycin + cyclophosphamide combination chemotherapy
ALP	Alkaline Phosphatase
APC	Allophycocyanin
AR	Androgen Receptor
AR-V7	Androgen Receptor splice Variant 7
AR-WT	Wild-Type Androgen Receptor
BR	Bloom & Richardson grade
BRAF	B-RAF Proto-oncogene, serine/threonine kinase
GUSB	Gluceronidase beta; gene involved in regular cell metabolism
CD	Cluster of Differentiation molecule
cDNA	Complementary DeoxyriboNucleic Acid
CEC	Circulating Endothelial Cell
CEER	Collaborative Enzyme Enhanced Reactive (assay)
cfDNA	Cell-Free DeoxyriboNucleic Acid
CI	Confidence Interval
CK	CytoKeratin
cKIT	KIT proto-oncogene receptor tyrosine kinase
CMF	Cyclophosphamide, Methotrexate and Fluorouracil combination chemotherapy
CRPC	Castration-Resistant Prostate Cancer
C_q	Cycle threshold for quantification
CT	Computed Tomography
CTC(s)	Circulating Tumor Cell(s)
ctDNA	Circulating Tumor DeoxyriboNucleic Acid
DAPI	4',6-DiAmidino-2-Phenylindole
DFS	Disease-Free Survival

DMFS	Distant Metastasis-Free Survival
DNA	DeoxyriboNucleic Acid
DSB	Double Strand Break
DTC	Disseminated Tumor Cell
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor alpha
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2, gene coding for HER2
ERCC1	Excision Repair Cross-Complementing protein 1
ESR1	Estrogen Receptor 1, gene coding for the ER-alpha protein
FEC	Fluorouracil, Epirubicin and Cyclophosphamide combination chemotherapy
FDA	Food and Drug Administration (United States)
FGFR	Fibroblast Growth Factor Receptor
FITC	Fluorescein IsoThioCyanate
FISH	Fluorescence *In Situ* Hybridization
FF	Fresh-Frozen
FFPE	Formalin-Fixed Paraffin-Embedded
FU	Follow-Up
GIST	Gastro-Intestinal Stromal Tumor
HBD	Healthy Blood Donor
HE	hematoxylin and eosin (staining)
HER2	Human Epidermal growth factor Receptor 2
HMBS	Hydroxymethylbilane synthase; gene involved in regular cell metabolism

HPRT1	Hypoxanthine Phosphoribosyltransferase 1; gene involved in regular cell metabolism
HR	Hazard Ratio
IF	ImmunoFluorescence
IQR	InterQuartile Range
KRT19	Gene coding for cytokeratin-19
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LABC	Locally-Advanced Breast Cancer
LDH	Lactate DeHydrogenase
LHRH	Luteinizing Hormone-Releasing Hormone
LNM	Lymph Node Metastasis
M	Metastasis
MAI	Mitotic Activity Index
MBC	Metastatic Breast Cancer
MCAM	Melanoma Cell Adhesion Molecule
MCRC	Metastatic ColoRectal Cancer
MCRPC	Metastatic Castration-Resistant Prostate Cancer
MET	Mesenchymal-to-Epithelial Transition
Metachr	Metachronous (metastasis)
mRNA	Messenger RiboNucleic Acid
MUC1	Mucin 1
NAC	NeoAdjuvant Chemotherapy
NR	Not Reported
NRAS	Neuroblastoma RAS Viral Oncogene Homolog
OS	Overall Survival
PBC	Primary Breast Cancer

pCR	Pathological Complete Response
PCWG2	Prostate Cancer Working Group 2
PD	Progressive Disease
PE	PhycoErythrin
PFS	Progression-Free Survival
pHER2	Phosphorylated HER2
PI3K	Phosphoinositide 3-Kinase
PR	Progesterone Receptor
PSA	Prostate-Specific Antigen
PT	Primary Tumor
RECIST	Response Evaluation Criteria in Solid Tumors
ROC	receiver operating characteristics
RR	Response Rate
RT-(q)PCR	Reverse Transcription (Quantitative) Polymerase Chain Reaction
sd	standard deviation
Synchr	Synchronous (metastasis)
TAC	Taxotere (docetaxel)/Adriamycine (doxorubicin)/ Cyclophosphamide combination chemotherapy
TTS	Time-to-Treatment Switch
Uk	Unknown
ULN	Upper Limit of Normal
WHO	World Health Organization
WT	Wild-type
ZA	Zoledronic Acid

