Characterization of Tumor Cells:

The gear for personalized medicine

I.E. de Kruijff

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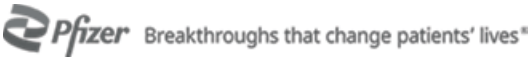

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The gear for personalized medicine

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However difficult life may seem, there is always something you can do, and succeed at.
It matters that you don't just give up.

Stephen Hawking

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Chapter 1
Introduction

GENERAL INTRODUCTION TO CANCER

For years, all types of cancer are subject of clinical investigation. However, in the Netherlands and worldwide in countries of all income levels, cancer is still the leading cause of death (1, 2). Although screening programs and improvements in care of patients presenting with primary cancer have substantially reduced mortality, many patients with cancer still die from developing metastatic disease. Taking breast cancer (BC) as example, 5-18% of the patients with *de novo* BC presents with stage IV cancer (3, 4) and patients without metastatic disease at diagnosis have around 30% chance of developing metastasis during lifetime (5). Fortunately, due to combined research efforts, new therapies have become available for patients with metastatic disease as well. But although these therapies prolong survival and promote quality of life in metastatic patients, curation of metastatic disease is still not possible in most cases, while patients are exposed to the side effects of treatment impairing their quality of life. Therefore, it is important to select the right drug for the right patient at the right time in their disease course, since generating the highest survival benefit by avoiding side effects is trivial during palliative care of metastatic patients.

A large variety of therapies is available for most types of cancer nowadays and selecting the right therapy for patients depends on multiple factors. Probably the most important factor to consider is the treatment wish of the patients. Patients can decide not to be treated at all, or if multiple treatment options are available, they have an option to choose for the therapy with the least side effects for example (6). This treatment decision making is in most cases also dependent on the age and performance status of the patients. But these are not the only factors to consider: the medical history, comorbidity, co-medication and organ function of the patients should also be taken into consideration when selecting a therapy for a patient. If there are no restrictions in general health, treatment wish or other factors, tumor characteristics can be determined to select the right therapy. Since cancer is a very heterogeneous disease, the molecular characteristics of each tumor are different (7, 8). Also within one patient, the characteristics of tumor cells can differ (9, 10). Therefore, it is important to know the up-to-date characteristics of a patient's tumor before the start of a treatment.

WHAT IS KNOWN ABOUT CHARACTERIZATION OF TUMOR CELLS

Identifying tumor characteristics is usually performed at diagnosis on the primary tumor. Based on tumor characteristics treatment of patients can be optimized. Examples are the successful application of drugs targeting the estrogen-receptor (ER) or human epidermal growth factor receptor 2 (HER2) in BC, which have substantially increased

overall survival (OS) (11, 12). Apart from the presence of receptors on the tumor cells, mutations in driver genes can also provide direction to which therapy is most suitable to target a tumor cell. An example are PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha) mutations, that occur in 40% of the ER-positive and HER2-negative BC. By using a combination of alpelisib, a PI3Kα-specific inhibitor, with fulvestrant in PIK3CA-mutated, ER+, HER2- advanced BC patients who received endocrine therapy previously, progression-free survival can be prolonged compared to fulvestrant only (13, 14).

A disadvantage of determining tumor characteristics on the primary tumor, is the fact that these characteristics can change over time and under treatment pressure. Focusing at BC again, the median discordance rate between the primary tumor and a paired metastasis within one patient is about 10% (interquartile range (IQR) 4-17%) for HER2 and 14% (IQR 9-25%) for ER (15). Most patients lose receptor expression over time, however a gain in receptor expression is also possible. Therefore, when starting therapy in metastatic patients, it should be obligatory not only to look at the characteristics of the primary tumor, which most often has been removed years before the development of metastatic disease, but also at the characteristics of the metastatic disease. However, taking a biopsy of solid metastatic lesions can be a cumbersome procedure. Metastasis can develop in many different places, which are not always accessible for taking a biopsy. In addition, there is always a risk of bleeding and infection when taking a biopsy of a metastatic lesion. Therefore, new methods that determine the characteristics of tumor cells at the moment a new therapy is initiated are necessary, and circulating tumor cells (CTCs) might serve this need.

CIRCULATING TUMOR CELLS

CTCs are tumor cells detached from the primary or metastatic tumor site that circulate in the peripheral blood. In all types of epithelial tumors, these cells can be isolated from the blood after taking blood with a venipuncture (16). Since in this way tumor cells can be isolated from the blood in a minimally invasive manner, numerous studies have been conducted on CTCs. At this moment, most commonly the CellSearch system (Menarini-Silicon Biosystems, Huntington Valley, PA, USA), which uses the specific immunogenic properties of CTCs, is used to enrich for CTCs. Through the application of magnetic beads with anti-epithelial cell adhesion molecule (EpCAM) antibodies attached to them, CTC are isolated from the blood using a magnet. Using this approach, the CTCs in the enriched fractions are accompanied by a background of leukocytes. Therefore, isolated cells are stained for cytokeratin (CK), 4′,6-diamidino-2-phenylindole (DAPI), to stain double-stranded DNA, and cluster of differentiation molecule 45 (CD45), which is a

marker for leukocytes, to distinguish the CTCs from the leukocyte background. The definition of a CTC isolated with the CellSearch technique is a EpCAM-positive, CK-positive, DAPI-positive, but CD45-negative cell. Following the landmark paper that showed that CellSearch-enriched CTCs were associated with poor prognosis in metastatic BC (mBC) patients (17), the CellSearch system received clearance from the United States Food and Drug Administration (FDA) for clinical use. The enumeration of CTCs showed that mBC patients with ≥5 CTCs had a worse progression-free survival (PFS) and OS than patients with <5 CTCs at the start of a new therapy. Also, when patients switched from <5 CTCs to ≥5 CTCs during therapy their PFS and OS decreased and patients with ≥5 CTCs at the start of therapy that dropped to <5 CTCs during therapy had better PFS and OS (18). In the meantime, the prognostic value of CTC enumeration has not only been shown for mBC patients, but also for patients with metastatic castration-resistant prostate cancer (mCRPC) (19), metastatic colorectal cancer (mCRC) (20), esophageal cancer (21), advanced gastric cancer (22), primary breast cancer (23), bladder cancer (24, 25), melanoma (26), lung cancer (27, 28), advanced pancreatic cancer (29), hepatocellular carcinoma (30) and ovarian cancer (31).

Besides enumeration of CTCs, it is also possible and critically important to examine the characteristics of CTCs. Since CellSearch-isolated CTCs are always present in low numbers and in a background of leukocytes, studying CTC characteristics demands assays that are highly sensitive and very specific. Nonetheless, it is possible to determine, for example, the ER- and HER2-status of CTCs in mBC patients (32), but also to examine the presence of point mutations (33), copy number alterations (CNAs) (34) and protein expression (35) on CellSearch-enriched CTCs. Furthermore, the possibility to measure individual transcripts makes it possible to determine expression profiles of these cells (36, 37), but also allows for different splice variants of a particular gene to be determined (38-40). Literature has shown that the characteristics of CTCs resemble those of the metastasis better than those of the primary tumor (41). Therefore, looking at the characteristics of CTCs is a promising tool to characterize a tumor at the moment of the initiation of a new line of therapy.

LIQUID BIOPSIES

Since the enumeration and characterization of CTCs are a sort of biopsies that are taken from the blood, instead of from the primary tumor or metastatic tissue, they are part of the liquid biopsies. Another type of liquid biopsy that is frequently subject of investigation, is circulating cell-free DNA (cfDNA). When cells undergo programmed cell death or apoptosis, they release DNA fragments into the bloodstream (42). This extra-cellular DNA can be detected in the blood serum or plasma after venipuncture. In healthy individuals,

cfDNA is mostly derived from hematopoietic cells (43). In patients with cancer, DNA is also released from tumor cells. Therefore, in these patients, the fraction of cfDNA that consists of tumor DNA, which is called circulating tumor DNA (ctDNA), can be measured. Besides in blood, ctDNA can also be detected in other body fluids, like cerebrospinal fluid or pleural effusions (44). However, since the ctDNA fraction is only a small portion of the total cfDNA fraction, which mostly consists of germline DNA, the detection of ctDNA can be challenging (45). Therefore, techniques as digital PCR (dPCR) and next-generation sequencing (NGS) are necessary, since only these techniques are sensitive enough to detect ctDNA in blood. With these techniques, two sequencing strategies exist for the detection of ctDNA: targeted and non-targeted. In case of known DNA aberrations, targeted sequencing is used, while non-targeted whole-genome sequencing (WGS) is used in the search for novel aberrations (44).

SCOPE OF THIS THESIS

In this thesis, the focus is on tumor characterization. This will include characterization of primary tumor tissue cells, characterization of circulating tumor cells and characterization of cell-free DNA and circulating tumor DNA. This characterization will not only be performed on breast cancer, but also on different tumor types like bladder cancer and prostate cancer. In **chapter 2**, a study is described in which CD146, a marker involved in epithelial-to-mesenchymal transition (EMT), is measured on primary BC tissues. As described above, the detection of CTCs with the CellSearch system depends on EpCAM. However, in mBC only in 61% of the patients CTCs are detected (17). It is currently not known if in the other patients CTCs are absent, or if they cannot be detected by systems that use EpCAM for isolation of CTCs. Research shows that especially normal-like breast cancer cell lines are not isolated by the CellSearch system, since they lack EpCAM expression (46). Therefore, in an attempt to improve CTC detection, there is a continuous search for other markers than EpCAM that can be used for the isolation of CTCs from blood. Since breast cancer cell lines that lack EpCAM expression seem to frequently express CD146 (47), this study was preformed to investigate the role of CD146 in the biology of BC. Considering the connection of CD146 to EMT, it was assumed this marker was linked to cancer aggressiveness. Therefore, it could also be important to determine CD146 on primary breast cancers, since this could possibly affect therapy decision making. **Chapter 3** describes a systematic review about the use of liquid biopsies in muscle-invasive bladder cancer (MIBC) patients. In MIBC patients it is recommended to offer platinum-based neo-adjuvant chemotherapy (NAC) prior to local definitive treatment. However, the overall survival benefit of NAC is modest and this therapy can give significant toxicity. Most optimal, NAC is only administered to patients who benefit from this therapy, to pre-

vent unnecessary side effects in patients who do not benefit from this treatment. Since CTCs are proven prognostic markers, which probably determine the aggressiveness of cancer, and liquid biopsies can characterize tumor cells and tumor DNA from blood and / or urine in a minimally invasive manner, liquid biopsies could be an attractive option to guide the administration of perioperative chemotherapy in MIBC patients. In **chapter 4** CTCs are determined in patients with metastatic castration-resistant prostate cancer (mCRPC). The number of treatment options for mCRPC have increased rapidly in the last decade. Therefore, biomarkers that can guide the selection for a particular treatment are necessary. Most mCRPC patients received one of the androgen-signaling-targeted inhibitors (abiraterone or enzalutamide) and docetaxel in early stages of their disease. After these therapies, the options are to treat these patients with cabazitaxel or the alternative androgen-signaling-targeted inhibitor. Although cabazitaxel is the better option (48), the main goal remains to administer cabazitaxel only to patients who will indeed benefit from this therapy and to prevent toxicity in the patients who do not have this benefit. Especially, since there are other treatment options, it is important to have tools that will select the right patients for the right therapy in this setting. By enumerating and characterizing CTCs in mCRPC patients who receive cabazitaxel therapy, the prognostic value of the CTC count for cabazitaxel is evaluated and it is investigated if particular characteristics can be found that are associated with outcome to cabazitaxel. **Chapter 5** shows the determination of the androgen receptor (AR) in CTCs of BC patients. AR is a known drug target for prostate cancer, but research has shown that its expression is also associated with endocrine resistance in metastatic BC (mBC) patients and that it might be a target for therapy for mBC patients expressing AR in their tumor. Determination of the AR in CTCs provides a minimally invasive manner to determine an up-to-date AR-status in these patients. This gives insight into the incidence of AR in mBC patients in total and across the different breast cancer subtypes. Also, by determination of the AR-status in CTC samples and matched primary tumors the discrepancy rate in AR-status was analyzed. Lastly, it was investigated if the AR-status of CTCs was associated with outcome to endocrine therapy. These data could give insight if the CTC-AR-status can be used as screenings tool for studies that investigate AR-targeting agents in BC. Finally, in **chapter 6** a study is described in which CTCs are enumerated and characterized in heavily pretreated mBC patients who are treated with cisplatin (cDDP). In this study, it is investigated if predefined molecular characteristics of CTCs are able to identify patients responding to cDDP-based chemotherapy. Furthermore, clinical data of these patients and side effects of the therapy are described.

Chapter 2

The Prevalence of CD146 Expression in Breast Cancer Subtypes and Its Relation to Outcome

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ABSTRACT

CD146, involved in epithelial-to-mesenchymal transition (EMT), might affect cancer aggressiveness. We here investigated the prevalence of CD146 expression in breast cancer subtypes, its relation to prognosis, the relation between CD146 and EMT and the outcome to tamoxifen. Primary breast cancer tissues from 1342 patients were available for this retrospective study and immunohistochemically stained for CD146. For survival analyses, pure prognosis was studied by only including lymph-node negative patients who did not receive (neo)adjuvant systemic treatment (n = 551). 11% of the tumors showed CD146 expression. CD146 expression was most prevalent in triple-negative cases (64%, $p < 0.001$). In univariable analysis, CD146 expression was a prognostic factor for both metastasis-free survival (MFS) ($p = 0.020$) and overall survival (OS) ($p = 0.037$), but not in multivariable analysis (including age, tumor size, grade, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) and Ki-67). No correlation between CD146 and EMT nor difference in outcome to first-line tamoxifen was seen. In this large series, our data showed that CD146 is present in primary breast cancer and is a pure prognostic factor for MFS and OS in breast cancer patients. We did not see an association between CD146 expression and EMT nor on outcome to tamoxifen.

1. INTRODUCTION

CD146, also known as melanoma cell adhesion molecule (MCAM, M-CAM and MUC18), was first described in malignant melanomas as a cell adhesion molecule (1). Since then, several different (patho)physiological roles have been described for CD146 in other types of cancer, including breast cancer. The most prominent role of CD146 in breast cancer is its involvement in the induction of epithelial-to-mesenchymal transition (EMT) (2-5). EMT is a developmental process (6), which is frequently involved in cancer dissemination. During EMT, the morphology of the tumor cells dramatically changes, so cells gain invasive capabilities and migratory functions (7), and epithelial cell adhesion molecule (EpCAM) expression is decreased (8). In certain instances, cancer cells also acquire stem cell-like properties after EMT (7, 9). EMT is amongst others a means for solid tumors to shed tumor cells, allowing them to intravasate into the bloodstream, where they are called circulating tumor cells (CTCs), and subsequently to extravasate to form distant metastases (10, 11). In line with its role in EMT, CD146 expression is predominantly observed in breast cancer cell lines with mesenchymal features (12-14). CD146 has been associated with a poor prognosis in many types of cancer, including melanoma, prostate cancer, hepatocellular carcinoma and ovarium cancer (15-18). Also in breast cancer, CD146 has been related to a poor prognosis (19, 20), but none of the data was obtained in patients who did not receive adjuvant therapy. Furthermore, CD146 expression has been associated with high grade tumors, estrogen receptor (ER)- and progesterone receptor (PR)-negative tumors and the triple-negative subtype (ER-/PR-/human epidermal growth factor receptor 2 (HER2)-) (3, 5, 19) while down modulation of CD146 leads to a less aggressive phenotype tumor (19).

Apart from a role in tumor aggressiveness, it has been demonstrated in breast cancer cell lines that CD146 expression can confer resistance against hormonal treatment. CD146 is overexpressed in cell lines which are resistant to 4-OH-tamoxifen compared to tamoxifen-sensitive cell lines and silencing of CD146 in these resistant cell lines reversed tamoxifen resistance. In addition, in a subset of the breast cancer cell lines, CD146 expression suppressed ER expression and overexpression of CD146 in breast cancer cells induced Akt (also known as protein kinase B) activity, which is recognized as one of the mechanisms contributing to endocrine resistance (2, 12, 21, 22). Lastly, patients treated with adjuvant tamoxifen had significant shorter overall survival (OS), recurrence-free survival (RFS) and distant metastasis-free survival (DMFS) when CD146 expression was increased in their primary tumors (12).

Based on the above, CD146 shows promise as a useful marker for predicting disease progression and treatment response in breast cancer patients. However, regarding disease progression, previous studies included adjuvant treated patients, which clouds the issue whether or not CD146 is a prognostic or predictive marker. Thus, to gain further

insight into the exact importance of CD146 in breast cancer we assessed the relation of CD146 expression, determined with immunohistochemistry (IHC), in primary breast cancer tissues with different molecular and histological subtypes. To the best of our knowledge, a pure prognostic evaluation of CD146 has not been executed. To enable this, we assessed CD146 expression in primary breast cancer tissues of patients with lymph-node negative disease who did not receive any (neo-)adjuvant systemic treatment. We also studied treatment response in a group of hormonal treatment-naive patients with recurrent breast cancer who were treated for metastatic disease with first-line tamoxifen monotherapy. In addition, EpCAM expression was determined in these tumors to assess whether gain in CD146 expression shows a loss of EpCAM expression. Lastly, we assessed the relation between CD146 expression and the expression of EMT markers at gene expression level in cell lines and primary breast cancer tissues.

2. RESULTS

2.1. CD146 Expression and the Relationship with Patient and Tumor Characteristics

CD146 showed membranous staining >1% of the epithelial tumor cells in 113 out of the 1025 tumors (11%). In many of these tumors (n = 49) >50% of the tumor cells showed CD146 expression. In 17 tumors, 26–50% of the tumor cells were CD146-positive, in 23 tumors this was 11–25% and in 24 tumors 1–10% of the tumor cells. An example of positive and negative CD146 staining is depicted in ***Supplementary Figure 1***.

In ***Table 1***, the association between the baseline characteristics of the patients and CD146 expression is shown. CD146-positive staining was more likely in tumors from patients who were younger and pre-menopausal. Also, CD146-positive tumors were associated with higher tumor grade, ER-negative, PR-negative and Ki-67-high tumors (all $p < 0.001$). Lastly, a higher T-stage at diagnosis was associated with more CD146-positive tumors ($p = 0.016$).

In the histological breast cancer subtypes (***Table 2***), the medullary subtype had the highest CD146 expression with 47.8% CD146-positive tumors. This subtype had significantly higher CD146 expression than tumors with an invasive lobular carcinoma ($p < 0.001$), mucinous subtype ($p = 0.001$), tubular subtype ($p = 0.027$) and invasive ductal carcinoma ($p < 0.001$). Finally, the invasive ductal carcinomas had higher CD146 expression than the invasive lobular carcinomas ($p < 0.001$).

With respect to the molecular subtypes (***Table 2***), the highest CD146 expression was present in the triple-negative subtype, with 63.9% CD146-positive tumors. This subtype had significantly higher CD146 expression than the other molecular subtypes (all $p < 0.001$). The HER2+ subtype has significantly higher CD146 expression than the Luminal

A ($p < 0.001$), Luminal B HER2- ($p = 0.022$) and Luminal B HER2+ ($p = 0.026$) subtypes. Lastly, the Luminal B HER2- subtype had higher CD146 expression than the Luminal A subtype ($p = 0.008$).

Table 1. Baseline characteristics and the relation to CD146 expression ($n = 1025$)

Characteristics	*N*	CD146-Negative	CD146-Positive	*p*-value
Age				$p < 0.001$ *
≤40	127	96	31	
41–55	431	383	48	
≥55	467	433	34	
T-Stage				$p = 0.016$
T1	602	547	55	
T2–T4	408	351	57	
N-Stage				$p = 0.577$ *
N0	576	511	65	
N1	347	308	39	
N2	102	93	9	
Menopausal status				$p < 0.001$
Pre-menopausal	477	405	72	
Post-menopausal	548	507	41	
Tumor grade				$p < 0.001$ *
Grade I	211	205	6	
Grade II	473	451	22	
Grade III	341	256	85	
Ki-67 status				$p < 0.001$
Low (<10%)	621	595	26	
High (≥10%)	404	317	87	
ER status				$p < 0.001$
Positive	861	831	30	
Negative	164	81	83	
PR status				$p < 0.001$
Positive	657	639	18	
Negative	368	273	95	
HER2 status				$p = 0.200$
Positive	119	110	9	
Negative	906	802	104	

Chi-square test performed for tumor grade, N-stage and Age (*): test for trend performed. In the T-stage analysis 15 tissues were removed due to unknown T-stage status. Of these 14 were CD146-negative and one was CD146-positive.

Table 2. CD146 expression in histological and molecular breast cancer subtypes

	CD146 Expression		
Tumor types	**Negative *N* (%)**	**Positive *N* (%)**	**p-value**
All tumors (*n* = 1025)	912 (89.0)	113 (11.0)	
Histological subtype			$p < 0.001$
Invasive ductal carcinoma	751 (88.5)	98 (11.5)	
Invasive lobular carcinoma	117 (99.2)	1 (0.8)	
Medullary	12 (52.2)	11 (47.8)	
Mucinous	18 (100)	0 (0.0)	
Tubular	11 (91.7)	1 (8.3)	
Papillary	3 (60.0)	2 (40.0)	
Molecular subtype			$p < 0.001$
Luminal A	437 (98.0)	9 (2.0)	
Luminal B HER2-negative	322 (94.4)	19 (5.6)	
Luminal B HER2-positive	72 (97.3)	2 (2.7)	
HER2-positive	38 (84.4)	7 (15.6)	
Triple negative	43 (36.1)	76 (63.9)	

Number and percentage of CD146-positive and -negative tumors. Also divided by histological and molecular subtypes. The reported p-values are the comparison within the molecular subtypes and within the histological subtypes.

2.2. CD146 and EpCAM Expression in the Diverse Breast Cancer Subtypes

Since CD146 has been associated with EMT, the expectation is that EpCAM expression is low in tumors with high CD146 expression. Of all tumors, 58% were EpCAM-high. CD146 staining was more frequently observed in EpCAM-high tumors (14.6%) compared with EpCAM-low tumors (6.0%, $p < 0.001$). In the molecular subtypes, the triple-negative group had the highest percentage of CD146-positive/EpCAM-positive staining (47.9%) and also the highest CD146-positive/EpCAM-negative staining (16.0%). For the histological subtypes, the medullary subtype had the highest percentage of CD146-positive/EpCAM-positive tumors (30.4%). For the CD146-positive/EpCAM-negative tumors, this was the papillary subtype (20.4%) (***Supplementary Table 1***).

2.3. Relationship of CD146 Expression with Expression of EMT-Related Genes

Since CD146 has the highest expression in the breast cancer cell lines with mesenchymal features, in line with its potential role in EMT, we expect that mesenchymal genes also have a higher expression in CD146-positive tumors. To study this, first the association between IHC staining of CD146 and its expression at the mRNA level (Affymetrix probe 211340_s_at) was established in a subset of 105 primary tumors. Of these patients, 25 had positive staining and showed a significant (1.7 fold) higher CD146 mRNA expression compared to patients who had CD146-negative tumors (Mann-Whitney $p < 0.0001$).

Considering the relation between CD146 and mesenchymal features in breast cancer cell lines, the correlation of all genes on the array with CD146 mRNA expression was performed in 52 breast cancer cell lines. The genes with the highest correlations (R > 0.6) were reviewed in DAVID (database for annotation, visualization and integrated discovery), an online tool to discover enriched functional-related gene groups in gene lists. Indeed, EMT was a significant enriched function (multiple testing corrected p = 6.8 × 10−4). A similar analysis in a cohort of 867 primary tumors resulted in correlated genes that showed for example angiogenesis and cell adhesion as overrepresented functions, but not EMT. When comparing the genes that correlated with CD146 in both the cell lines and in the tumors, there was an overlap of 24 genes (***Supplementary Figure 2***). These overlapping genes are amongst others, known for their function in caveolae formation and focal adhesion. Repeating the analyses for overrepresented functions in TCGA (23) and METABRIC (24) datasets also did not yield EMT as overrepresented function.

2.4. Relationship of CD146 Expression with Prognosis

For survival analysis, only patients without locoregional or distant metastases at diagnosis, N0 disease at diagnosis and who did not receive (neo-)adjuvant systemic treatment were included. Furthermore, 25 patients were lost to follow up. In total, survival analysis was performed on 551 patients. The median follow up of these patients was 98 months (range 2–334 months). For metastasis-free survival (MFS), all common prognostic factors were included (***Table 3***). Age > 55 year was associated with longer MFS, whereas patients with a higher tumor grade, higher T-stage and a HER2-positive or Ki-67-high tumor had shorter MFS. CD146 was associated with shorter MFS in univariable analysis (Fine & Gray model), with a hazard ratio (HR) of 1.77 (95% CI 1.09–2.87, p = 0.020). There was no relation between the amount (%) of CD146-positive cells and MFS. All factors in the univariable analyses are proven prognostic factors and were included in the multivariable Fine & Gray model. This model is set up to search for the best fitting model (i.e., the strongest predictive model). It showed that higher T-stage and HER2-positivity were independent predictors of shorter MFS. Age, PR-status, Ki-67-status and also CD146 (HR 1.51, 95% CI 0.79–2.87, p = 0.210) contribute to the quality of the model, but are not independent predictors of MFS, i.e., they make the model stronger, but are not significant predictors of MFS in multivariable analyses. The cumulative incidence function (CIF, of the Fine & Gray model) of MFS in relation to CD146 is shown in ***Figure 1A***.