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PHD PORTFOLIO, CURRICULUM VITAE & LIST OF PUBLICATIONS

General Courses	Year	ECTS
BROK course on clinical research, legislation and organization	2008	1.5
BROK recertification	2014	1
Biomedical English Writing	2012	2
NiHes Winter Programme: • Clinical epidemiology • Biostatistics for clinicians • Regression analysis for clinicians • Survival analysis for clinicians	2012	5
Workshop Successful Grant Writing	2012	0.3
BKO training Teach the Teacher	2012	1
Workshop Adobe Photoshop and Ilustrator CS6	2013	0.15
Workshop Adobe Indesign CS6	2014	0.15
Workshop How to teach groups of students	2014	0.15
Workshop Individual Interviews with Students	2014	0.15
Masterclass Cambridge Advanced General English (two semesters)	2015 2016	3

Specific Courses	Year	ECTS
MolMed Course Introduction to Biomedical Research Techniques	2011	1.6
NVVO Introduction course into Fundamental and Clinical Oncology	2011	1.5
Veridex CellSearch Training	2011	1.5

Oral presentations	Year	ECTS
Medical Oncology Research Meeting, EMC, Rotterdam	Annually	1
Josephine Nefkens Institute Oncology Meeting, Rotterdam	Annually	1
A Sister's Hope Brilliant Minds Together Meeting, Amsterdam	2012	0.2
Dutch Uro-Oncology Studygroup (DUOS) symposium, Utrecht	2012	0.2
Molecular Tools Group meeting, Uppsala University, Sweden	2012	0.2
European Organization for Research and Treatment of Cancer (EORTC) Gynaecologic Cancer Group General Assembly and Business Meeting, Brussels	2013	0.2
Scientific meeting of Department of Medical Oncology, EMC, Rotterdam	2013	0.2
DUOS symposium, Utrecht	2012	0.2
Sint Franciscus Gasthuis Research Meeting, Rotterdam	2015	0.2
Janssen Academy, Rotterdam	2016	0.2

Poster presentations	Year	ECTS
The European Cancer Congress, Amsterdam	2013	1
American Society of Clinical Oncology, Chicago, Illinois	2014	1
The European Cancer Congress, Vienna, Austria	2015	1

(Inter)National conferences and Symposia	Year	ECTS
Scientific Meeting of the Department of Medical Oncology, Rotterdam	Annually	0.5
Novel Treatments in Gynaecological Cancer, Amsterdam	2011	0.2
EORTC 50th Anniversary Meeting, Leuven, Belgium	2012	0.4
Advances in Circulating Tumor Cells, Athens, Greece	2012	1
Borstkanker Behandeling Beter, Rotterdam	2013	0.2
American Society of Clinical Oncology, Chicago, Illinois	2013	1
Scientific Meeting of the Erasmus Medical Center, Rotterdam	2014	0.2
Center for Personalized Cancer Treatment (CPCT) Symposium	2015	0.2

Teaching	Year	ECTS
Lecture Junior Med School class	Annually	0.2
Lecture third-year medical students, Minor Oncology	Annually	0.2
Supervision of four-week Junior Med School Medical Oncology lab program	2011	1.5
Development and coordination of four-week Junior Med School Medical Oncology lab program	2013	2
Co-supervision medical master student	2012	2
Supervision University College student	2014	0.5
Tutor first-year medical students	2014	1
Lecture second-year medical students in Honours Class	2015	0.2
Extended tutorate first-year students (Kennismaking Beroeps Praktijk)	2016	0.5