In univariable Cox regression analysis for overall survival (OS) the same prognostic factors were included (***Table 4***). T-stage, tumor grade, ER, PR, HER2 and Ki-67 were associated with OS. CD146 expression is associated with shorter OS in univariable analysis (HR 1.67, 95% CI 1.03–2.69, p = 0.037). No relation was found between the amount (%) of CD146-positive cells and OS. In the multivariable model, T-stage and HER2 remained independent prognostic factors for OS. Ki-67 was also an independent factor, but vio-

lated the proportional hazards assumption (tested with Schoenfeld residuals), and was added to the model as stratum variable. CD146 was not a significant addition to the multivariable model (HR 1.42, 95% CI 0.84–2.38, p = 0.191). A Kaplan-Meier curve for OS in relation to CD146 is shown in ***Figure 1B***.

The comparison of ER-positive and ER-negative patients, showed that the ER-positive patients have a significantly shorter MFS and OS when they are CD146-positive (respectively HR 2.67, 95% CI 1.32–5.41, p = 0.006 and HR 2.69, 95% CI 1.30–5.55, p = 0.007), but in the ER-negative group there is no difference in MFS and OS between the CD146-positive and negative patients (respectively HR 1.11, 95% CI 0.51–2.45, p = 0.79 and HR 0.80, 95% CI 0.38–1.72, p = 0.57) (***Supplementary Figure 3***). Due to small numbers, regression analysis was not performed in the other subgroups. For the number of unfavorable events in the different subgroups see ***Supplementary Table 2***.

Table 3. Fine & Gray regression analysis for metastasis-free survival (MFS)

	Univariable Analysis			Multivariable Analysis		
Characteristics	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age						
40–55 vs. <40	0.69	0.41–1.17	0.170			
>55 vs. <40	0.48	0.28–0.81	0.006	0.63	0.35–1.15	0.130
T-stage						
T2–T4 vs. T1	1.94	1.36–2.76	<0.001	1.77	1.22–2.59	0.003
Tumor grade						
II vs. I	2.44	1.38–4.30	0.002			
III vs. I	2.94	1.64–5.27	<0.001			
ER						
Pos vs. neg	0.74	0.47–1.18	0.210			
PR						
Pos vs. neg	0.85	0.59–1.23	0.390	1.29	0.83–1.99	0.260
HER2						
Pos vs. neg	2.92	1.92–4.46	<0.001	2.64	1.65–4.22	<0.001
Ki-67						
Pos vs. neg	1.82	1.28–2.59	<0.001	1.37	0.92–2.05	0.120
				Additions to the base model		
CD146						
Pos vs. neg	1.77	1.09–2.87	0.020	1.51	0.79–2.87	0.210

Univariable and multivariable regression analysis for MFS during 120 months of follow up. Only patients who were N0, M0 at baseline and did not receive neo-adjuvant or adjuvant therapy (N = 551) were included.

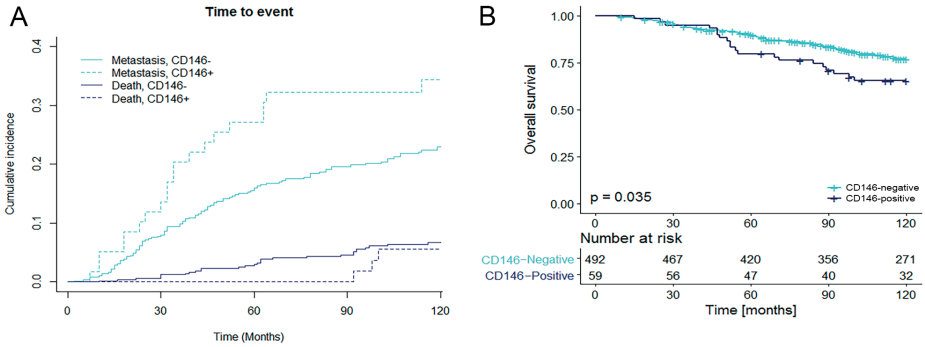

Figure 1. MFS and OS as function of CD146 expression.
(**A**) MFS (depicted with cumulative incidence function) and (**B**) OS (depicted with Kaplan-Meier method) as function of CD146 expression. Only patients who were N0, M0 at baseline and did not receive neo-adjuvant or adjuvant therapy (N = 551) were included. For MFS (**A**) the patients who developed a metastasis are depicted in the light blue lines, the dark blue lines (death) are the patients who died without any evidence of disease.

Table 4. Cox regression analysis for overall survival (OS)

	Univariable Analysis			Multivariable Analysis		
Characteristics	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Age						
40–55 vs. <40	0.68	0.39–1.18	0.170			
>55 vs. <40	0.76	0.44–1.30	0.311			
T-stage						
T2–T4 vs. T1	1.98	1.39–2.83	<0.001	1.86	1.30–2.66	<0.001
Tumor grade						
II vs. I	2.06	1.19–3.59	0.010			
III vs. I	2.51	1.43–4.41	0.001			
ER						
Pos vs. neg	0.63	0.41–0.97	0.035			
PR						
Pos vs. neg	0.63	0.44–0.90	0.011			
HER2						
Pos vs. neg	2.55	1.68–3.87	<0.001	2.15	1.39–3.31	<0.001
Ki-67						
Pos vs. neg	1.71	1.20–2.43	0.003			
				Additions to the base model		
CD146						
Pos vs. neg	1.67	1.03–2.69	0.037	1.42	0.84–2.38	0.191

Univariable and multivariable regression analysis for OS during 120 months of follow up. Only patients who were N0, M0 at baseline and did not receive neo-adjuvant or adjuvant therapy (N = 551) were included. The multivariable regression analysis has been stratified for Ki-67, both the base model as the base model with addition of CD146.

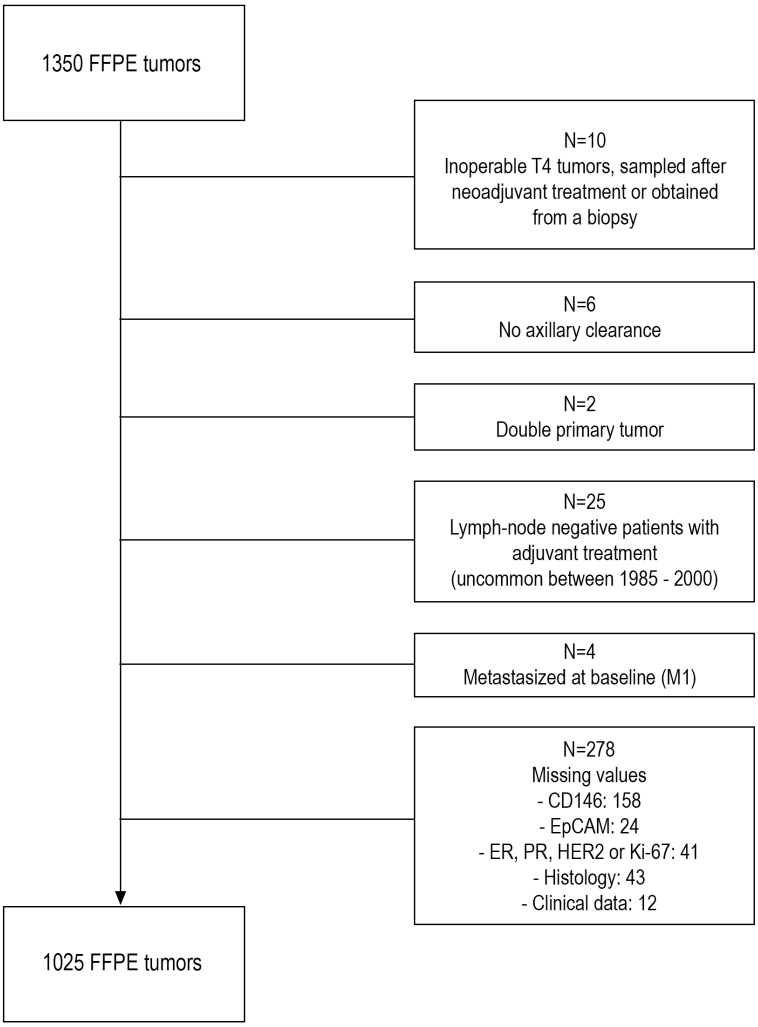

Figure 2. Flowchart of formalin-fixed paraffin-embedded (FFPE) tissues included in the study (n= 1350).

2.5. CD146 Status and Outcome to Tamoxifen Treatment

The CD146 status of the primary tumor was also determined in a cohort of patients (n = 317) who received tamoxifen as first-line treatment in the palliative setting. All patients were ER-positive and did not receive any (neo)adjuvant endocrine treatment after diagnosis. Of these patients, only 8 patients had a CD146-positive tumor (2.5%). Although 6 out of 8 (75%) CD146-positive patients showed clinical benefit, as expected with this low number, there was no statistical difference in response on tamoxifen in the CD146-positive and -negative patients (p = 0.368). From the CD146-negative patients, 200 patients (65%) had clinical benefit (***Supplementary Table 3***).

3. DISCUSSION

In this study, we aimed to gain more insight into the clinical significance of CD146 expression in breast cancer and to determine if CD146 is a pure prognostic marker. We showed that 11% of the primary breast cancer tissues express CD146. This is in line with a previously published paper, in which 7% of all tumors (n = 635) had CD146 expression (19), though in another study a percentage of 35% CD146-positive tumors was described (n = 505), but this study contained 29% triple negative tumors, while in our study only 11% was triple-negative (3).

In literature, CD146 expression was predominantly seen in breast cancer cell lines with mesenchymal features (12-14). In our study, we could confirm this and additionally found a relation between CD146 and EMT in the breast cancer cell lines. It is also known that CD146 expression varies across the different breast cancer subtypes, especially the triple-negative breast cancer subtype has been associated with CD146 expression (3, 5). Our data confirms that CD146 expression is more prevalent in triple-negative breast cancers than in other breast cancer subtypes (63.9% vs. 4.1%). The triple-negative subtype can be divided into six different subtypes, of which one is the mesenchymal subtype (25). However, in the primary breast cancer tumors of this dataset and in the publicly available TCGA and METABRIC data, there was no correlation between genes that are associated with EMT and CD146 expression. Another observed phenomenon of tumors that undergo EMT is loss of EpCAM (8). Therefore, we expected tumors with CD146 expression to have loss of EpCAM. Our data did not show an inverse relationship between CD146 and EpCAM expression in any of the investigated subtypes. CD146 expression was even more frequently observed in EpCAM-high compared to EpCAM-low tumors. Thus, while in cell lines it is clear there is an association between CD146 and EMT, it is not clear-cut that CD146 expression is indicative for EMT in primary breast cancers. In previously published papers, CD146 expression has also been linked to EMT in cell lines and mouse models (2, 3), but our data cannot confirm this relation between CD146 expression and EMT in primary breast cancers. Therefore, while others have shown in preclinical experiments that CD146 expression is necessary for the induction of EMT, our data in primary breast tumors suggests that CD146 expression alone is insufficient to drive a breast cancer tumor cell into EMT.

With respect to the histological subtypes, the medullary subtype had the highest CD146 expression. Of the 23 medullary tumors included in this study, 11 of these tumors were of the triple-negative molecular subtype. So more than half of the medullary tumors are of another molecular subtypes (mainly luminal B HER2-subtype (n = 7)).

In other tumor types, CD146 expression is associated with tumors with a higher tumor grade and an increased metastatic potential (18, 26, 27). The data shown here confirms that this is also the case in breast cancer. In addition, CD146 expression was more preva-

lent in tumors from younger patients. Regarding prognosis, this data shows that CD146 is a pure prognostic factor in the first 10 years of follow-up, a result not confounded by including patients who were treated with adjuvant systemic therapy, as others have done previously. In the study of Zabouo et al. (19), 51% of the patients received adjuvant chemotherapy and 52% adjuvant endocrine therapy, which obscures the assessment of the true prognostic value of a marker. It is not clear if the patients in the study of Zeng et al. (3) received any type of systemic (neo)adjuvant treatment. While Zeng et al. also showed worse MFS and OS for CD146-positive patients with a follow-up period of 100 months, Zabouo et al. showed that CD146-positive patients had significantly lower OS in the first 5 years, but not in the period thereafter (3, 19). The data presented here (***Figure 1***) shows that the effect of CD146 expression is more distinct as time progresses for OS. OS does not differ in the first 45 months with respect to CD146, but between 45 and 120 months the CD146-positive patients have a worse prognosis (HR 2.16, 95% CI 1.25–3.74, p = 0.005, see ***Supplementary Figure 4***). When correcting for other prognostic factors of breast cancer in multivariable analysis, which has not been done before, CD146 does add to the quality of the model for MFS, but it is not a significant independent prognostic factor for MFS nor OS.

We divided the group of patients in an ER-positive and ER-negative subgroup to explore what the difference in prognostic impact of CD146 is between these two subgroups. It is striking that in the ER-negative subgroup, there is no difference in MFS and OS between the CD146-positive and CD146-negative patients, while in the ER-positive subgroup both MFS and OS are shorter in the CD146-positive group, although the numbers of CD146-positive patients is somewhat low. In ***Supplementary Figure 3*** it was found that 80% of the ER-positive, CD146-negative patients were alive after 10 years, while the CD146-positive patients, with probably more aggressive tumors, only 53% were still alive. In the ER-negative patients this was around 70% for both the CD146-positive and -negative subgroup. So, although CD146 expression is rare in ER-positive patients, CD146 has the potential to be of value as a potential prognostic marker in this subgroup. It should be recommended to validate this in a larger independent series, preferably prospectively, since this could potentially have consequences for ER-positive/CD146-positive patients, for example in more extensive monitoring or prolonging of the duration of adjuvant therapy.

As shown previously, CD146 expression is associated with tamoxifen resistance in breast cancer cell lines (12). To examine whether this also holds true in patients, we investigated this in a set of primary breast cancer tissues of patients who did not receive (neo) adjuvant endocrine treatment and were treated in the first-line palliative treatment with tamoxifen. In total, only 2.5% (8/317) of these patients were CD146-positive. Though no difference in outcome to tamoxifen was seen in this set of patients, the number of CD146-positive patients is too small to draw firm conclusions.

CD146 expression is present in 11% of all primary breast cancer tissues and is predominantly present in the medullary and triple-negative subtypes. We found no strong evidence of CD146 expression and EMT in primary breast tumors, suggesting that CD146 is maybe necessary, but not sufficient for EMT in breast cancer. CD146 expression was associated with more aggressive tumors and patients who are CD146-positive had a shorter MFS and OS, without the confounding effect of adjuvant treatment.

4. MATERIALS AND METHODS

4.1. Patient and Tissue Samples

Formalin-fixed paraffin-embedded (FFPE) tissue of the primary tumor was collected from all patients with breast cancer who entered the Erasmus University Medical Center (Rotterdam, The Netherlands) for local treatment of their primary disease during the period of 1985 to 2000. This study was approved by the Erasmus MC medical ethics committee (MEC 02-953, approved 11th of April 2002). In total, from 1350 patients FFPE tissues and complete clinical follow-up information was collected for the primary objective. Due to missing values and several other reasons that are listed in ***Figure 2***, 325 tissues were excluded, leaving a total of 1025 FFPE tissues for analysis. All tissues had a known histological subtype and molecular surrogate subtype and could therefore be used for subtype analysis. 551 tissues were from patients who were lymph-node negative and did not receive (neo-)adjuvant treatment and hence could be used to assess the pure prognostic value of CD146. To assess the role of CD146 in response to tamoxifen treatment, an additional set of 462 FFPE tissues of the primary tumor of breast cancer patients who received tamoxifen as first-line palliative treatment was collected. Of these, CD146 status was determined of 317 ER-positive tumors from patients who did not receive (neo)adjuvant endocrine treatment.

4.2. Tissue Microarray and Immunohistochemistry

Pathologists (MdB, CvD) from the Erasmus Medical Center assessed the histology of all eligible tumors and also scored tumor grade according to the modified method of Scarff, Bloom & Richardson (28) prior to preparing the tissue microarray (TMA). Thereafter, representative areas of the tumor for inclusion in the TMA were marked. From these areas, three cores were taken with a 0.6mm needle and added to the TMA. These TMAs were stained with standard protocols for ER, PgR, HER2, Ki-67, EpCAM and CD146 and scored manually for staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and quantity (percent of stained breast tumor cells). See ***Supplementary Table 4*** for more information about IHC staining methods, cut-offs and used subtypes (29, 30).

4.3. Epithelial-to-Mesenchymal Transition

To study the role of CD146 in EMT, gene expression data was used of 52 breast cancer cell lines (available as entry GSE41313at the Gene Expression Omnibus (GEO) http://www.ncbi.nlm.nih.gov/ geo/), 105 tissues from our dataset and 867 primary breast cancer tumors (in-house data plus publicly available data. GEO entries GSE2034, GSE5327, GSE12276, GSE27830 and GSE47389 (in-house) and GSE2990, GSE7390 and GSE11121). The data were normalized using fRMA (31) and were corrected for batch effects using ComBat (32) before analyses. First, correlation of all available probes (plus2-PM chip, Affymetrix, Santa Clara, CA, USA) to the CD146 probe 211340_s_at was assessed in the 52 cell lines. An arbitrarily chosen cutoff of $R > 0.6$ was used to ensure both a decent correlation with CD146 and a sufficient number of genes ($n = 342$) were then available for overrepresentation analysis using DAVID (33, 34). The same analysis was performed in the primary tumor cohort. However, since correlations in the tumor cohort ranged between −0.35 and +0.6, another cutoff was established. The correlation coefficients were normally distributed and the average and standard deviation (SD) were used to calculate a cutoff for the extreme end of the distribution ($p < 0.001$). This gave an R of 0.35 and yielded 200 genes for overrepresentation analysis. This method was also used for TCGA (23) and METABRIC (24) data (downloaded from cBioPortal—http://www.cbioportal.org/), yielding a cutoff of $R > 0.502$ and 37 genes for analysis (TCGA) and $R > 0.276$ and 47 genes (METABRIC). So although the absolute correlation is not that high in the primary tumor data, the selected genes are the highest correlating genes with CD146.

4.4. Statistical Analysis

The relation between patient characteristics and the immunohistochemical profile of the tumor were analyzed with the Pearson chi-square test. For subtype analysis, the Fisher's exact test is used in case of small subtype groups. For all patients, time from diagnosis to the first distant metastasis (MFS) and time from diagnosis until patient's death (OS) was determined. Patients who did not experience an event were censored at the last date of contact (with a maximum of 10 years, since patients usually return to the general practitioner for follow-up after 10 years). For MFS, all distant metastases were counted as an event, but local-regional relapses were not. Patients diagnosed with secondary contralateral breast cancer were censored for MFS at the date of diagnosis of the secondary breast cancer. A total of 97 patients died without evidence of disease and were censored at last follow-up in the analysis of MFS. During follow up, 316 patients died with evidence of disease. To assess the prognostic role of CD146, only data from patients who were lymph-node negative and did not receive (neo)adjuvant treatment were used ($n = 551$). For the patients who were treated with tamoxifen, clinical benefit (CB) is defined as patients who show complete response (CR), partial response (PR) or stable disease (SD) for more than 6 months on tamoxifen treatment. Survival analyses

for MFS were performed using the competing risks model of Fine & Gray to account for the competing risk of death of patients who did not have evidence of disease at the moment of death. MFS is visualized by means of the cumulative incidence function (CIF). For OS the Cox proportional hazards model was used and the Kaplan-Meier method for visualization. All factors from the univariable analyses were added to the multivariable analysis where a stepwise backward selection procedure was used to study which factors are independently related to the outcomes. In the Cox models this was performed with a threshold for significance of $p < 0.05$. For the Fine & Gray model this procedure was based on the Akaike information criterion (AIC). This method is not p-value driven, but compares the relative quality of the models with different combinations of prognostic factors to each other and selects the best fitting model. The results are presented with a hazard ratio (HR) and its 95% confidence interval (95% CI). All computations were performed using R and all reported p-values are two-sided.

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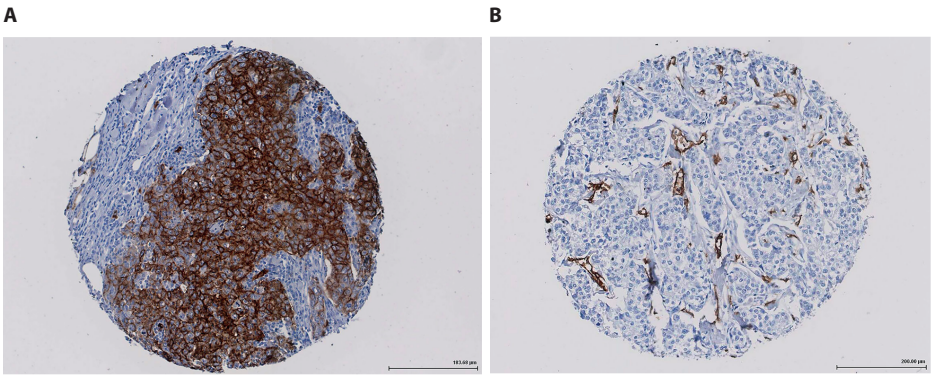
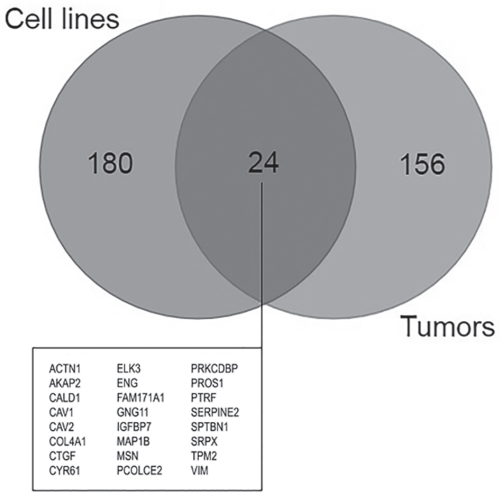
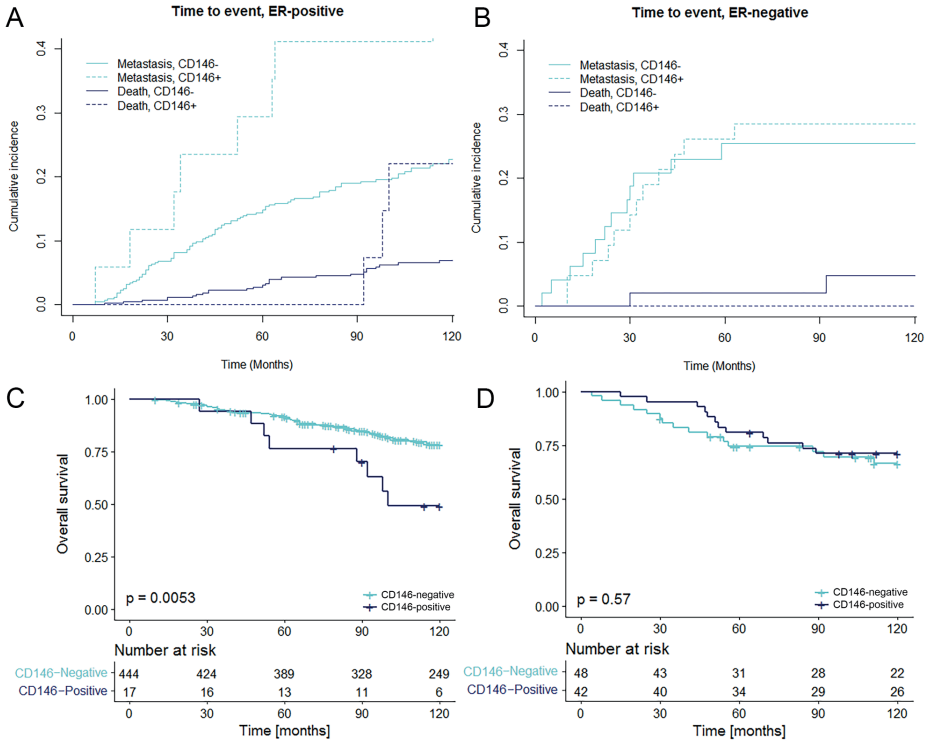
SUPPLEMENTARY MATERIALS



Supplementary Figure 1. Immunohistochemical staining for CD146
Immunohistochemical staining of CD146 in a (**A**) CD146-positive and (**B**) CD146-negative primary breast cancer tumor (only CD146 staining in vessels)

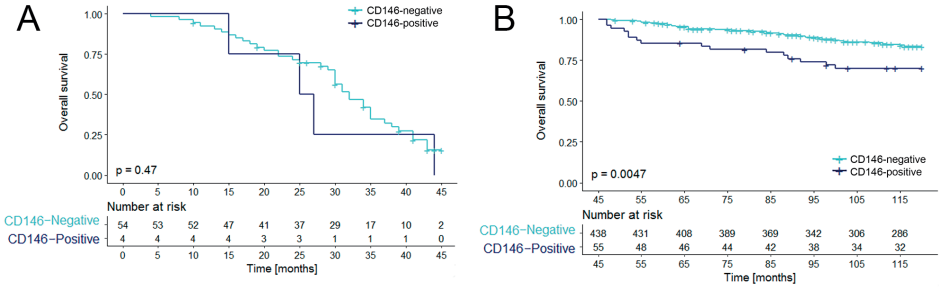


Supplementary Figure 2. Overlapping genes between cell lines and primary tumors
From the highest correlating genes in the cell lines and the primary tissues, these are the 24 overlapping genes.