Wendy Onstenk werd geboren op 13 april 1984 te Schiedam. Zij volgde middelbaar onderwijs aan het Stedelijk Gymnasium te Schiedam. Vanaf de vierde klas in 2000 participeerde zij één à twee dagen per week in onderzoek naar embryogenese en congenitale afwijkingen aan de afdeling Plastische en Reconstructieve Chirurgie van het Erasmus MC onder supervisie van dr. A.J.M. Luijsterburg en dr. C. Vermeij-Keers. Dit resulteerde in een profielwerkstuk over craniosynostoses, waarvoor zij een prijs ontving. In 2002 behaalde zij *cum laude* haar VWO-diploma aan het Stedelijk Gymnasium te Schiedam, waarna zij direct begon met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Vanaf 2004 werkte zij als medisch student bij de afdeling Interne Oncologie van het Erasmus MC, in eerste instantie als medisch typiste en vanaf 2007 als datamanager bij het trialbureau Interne Oncologie. Hier werkte zij als lokaal en regionaal datamanager aan verschillende fase I, II en III studies binnen de oncologie. In dit kader behaalde zij ook haar *Good Clinical Practice* diploma in 2008. In 2009 deed zij haar wetenschapsstage van 21 weken aan het Laboratorium *Medische Tumor Immunologie* in de Daniël den Hoed Kliniek onder supervisie van dr. Michiel Strijbos en dr. Jan-Willem Gratama. Na afronding van de scriptie *Biomarkers in Clinical Oncology* behaalde zij haar doctoraal diploma. Vanaf 2009 tot 2011 volgden twee jaar co-schappen, welke afgesloten werden met een oudste co-schap bij de afdeling Interne Geneeskunde in het Ikazia Ziekenhuis onder supervisie van dr. A. Dees. Gedurende de co-schappen werkte zij door aan een eerder gestart onderzoek bij patiënten met eierstokkanker in samenwerking met dr. M.E.L. van der Burg en prof.dr. P.M.J.J. Berns, wat resulteerde in twee publicaties. Na de co-schappen legde zij in 2011 het artsexamen *cum laude* af en startte zij met het promotie-onderzoek aan de afdeling Interne Oncologie van het Erasmus MC Kankerinstituut onder supervisie van prof.dr. S. Sleijfer, prof.dr. J.A. Foekens en dr.ir. J.W.M. Martens, zoals beschreven in dit proefschrift. Tijdens het promotie-onderzoek heeft zij in 2012 een laboratorium stage van twee maanden gevolgd bij de *Molecular Tools Research Group* aan de Universiteit van Uppsala in Zweden onder supervisie van dr. O. Söderberg. Deze stage werd mogelijk gemaakt door het verwerven van een persoonsgebonden reisbeurs via het *Koningin Wilhelmina Fonds*. In 2014 won zij een *Conquer Cancer Foundation of the Americal Society of Clinical Oncology Merit Award* voor het ingediende onderzoek voor het ASCO congres van dat jaar. Gedurende het promotie-onderzoek heeft zij mogen presenteren op (inter)nationale congressen en schreef zij mee aan enkele gehonoreerde subsidie aanvragen, onder andere door *Pink Ribbon* en *A Sister's Hope*. Tevens was zij actief betrokken bij het onderwijs aan *Junior Med School*, eerste-, tweede- en derdejaars geneeskunde en master studenten. Per 1 april 2015 startte zij als postdoctoral fellow aan de afdeling Interne Oncologie van het Erasmus MC Kankerinstituut en werkte zij aan de *Center for Personalized Cancer Treatment* (CPCT)-02 studie. Vanaf 1 april 2016 werkt zij tevens in dienst van *Hartwig Medical Foundation* om grootschalige moleculaire analyse van tumoren te faciliteren.

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