Supplementary Figure 3. MFS and OS as function of CD146 expression in the ER-positive and -negative subgroup

MFS and OS as function of CD146 expression. (**A**) MFS in ER-positive subgroup and (**B**) ER-negative subgroup (depicted with the cumulative incidence function). (**C**) OS in ER-positive subgroup and (**D**) ER-negative subgroup (depicted with Kaplan-Meier). For (**A**) and (**B**) the light blue lines (metastasis) are the patients who developed a metastasis, the dark blue lines (death) are the patients who died without any evidence of disease.



Supplementary Figure 4. OS as function of CD146 expression divided in two time periods

OS (depicted with Kaplan-Meier method) as function of CD146 expression in patients who were N0, M0 at baseline and did not receive neo-adjuvant or adjuvant therapy (N = 551). (**A**) shows the OS of these patients in the first 45 months (months 0-45) and (**B**) the OS from month 46 till 120 (months 46-120).

Supplementary Table 1. Relationship between EpCAM and CD146 expression

	EpCAM-high	**EpCAM-low**	*Total*	
CD146-positive	87	26	*113*	
CD146-negative	508	404	*912*	
Total	*595*	*430*	*1025*	
	CD146+/EpCAM+	**%**	***CD146+/EpCAM-***	**%**
Total	87/1025	8.5	26/1025	2.5
		Molecular subtype		
Luminal A	8/446	1.8	1/446	0.2
Luminal B HER2-	15/341	4.4	4/341	1.2
Luminal B HER2+	2/74	2.7	0/74	0.0
HER2+	5/45	11.1	2/45	4.4
TNBC	57/119	47.9	19/119	16.0
		Histological subtype		
Invasive ductal carcinoma	77/849	9.1	21/849	2.5
Invasive lobular carcinoma	1/118	0.8	0/118	0.0
Mucinous	0/18	0.0	0/18	0.0
Tubular	1/12	8.3	0/12	0.0
Medullary	7/23	30.4	4/23	17.4
Papillary	1/5	20.0	1/5	20.0

A) Relationship between EpCAM and CD146 expression within all tumors. B) Number of tumors with CD146 expression and with or without EpCAM expression, divided in the molecular and histological subtypes.

Supplementary Table 2. Number of unfavorable events in the histological and molecular breast cancer subtypes

	Total (n)	Number of events (n, %)	p-value
CD146-positive	59	20 (34)	p=0.024
CD146-negative	492	103 (21)	
Histological subtype			*p=0.213*
Invasive ductal carcinoma	458	109 (24)	
Invasive lobular carcinoma	61	8 (13)	
Medullary	10	2 (20)	
Mucinous	9	0 (0)	
Tubular	8	2 (25)	
Papillary	5	2 (40)	
Molecular subtype			*p<0.001*
Luminal A	248	39 (16)	
Luminal B HER2-negative	171	40 (23)	
Luminal B HER2-positive	42	17 (40)	
HER2-positive	28	12 (43)	
Triple negative	62	15 (24)	

Number of unfavorable events in all tumors and divided by histological and molecular subtypes. P-value determined with chi-square test. Only patients who were M0 at baseline and did not receive neo-adjuvant or adjuvant therapy (N = 551) were included

Supplementary Table 3. CD146 status in relation to the type of response on tamoxifen in the first line

	CD146-negative	CD146-positive	*Total*
Complete response	7 (2%)	1 (13%)	8
Partial response	52 (17%)	2 (25%)	54
Stable disease > 6 months	141 (46%)	3 (37%)	144
Stable disease <= 6 months	24 (8%)	0 (0%)	24
Progressive disease	85 (27%)	2 (25%)	87
Total	309	8	317

Response on first-line tamoxifen treatment in relation with CD146 status. Stable disease is divided by stable disease shorter than 6 months or longer than 6 months.

Supplementary Table 4. Methods of the TMA immunohistochemistry and breast cancer subtypes.

A	Antigen	Antibody Clone	Company	Used concentration	Heat-induced antigen retrieval	Cut-off for calling tumor positive / high
	ER	1D5	Dako	1:40	pH 9	Nuclear staining, > 10% positive nuclei
	PR	Pgr636	Dako	1:50	pH 9	Nuclear staining, > 10% positive nuclei
	HER2	Herceptest	Dako	Ready to use from kit		Moderate or strong intact membranous staining in > 10% of the tumor cells or FISH-amplified
	Ki-67	MIB-1	Dako	1:50	pH 6	Nuclear staining, > 10% positive nuclei (24)
	EpCAM	VU1D9	Cell Signaling	01:4.7	pH 6	Moderate or strong staining of the whole tumor cell membrane in ≥90% of the tumor cells
	CD146	N1238	Novocastra	01:5.5	pH 6	Whole membrane staining in >1% of the individual tumor cells

B	Subtype	
	Luminal A	ER+, HER2-, PR+ and Ki-67 low
	Luminal B HER2-	ER+, HER2- and either PR- or Ki-67 high
	Luminal B HER2+	ER+, HER2+ and any PR or any Ki-67
	HER2-positive	ER-, PR-, HER2+ and any Ki-67
	Triple negative	ER-, PR-, HER2-, any Ki-67

A) Antibodies used in immunohistochemistry. For the TMA the cores were put in a blank receive blok (80 cores per block) using the Automated Tissue Arrayer ATA-27 (Beecher Instruments Inc, Sun Prairie, WI, USA). Slides from the TMA were cut (4 µm) and stained with the EnVision™ method (Dako, Glostrup, Denmark). After staining, slides were converted to digital images with the Virtual Slide Scanner NanoZoomer 2.0-HT (Hamamatsu, Japan). These digital images were uploaded into the software program Distiller (Slidepath, Ireland) and all images were scored manually. Staining of all invasive breast tumor cells was recorded. The mean of the positive stained breast tumor cells was calculated from the total cell count of the 3 separate cores. The cut-off for when a tumor is scored positive is visible in the figure. In case of weak to moderate ('++') HER2-staining in > 10%, FISH was performed with the Dako HER2FISH pharmDx™ Kit and scored and interpreted according to the kit guidelines. B) Molecular subtypes used, determined with IHC.

Chapter 3

Liquid Biopsies to Select Patients for Perioperative Chemotherapy in Muscle-invasive Bladder Cancer: A Systematic Review

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ABSTRACT

Context: Neoadjuvant chemotherapy (NAC) is considered the standard treatment for muscle-invasive bladder cancer (MIBC). However, its overall survival benefit is limited and toxicity is significant; hence, NAC has not been adopted universally.
Objective: To systematically evaluate whether biomarkers can guide the administration of perioperative chemotherapy in MIBC patients.
Evidence acquisition: A systematic search of the PubMed database was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA). In total, 215 papers were screened and 22 were selected to assess the potential clinical value of circulating tumor cells (CTCs) and cell-free DNA (cfDNA) in selecting MIBC patients for perioperative chemotherapy.
Evidence synthesis: We found that the presence of one or more CTCs before radical cystectomy, as determined by the CellSearch technique, is a robust marker for poor recurrence-free and overall survival. Consequently, whether NAC can be withheld in patients without the presence of CTCs is a subject of ongoing investigation. Studies investigating various approaches to detect cfDNA showed that cfDNA is present in the blood of MIBC patients, but varying results on its prognostic value have been reported. Successful cfDNA-based approaches are likely to encompass at least a multitude of genes using next-generation sequencing, as there are generally few hotspot somatic mutations in MIBC.
Conclusions: Liquid biopsies hold promise in selecting MIBC patients for perioperative chemotherapy, but instead of more proof-of-principle studies, prospective studies investigating true clinical applicability for treatment decision making are urgently needed.
Patient summary: Liquid biopsies appear to be a promising tool to guide the administration of chemotherapy in patients with muscle-invasive bladder cancer; however, the optimal way to implement these remains to be determined.

1. INTRODUCTION

Bladder cancer (BC) is a commonly diagnosed malignancy in the Western World and was responsible for an estimate of 165 000 deaths worldwide in 2012 (1). Patients with muscle-invasive bladder cancer (MIBC) are at the highest risk of developing distant metastases. The standard treatment of nonmetastatic MIBC (nmBC) consists of pelvic lymph node dissection and radical cystectomy (RC) with urinary diversion. Despite extensive surgery, MIBC patients have poor 3-yr metastasis-free survival of approximately 50% (2). Perioperative chemotherapy can prevent distant metastases. Therefore, the current European Association of Urology (EAU) guideline (3) recommends offering platinum-based neoadjuvant chemotherapy (NAC) in nmBC patients prior to local definitive treatment. However, the benefit from NAC is relatively modest, with an absolute overall survival (OS) benefit of 5–6% after 10 yr (2,4). Given this limited OS benefit and the significant toxicity, platinum-based NAC is underutilized in current clinical practice (5). Aside from NAC, adjuvant chemotherapy (AC) can be considered (6), even after NAC (7). However, there is no evidence supporting that AC improves OS.

Given the small OS benefit from NAC, exposing all MIBC patients to NAC will result in overtreatment. Therefore, there is a clear clinical need for biomarkers that give insight into which MIBC patients will or will not benefit from NAC. Previously investigated predictive markers in the primary tumor of MIBC patients did not provide information about distant micrometastases, and none of them are currently used in the clinic. So-called "liquid biopsies", such as circulating tumor cells (CTCs) and cell-free DNA (cfDNA), are considered a reflection of tumor burden. These liquid biopsies are minimally invasively measured in blood or urine, rendering them attractive to guide perioperative management of MIBC patients. This review encompasses a systematic overview of liquid biopsy research in MIBC and puts into perspective the possible applications for driving treatment decision in the perioperative setting of MIBC.

2. EVIDENCE ACQUISITION

A systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines. A PubMed search for all eligible articles written in English up to October 2019 was performed. The search strategy and keywords used are shown in ***Figure 1***. All titles and abstracts were screened by two of the authors (I.K. and N.B.) to determine their eligibility. In total, the literature search retrieved 215 papers (***Supplementary Table 1***). After removing 56 duplicates, 159 titles and/or abstracts were screened and 32 full-text papers were reviewed. Only articles describing MIBC patients were included. All reviews, commentaries, and case studies

(reports ≤10 patients) were excluded. I.K. and N.B. reviewed the papers independently, and in the end 22 papers that met all the criteria for inclusion were selected (***Tables 1 and 2***). All CTC counts in this review are reported per 7.5 ml of blood.

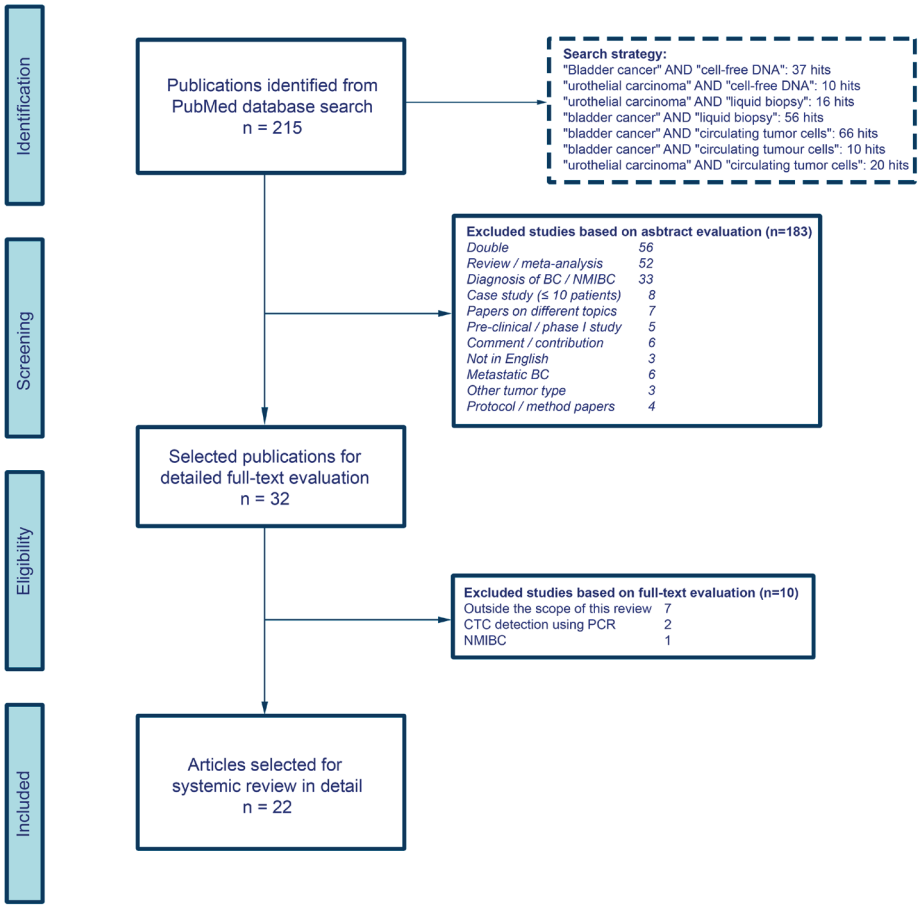

Figure 1. PRISMA flow diagram.
BC = bladder cancer; CTC = circulating tumor cell; NMIBC = non–muscle-invasive bladder cancer; PCR = polymerase chain reaction; PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-analyses.

3. EVIDENCE SYNTHESIS

3.1. Circulating tumor cells

3.1.1. CTC detection with the CellSearch system

CTCs are tumor cells circulating in the peripheral blood. Earlier research on CTCs in BC patients was mostly based on polymerase chain reaction (PCR)-based assays in which

a specific marker (CK20, hTERT, UP-II, or Survivin) was measured in whole blood or urine (8–10). However, most studies were limited by patient numbers and problems with false-positive and false-negative samples (8). The isolation of CTCs is nowadays performed with techniques using the immunofluorescence properties of CTCs to enrich for them, with the CellSearch method most frequently used (***Figure 2***). Using magnetic beads coupled to anti-epithelial cell adhesion molecule (EpCAM), CTCs are isolated, after which cells are stained and scored as CTCs or other cells (eg, leukocytes) by a trained user. CTCs detected using this assay were rarely present in patients with benign diseases (11), and have good inter- (12) and intrareader (13) agreement; moreover, after multiple reports describing robust prognostic value, the system received Food and Drug Administration clearance for enumerating CTCs in patients with metastatic breast, prostate, and colorectal cancer.

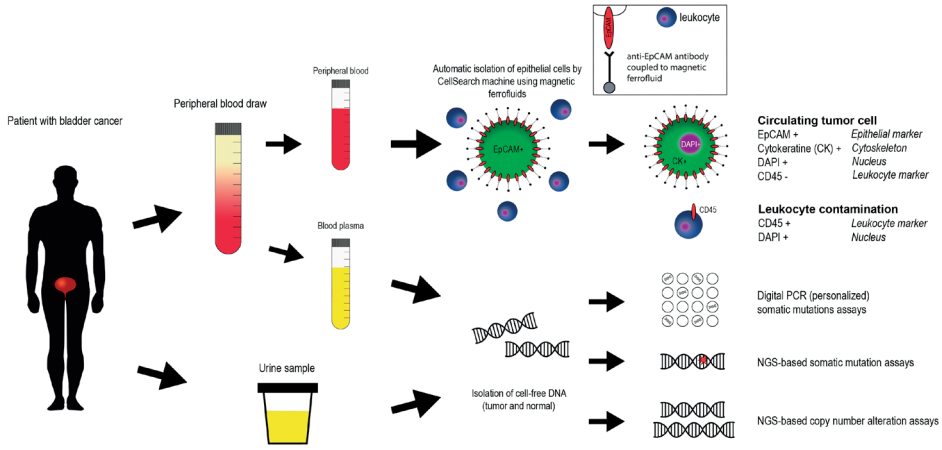

Figure 2. Various liquid biopsies in the current landscape.
Top: workflow of CellSearch system for the enumeration of CTCs. Bottom: options for isolation of cell-free DNA from blood or urine. CTC = circulating tumor cell; NGS = next-generation sequencing; PCR = polymerase chain reaction.

Following the landmark paper showing that CellSearch- enriched CTCs were associated with poor prognosis in metastatic breast cancer patients (14), Naoe and colleagues (15) found that CTCs could be detected in BC patients using CellSearch. They observed no CTCs in 12 nmBC patients versus one or more CTCs in 10 of 14 metastatic patients.

Guzzo et al (16) were the first to describe the presence of CTCs in nmBC patients. In nine of 43 patients (35/43 with MIBC), one or more CTCs were detected (median one CTC). A subset of the patients (37%) received NAC; however, the number of CTCs observed in these patients was not different from those observed in patients who had not received NAC. There was no relation between the presence of CTCs and clinicopathological characteristics, but analyses were underpowered.

Table 1. Overview of studies evaluating CTCs

Author	System used	N	Moment of sampling	Pathological tumor stage	Results (median, range in CTC+)	% CTC+	Clinical value of CTC+
Naoe [15]	CellSearch	12	Preoperative	pT0-1: 8 pT2-4: 4	0/4 CTC+	0	ND
Guzzo [16]	CellSearch	43	Preoperative	pT0-1: 13 pT2: 6 pT3: 12 pT4: 12	9/43 CTC+ (1, 1-9)	21	No relation between CTCs and clinicopathological characteristics
Flaig [17]	CellSearch	28	Preoperative	pT0-1: 8 pT2: 6 pT3: 8 pT4: 4	5/28 CTC+ (1-6)	18	ND
Winters [18]	CellSearch	16	Before NAC	cT2-4: 16	3/16 CTC+ (all 1 CTC)	19	ND
Abrahamsson [19]	CellSearch	88	Preoperative	pT0-1: 32 pT2: 18 pT3: 15 pT4: 10	17/88 CTC+ (3, 1-105)	19	Increased risk of radiological metastatic disease; independent predictor earlier progression
Rink [20]	CellSearch	50	Preoperative	pT0-1: 16 pT2: 10 pT3: 15 pT4: 9	15/50 CTC+ (1, 1-11)	30	Association with RFS, CSS, and OS
Rink [21]	CellSearch	100	Preoperative	pT0-1: 34 pT2: 20 pT3: 32 pT4: 14	23/100 CTC+ (1, 1-100)	23	Independent predictor of RFS, CSS, and OS

Table 1. Overview of studies evaluating CTCs (continued)

Author	System used	N	Moment of sampling	Pathological tumor stage	Results (median, range in CTC+)	% CTC+	Clinical value of CTC+
Soave [22]	CellSearch	188	Preoperative	pT0–1: 57 pT2: 45 pT3: 61 pT4: 25 NB: 47/188 variant UCB histology	42/188 CTC+ (1, 1-163) 12/47 CTC+ with variant UCB	22 26	Independent predictor of RFS, CSS, and OS independent of variant UCB histology
Soave [23]	CellSearch	185	Preoperative	pT0–1: 57 pT2: 45 pT3: 59 pT4: 24	41/185 CTC+ (1, 1-163)	22	Independent predictor of RFS, CSS, and OS
Alva [28]	Isoflux	20 And 13 HBDs	Before NAC	pT0–1: 7 pT2: 6 pT3: 4 pT4: 3	18/20 CTC+ (13, 0-358) 9/13 HBDs CTC+ (2, 0-8)	90 69	ND
Chalfin [29]	AccuCyte / Cytefinder	12	Preoperative	pT2-4: 12	2/12 EpCAM-positive CTC+ (0, 0-1) 7/12 EpCAM-negative CTC+ (1, 0-4)	17 58	ND
Todenhofer [31]	AdnaTest	40	Preoperative	pT2: 15 pT3: 19 pT4: 6	12/40 CTC mRNA positivity	30	Association with clinical and pathological tumor stage
Fina [32]	AdnaTest	19	Before NAC	pT2-4: 19	4/16 CTC mRNA positivity	25	None

CSS = cancer-specific survival; CTC = circulating tumor cell; HBD = healthy blood donor; NAC = neoadjuvant chemotherapy; ND = not described; OS = overall survival; RFS = recurrence-free survival; UCB = urothelial bladder cancer. Only patients with nonmetastatic bladder cancer are described. Clinical value of CTC + described the most important parameters as discussed in the manuscript.

Table 2. Overview of studies investigating the detection of cfDNA

Author	Source of cfDNA	Technique used	N	Moment of sampling	Pathological tumor stage	Results
Ellinger [34]	Serum	qRT-PCR	45	Preoperative	pT0-1: 12 pT2: 6 pT3: 19 pT4: 8	Short and large fragments of cfDNA increased in BC patients compared to BPH patients Increased ratio of small/large cfDNA associated with poor BC-specific survival after RC
Christensen [38]	Plasma & urine	ddPCR FGFR3 & PIK3CA	403	Preoperative 31/403 followed in plasma and/or urine	pT0-1: 2 pT2-4: 29	High ctDNA level associated with recurrence, lower RFS and OS (all p<0.05) Recurrence in 8/9 patients with ctDNA and in 6/18 patients without ctDNA detectable Higher level of urine cfDNA associated with RFS (p=0.031), OS (p=0.035) Higher level of plasma cfDNA associated with RFS (p<0.01), OS (p=0.02)
Birkenkamp-Demtroder [39]	Plasma & urine	Personalized ddPCR	60	Before and during NAC, 26 followed in plasma and/or urine	pT0-1: 4 pT2: 21 pT3-4: 1	Relapse after RC (median 275 days): ctDNA detectable at median 137 days cfDNA detectable in 50% of patients without evidence of relapse
Christensen [42]	Plasma	Targeted 51-gene NGS panel vs ddPCR	38	Before NAC	pT2-4: 38	24/38 mutations found with dPCR were found back with NGS (63.2%) 4 mutations only detected with NGS and not dPCR (all present in tissue sample)
Vandekerkhove [43]	Plasma	Targeted 52-gene NGS panel	14	Before or during chemotherapy	pT2-4: 14	ctDNA levels above threshold (>2% VAF) in 1/7 representative MIBC patient samples
Patel [44]	Plasma & urine	Targeted 8-gene NGS panel & sWGS	17	Before NAC	pT2: 2 pT3: 14 pT4: 1	ctDNA (mutation or CNA) in 6/17 plasma samples before NAC ctDNA in 9/17 urine samples before NAC Longitudinal sampling during NAC: mutant ctDNA in 35% of urine and 10% of plasma samples (p<0.001)

Table 2. Overview of studies investigating the detection of cfDNA (continued)

Author	Source of cfDNA	Technique used	N	Moment of sampling	Pathological tumor stage	Results
Soave [45]	Serum	Multiplex 37-gene PCR (MLPA)	72	Preoperative	pT0-1: 26 pT2: 20 pT3: 15 pT4: 11	cfDNA CNAs detected in 49%, presence not associated with RFS, CSS or OS Reduced CSS if copy number gain in either *KLF5, ZFHX3* or *CDH1* (all $p<0.05$)
Christensen [46]	Plasma	Patient-specific 16-gene NGS panel	68	Before and during NAC and before and after RC	pT1: 4 pT2-4: 64	ctDNA positivity versus negativity before NAC, before RC and after RC were all associated with shorter RFS and OS (all $p<0.001$)
Togneri [47]	Urine	Microarray CNA assay with 74 hotspot SNVs	23	Preoperative	pT0-1: 9 pT2-4: 14	DNA aberrations from primary tumor found in 92% of urine cfDNA vs 37% urine cellular DNA

BC: bladder cancer, BPH: benign prostate hyperplasia, Clinical MIBC (cMIBC): cT2-T4/ pathological MIBC (pMIBC): pT2-T4, CNA: copy number alteration, SNV: single nucleotide variant, CSS: cancer-specific survival, dPCR: digital droplet PCR, MIBC: muscle-invasive bladder cancer, mBC: metastatic bladder cancer, MLPA: multiplex ligation-dependent probe amplification, NAC: neoadjuvant chemoterapy, NR: not reported, OS: overall survival, PCA: prostate cancer, RC: radical cystectomy, RFS: recurrence-free survival, sWGS: shallow whole genome sequencing, WES: whole exome sequencing, qRT-PCR: qualitative real time PCR. Only patients with non-metastatic bladder cancer are described.

Multiple groups confirmed the detection of CTCs in nmBC patients in small patient sets (17–19). The largest dataset on CTCs in nmBC patients to date has been published by the Hamburg group on several occasions (20–23). Their most recent analysis encompassed 185 nmBC patients (69% with pathological MIBC) undergoing RC without NAC, of whom 50 patients received AC (patients with ≥pT3 tumors and/or lymph node metastasis) (23). CTCs were found in 41 of 185 (22%) patients. The presence of CTCs was associated with lymphovascular invasion, microvascular invasion, and positive soft tissue surgical margins, but not with lymph node metastasis. A weak association was found with pathological T stage ($p = 0.065$), and in a later paper (encompassing three extra patients) (22) the association became significant ($p = 0.041$). CTC-positive patients who underwent RC, with or without AC, had poorer recurrence-free survival (RFS; either local recurrence or metastatic recurrence) than CTC-negative patients (2-yr RFS 30% (95% confidence interval (CI) 14– 46%) vs 69% (59–79%), $p < 0.001$), poorer cancer-specific survival (CSS; 2-yr CSS 45% (27–63%) vs 74% (66–82%), $p < 0.001$) and OS (2-yr OS 41% (23–59%) vs 70% (60–80%), $p < 0.001$). CTC positivity remained associated with RFS, CSS, and OS in a subanalysis of patients who had not received AC. The presence of CTCs was not associated with prognosis in patients receiving AC ($n = 50$). While this discrepancy was probably due to the small number of patients (17 CTC-positive vs 33 CTC-negative patients) and even fewer events, one might speculate that AC eradicates CTCs from the blood in BC patients. Accordingly, a report (18) describing four BC patients found a decrease in postchemotherapy CellSearch CTCs in all patients.

3.1.2. CTC detection using other systems

Since the CellSearch system depends on EpCAM and since EpCAM is not expressed in all nmBC tissues (24) or EpCAM expression may be lowered or lost during epithelial-to-mesenchymal transition (25,26), many alternative assays have been developed (an in-depth overview in the study of Mamdouhi et al (27)). However, generally, the prognostic value of CTC assays alternative to CellSearch has been more or less anecdotal and less rigorous than that of CellSearch.

Alva and colleagues (28) evaluated 20 MIBC patients receiving NAC and enriched CTCs with CellSearch and Isoflux. Isoflux has a similar definition of CTCs as CellSearch and uses a microfluidic EpCAM-based system to isolate the CTCs, for which the manufacturer claims that CTCs with a low EpCAM expression are also enriched. Before NAC, Isoflux CTCs were detected in 18 of 20 patients, and also in nine of 13 healthy donors (median 2, range 0–8). Using a cutoff of ≥10 CTCs, 60% of patients had CTCs before NAC, which decreased to 33% after one cycle. In four of nine samples, ≥10 Isoflux CTCs were observed, while no CellSearch CTCs were detected. Given the presence of the Isoflux-enriched "CTCs" in young male healthy donors (median age 30 yr, range 22–54 yr), these findings should be interpreted with caution.

Other systems with various phenotypic definitions for CTCs were also used: Chalfin et al (29) found nucleated EpCAM-negative, pan-CK–positive cells on buffy coat divided over glass slides in seven of 12 MIBC patients. However, as other cells such as circulating endothelial cells may also express CK18 (30) and are therefore captured by pan-CK, specificity may be compromised when excluding EpCAM as a marker.

Two small studies evaluated CTC mRNA detected by AdnaTest in MIBC patients before NAC or RC (31,32). AdnaTest first enriches for CTCs using anti- EpCAM, anti-HER2, and anti-EGFR magnetic antibodies, and then investigates mRNA expression for epithelial markers. These studies found mRNA expression in 25–30% of all MIBC patients, and positivity was associated with more advanced disease.

3.2. Cell-free DNA

3.2.1. Cell-free DNA in plasma

While cfDNA in healthy persons is derived from nontumor cells, in cancer patients, cfDNA can be released from apoptotic and necrotic cancer cells (33). Ellinger et al (34) were the first to describe cfDNA detection in 45 nmBC patients undergoing RC and in 45 patients with benign prostate hyperplasia (BPH). The number of small (124 bp) and large (271 bp) cfDNA fragments measured with quantitative real-time PCR was higher in BC patients than in BPH patients, as well as the ratio between the small and large fragments (apoptotic index). Using a receiver operating characteristic curve, they found an "apoptotic index" of >12.1 to be associated with poorer CSS after RC (hazard ratio 1.07, 95% CI 1.02–1.13, p = 0.011). However, the paper lacked a validation cohort.

While this study demonstrated that increased amount of cfDNA is associated with prognosis in BC patients, it should be realized that cfDNA comprises all DNA (tumor and nontumor) in blood. Specifically analyzing the tumor fraction, called circulating tumor DNA (ctDNA), is therefore of great interest. However, ctDNA sometimes comprises <1% of the total cfDNA (35,36), making its detection challenging.

3.2.2. Personalized digital PCR assays

Birkenkamp-Demtroder and colleagues (37) published multiple studies in which they used personalized assays to detect ctDNA. This approach first investigates the primary tumor for specific mutations. Subsequently, cfDNA is interrogated using digital droplet PCR (ddPCR). After showing proof of principle of this personalized assay, even in localized disease, the approach was extended to 403 MIBC or high-risk NMIBC patients undergoing RC (38). Analyzing primary tumor tissue for hotspot mutations in PIK3CA and FGFR3, both frequently mutated in nmBC, 43 (11%) patients had one or more mutations. In 27 patients, these mutations were longitudinally followed in ctDNA. Based on the median mutant ctDNA levels, patients were dichotomized to either low or high ctDNA levels. A high preoperative ctDNA level was associated with disease recurrence (p =

0.016; local and metastatic), and lower RFS ($p < 0.001$) and OS ($p = 0.018$); eight of nine (89%) patients with detectable ctDNA in plasma experienced a recurrence, while six of 18 (33%) patients with no detectable preoperative ctDNA recurred.

An important limitation is that only PIK3CA and FGFR3 were screened, rendering patients not harboring mutations in these genes ineligible. In a follow-up paper (39), whole exome sequencing (WES) on 24 tumor tissues of 60 patients (50 with MIBC after NAC and 10 metastatic MIBC (mBC) patients before palliative chemotherapy) was performed to longitudinally follow ctDNA. Using 84 personalized ddPCR assays, ctDNA was detected in 75% of patients after RC. The levels of ctDNA differed significantly between patients who remained disease free and those who had a metastatic relapse. In half of the MIBC patients undergoing RC, metastatic relapse occurred after a median of 275 d (range 126–942 d), while ctDNA in these patients was already detectable at a median of 137 d (range 7–812 d). Interestingly, ctDNA was also detected in 50% of the MIBC patients without evidence of metastatic relapse. Their number of ctDNA copies was always <4 copies/ml, while only two of 12 patients with a metastatic relapse had such a small number of copies per milliliter. However, as follow-up for metastatic relapse was quite short (median 15 mo in nonrelapsing patients), patients with detectable ctDNA might have developed metastatic disease later than the writing of this paper, or the ctDNA assay may have suffered from specificity issues.

These papers demonstrated that ctDNA can be detected in nmBC and may be of use in guiding perioperative management. However, as there are few hotspot mutations in MIBC (40,41), sequencing the primary tumor on a patient-per-patient basis with the intent to identify patient-specific mutations to monitor in ctDNA is time consuming and labor intensive. In addition, new mutations occurring in ctDNA, not or infrequently present in the primary tumor, are missed. Therefore, there is a trend toward using new techniques to analyze ctDNA that do not interrogate only one mutation at a time.

3.2.3. Next-generation sequencing

Deep sequencing of the whole cfDNA genome is expensive, which is why most groups used next-generation sequencing (NGS) panels encompassing a select set of genes. Christensen and colleagues (42) developed a 51-gene NGS panel based on known mutational data in MIBC and included 38 patients with MIBC or mBC. In their mostly technical paper, they compared this NGS panel with ddPCR. Overall, NGS had inferior sensitivity to ddPCR, which is probably explained by NGS having insufficient genomic coverage for certain positions in which a mutation was present.

Vandekerkhove et al (43) also developed a 52-gene panel based on known genes containing somatic mutations or copy number alterations (CNAs). They included 51 patients (14 localized MIBC, and 37 nodal or distant mBC). In 42 patients without active treatment, ctDNA (threshold >2% variant allele frequency) was present in one of seven

patients (14%) with local disease, seven of 13 patients with lymph node metastases (54%), and in 16 of 22 (73%) patients with distant metastatic disease.

Patel and colleagues (44) analyzed longitudinal cfDNA samples of 17 MIBC patients before and during cisplatin-based NAC. They used a targeted NGS panel including eight genes commonly mutated in BC and also shallow whole-genome sequencing (sWGS) to assess CNAs. They found ctDNA (single nucleotide variants and CNAs combined) in 35% (6/17) of the plasma samples of patients before NAC; during NAC, five of six patients with detectable ctDNA experienced either local or metastatic recurrence, which did not occur in any of the six patients without detectable ctDNA.

Soave et al (45) analyzed cfDNA of 72 BC patients treated with RC. Using a multiplex PCR method to characterize CNAs in 37 mostly tumor-related genes, CNAs were detected in 48.6% (median 2). There was no difference in outcome based on the presence of CNAs. Reduced CSS was described for patients with copy number gains in KLF5, ZFHX3, or CDH1 ($p = 0.028$, $p = 0.026$, and $p = 0.044$, respectively). However, these analyses were performed with only few patients and without correction for multiple testing.

Christensen et al (46) presented promising results. They analyzed longitudinal cfDNA samples of 68 patients with localized MIBC before NAC, after NAC, and after RC. They first performed WES of tumor tissue to identify patient-specific somatic variants and then interrogated cfDNA for the presence of 16 patient-specific somatic variants using multiplex PCR NGS. A sample was considered ctDNA positive when two or more of 16 target variants were detected. The median follow-up was 21 mo after RC. The metastatic relapse rate for the whole cohort was 20% ($n = 13$). Positivity for ctDNA before NAC, before RC, and after RC was associated with shorter RFS and OS (all $p < 0.001$). In ctDNA-negative patients,12-mo metastatic recurrence rates of 3% (1/35), 7%, and 0% were found before NAC, after NAC, and after RC, respectively. In contrast, 12-mo metastatic recurrence rates in ctDNA-positive patients were 42%, 75%, and 59%, respectively. These data seem to offer sufficient proof of principle for initiating prospective studies investigating the influence of ctDNA status on treatment decision making. However, as it will be necessary to first sequence tumor tissue and subsequently construct NGS PCR panels on a patient-per-patient basis, the main question remains whether this labor-intensive approach will be feasible within an appropriate time frame for treatment decision making.

3.2.4. Cell-free DNA in urine

As bladder tumors are in direct contact with urine and ctDNA from blood may be partly cleared by the kidneys, ctDNA can also be isolated from urine. DNA in urine can be measured from the cell pellet (cellular DNA) or from the supernatant of the urine (cfDNA).

Togneri et al (47) compared cellular DNA and cfDNA from urine with formalin-fixed paraffin-embedded tissues in 14 MIBC patients before transurethral resection of the bladder (TURB). They used a microarray-based assay, which primarily detected CNAs, but

also 74 hotspot somatic mutations. Of the DNA aberrations (CNAs or mutations) found in the primary tumor, 92% was found in urine cfDNA, while only 37% was found in urine cellular DNA. The concordance between urine cfDNA and tumor tissue was higher than the results using plasma cfDNA as described above, although a direct comparison was not provided. Therefore, how urine cfDNA compared with plasma cfDNA and whether urine cfDNA had any prognostic value were still not clear.

Patel et al (44), as described above, investigated both plasma cfDNA and urine cfDNA samples in 17 MIBC patients prior to and during NAC using sWGS. Before NAC, they found tumor-associated DNA aberrations in nine of 17 urine cfDNA samples versus six of 17 plasma cfDNA samples. Interestingly, when analyzing all samples together, somatic mutations were more frequently detected in urine than in plasma cfDNA (34.5% vs 9.9%, $p < 0.001$) and at higher mutant allele frequencies. However, neither urine cfDNA nor plasma cfDNA captured all the somatic mutations detected across

the samples. Furthermore, the detection of mutant DNA in both plasma or urine cfDNA at the 2nd NAC cycle was associated with either local or metastatic disease recurrence; however, a small number of patients were analyzed.

Christensen and colleagues (38) investigated the occurrence of FGFR and PIK3CA mutations in urine cfDNA using ddPCR. A higher level of cfDNA in urine was not associated with recurrence ($p = 0.11$) in MIBC patients who had undergone RC. However, when dichotomized to low versus high urine cfDNA levels, there was a difference in RFS ($p = 0.031$) and OS ($p = 0.035$).

4. DISCUSSION

In this systematic review on liquid biopsies in MIBC patients, we found that liquid biopsies have the potential to guide perioperative treatment decision making in MIBC patients but that studies establishing their clinical utility are still lacking.

For CTCs, the largest study on the prognostic value of CellSearch CTCs in MIBC patients showed that their presence before RC was associated with worse RFS, CSS, and OS. This is consistent with findings in almost all other epithelial malignancies. However, despite their robust prognostic value, clinical decision making is still rarely based on CTC-derived data, not only in MIBC, but also in other tumor types (48). Studies that were using other assays to enumerate CTCs were much smaller, head-to-head comparisons with CellSearch were lacking, and specificity issues might have played a part as CTCs were detected in healthy donors.

The prognostic value of plasma cfDNA in MIBC patients is less clear, with studies including rather small patient cohorts and varying results that were often based on data-driven cutoff points without validation of the cutoffs. As all studies used different

assays to detect ctDNA, a direct comparison is not possible. However, one can speculate which assay is most likely to eventually have clinical utility. The most promising results to date have been obtained using personalized ddPCR or NGS assays based on somatic variants found in tumor tissue. However, to incorporate this strategy on a broad scale in a time frame acceptable for treatment decision making is challenging. The underlying problem is the lack of hotspot mutations in MIBC (41), hampering the development of a one-size-fits-all strategy based on somatic mutations in cfDNA. As CNAs are often present in MIBC (41), strategies using sWGS on ctDNA are also of interest (44,45). Detecting CNAs in ctDNA may also have significance in patients receiving immunotherapy, the clinical value of which as perioperative therapy is now investigated in several trials with MIBC patients (49). Recent explorative studies in patients with various cancer types receiving immunotherapy suggested that baseline cfDNA- assessed lower tumor mutational burden (50), higher amounts of CNAs (51), or stable copy number instability during treatment (52) may be linked to poor response to immunotherapy.

Several studies have looked at ctDNA detection in matched urine and plasma samples of patients with MIBC. In general, these data seem to point toward better detection of ctDNA in urine cfDNA than in plasma cfDNA in patients undergoing TURB or NAC. However, these results were generated in small patient groups, all of which had their primary tumor in situ. Interestingly, also after RC, urine cfDNA could be detected and an association with prognosis was described. Theoretically, detection would depend on the location of the recurrence: either in the bladder itself or at a distant metastatic site. It is not known whether previous treatment with bacillus Calmette-Guerin (BCG) might play a role and to what extent cfDNA is indeed cleared by the kidneys. A study investigating circulating fetal DNA found that 0.2–19% of fetal cfDNA is renally cleared (53). However, whether this also holds true in cancer patients, who are older and have a higher chance of renal comorbidity, is not known. Furthermore, it is not known whether the presence of carcinoma in situ or positive surgical margins after RC will affect the results of the urine assays. Therefore, the results as described with urine cfDNA in MIBC warrant the collection of urine to study cfDNA in future studies.

While ctDNA analyses in MIBC theoretically hold great promise, it is not ready for clinical utility. Especially when measuring ctDNA in the setting of nonmetastatic disease, such as MIBC, one has to scrutinize carefully when it comes to sensitivity, specificity, and reproducibility of the assay, and together with optimal preanalytical conditions, this is part of ongoing investigations (54).

Although the potential of liquid biopsies in the perioperative setting of MIBC is clear, clinical utility needs further prospective validation. For example, trials could investigate whether (1) patients with good prognosis based on liquid biopsies can safely omit NAC before RC or undergo combined-modality therapy with TURB and concurrent chemoradiotherapy instead of RC, (2) patients with poor prognosis based on liquid biopsies

could receive intensified NAC or AC, and (3) patients who have CTCs or cfDNA detectable during NAC or after RC should receive additional treatment.

The authors are aware of only two interventional trials in MIBC patients. The CirGuidance study (trial number: NTR4120, investigator initiated by the authors of this review; ***Figure 3***) enrolled patients with MIBC who are candidate for RC with the hypothesis that patients without CTCs have such a good prognosis, not justifying NAC. To this aim, CellSearch CTCs were enumerated in patients having clinical stage T2-T4aN0-N1M0 urothelial MIBC before treatment decision was made. Patients without detectable CTCs were not allowed to receive NAC, while in patients in whom one or more CTCs were present, NAC was encouraged. The primary endpoint of the study is the 2-yr OS. It was hypothesized that 188 CTC-negative patients were needed to show 2-yr OS of 75 ± 5%.

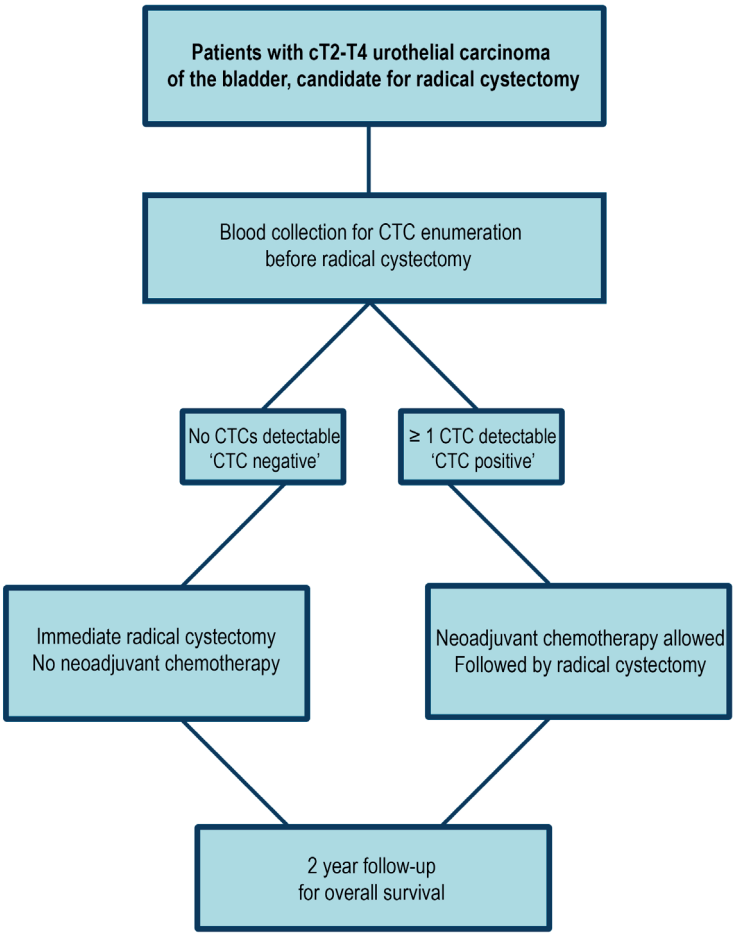

Figure 3. Flowchart of the CirGuidance study.
CTC = circulating tumor cell.

Accrual (n = 320) was recently completed and final trial results are expected in 2020. Recently, another interventional study in the form of the TOMBOLA study (NCT04138628) was initiated. This study enrolls patients who have undergone NAC and RC, and monitors for the presence of cfDNA after RC using personalized ddPCR (as also described above (39)). If patients have detectable cfDNA and/or evidence of relapse on imaging, they receive immunotherapy with atezolizumab with the hypothesis that early initiation of immuno-therapy based on the presence of cfDNA will result in better response rates and improved survival. The primary end-point is the number of patients reaching a complete response.

5. CONCLUSIONS

Liquid biopsies might potentially guide treatment decisions on perioperative chemotherapy in MIBC, but to date, studies have demonstrated only proof of principle. Besides further analytical validation, especially for cfDNA analyses, interventional studies investigating changes in treatment decision making based on liquid biopsies are still needed and are slowly being initiated.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. Overview of all reviewed articles

Author	Journal	Reason for exclusion
"Bladder cancer" AND "cell-free DNA": 37 hits		
Excluded: 10x review, 11x diagnosis of BC, 1x comment, 1x urine integrity, 1x meta-analysis, 1x mBC, 1x methylation, 1x mitochrondrial, 2x non-muscle invasive bladder cancer, 1x technical paper		
Birkenkamp-Demtroder	**Eur Urol 2018**	
Christensen	**Sci Rep 2018**	
Christensen	**Eur Urol 2017**	
Christensen	**J Clin Oncol 2019**	
Ellinger	**Cancer Letters 2008**	
Togneri	**Eur J Hum Genet 2016**	
Vandekerkhove	**Clin Cancer Res 2017**	
Birkenkamp-Demtroder	*Eur Urol 2016*	*NMIBC*
Abbosh	Urol oncol 2016	Review
Brisuda	Urol int 2016	Diagnosis of BC
Cao	Expert Rev Anticancer Ther 2019	Meta-analysis
Casadio	J Vis Exp 2017	Diagnosis of BC
Casadio	Urol Oncol 2013	Diagnosis of BC
Chang	Int j biol markers 2007	Diagnosis of BC
Cheng	Clin chem 2019	Diagnosis of BC
de Almeida	Clin Biochem 2016	Diagnosis of BC
Di Meo	Mol cancer 2017	Review
Ellinger	Urol Oncol 2012	Mitochondrial DNA
Emaus	Anal Chim Acta 2019	Technical paper
Ghanjati	Cancer biomarkers 2014	Methylation of DNA
Grivas	Eur Urol Oncol 2019	mBC
Hauser	Anticancer Res 2012	Diagnosis of BC
Kerr	Expert Rev Mol Diagn 2019	Review
Khetrapal	Cancer treat rev 2018	Review
Kim	Investig Clin Urol 2016	Diagnosis of bladder cancer
Li	J Clin Lab Anal 2019	Urine integrity
Maas	World J Urol 2019	Review
Maas	Expert Rev Mol Diagn 2018	Review
Rink	World J Urol 2019	Review
Rink	Transl Androl Urol 2019	Review
Santoni	Front Oncol 2018	Review
Stasik	Clin Biochem 2019	Diagnosis of BC
Wang	Am J Transl Res 2018	Review
Ward	Transl Androl Urol 2017	Commentary

Supplementary Table 1. Overview of all reviewed articles (continued)

Author	Journal	Reason for exclusion
Xu	Urol oncol 2019	Diagnosis of BC
Xu	Clin genitourin cancer 2019	NMIBC
Zancan	Int j biol markers 2009	Diagnosis of BC
"urothelial carcinoma" and "cell-free DNA": 10 hits		
Excluded: 7x double, 1x case study, 1x diagnosis of BC		
Soave	**Oncotarget 2017**	
Lee	Sci rep 2018	< 10 patients, so case study
Hayashi	Cancer Sci 2019	Diagnosis of BC
"bladder cancer" and "liquid biopsy": 56 hits		
Excluded: 11x double, 21x review, 1x protocol, 2x other tumor type, 1x contribution, 2x comment, 1x NMIBC, 10x diagnosis of BC, 2x mBC, 1x variant BC, 1x case series, 1x editorial, 1x outside scope		
Patel	**Sci Rep 2017**	
Wong	*J Exp Med 2018*	*Outside the scope of this paper*
Agarwal	Cancer 2018	mBC
Anantharaman	BMC cancer 2016	Case series (4 MIBC patients)
Awasthi	Asian Pac J Cancer Prev 2017	Gallbladder carcinoma
Bosschieter	PLoS One 2018	Protocol for urine collection
Chalfin	Eur Urol Oncol 2019	mBC
Chang	J Urol 2019	Comment
Chedgy	Urol Oncol 2016	Review
Cheng	Clin Chem 2019	Diagnosis of BC (and case serie)
Cimadamore	Curr Drug metab 2019	Contribution
Contreras-Sanz	Int J Urol 2017	Review
Ellinger	Expert Rev Mol Diagn 2015	Review
Herrington	J Pathol 2019	Review
Hirotsu	Cancer Sci 2019	Diagnosis of BC
Hofbauer	Urologe A 2017	Review
Hugen	Front Oncol 2017	Review
Kang	Transl Androl Urol 2017	Comment
Kang	Investig Clin Urol 2016	Short review
Kumari	Pathol Oncol Res 2017	Gallbladder carcinoma
Larsen	Int J Mol Sci 2019	Review
Lianidou	Crit Rec Clin Lab Sci 2014	Review
Liyanage	Anal Chem 2019	Early diagnostics
Lodewijk	Int J Mol Sci 2018	Review
Malentacchi	Urol Oncol 2016	Diagnosis of BC
Miyamoto	Lancel Oncol	Review

Supplementary Table 1. Overview of all reviewed articles (continued)

Author	Journal	Reason for exclusion
Murata	Environ Health Prev Med 2018	Review
Montironi	Front Oncol 2019	Editorial
Nagata	Dis Markers 2016	Review
Nicolazzo	J Cancer Res Clin Oncol 2017	Non muscle invasive bladder cancer
Palsgrove	Hum Pathol 2019	Variant UC
Pardini	Oncotarget 2018	Diagnosis of BC
Piao	Investig Clin Urol 2018	Review
Riethdorf	Transl Androl Urol 2017	Review
Springer	Elife 2018	Diagnosis of BC
Szarvas	Methods Mol Biol 2018	Review
Tan	BMC Cancer 2017	Diagnosis of MIBC from NMIBC
Thoma	Nat Rev Urol 2017	Review
Todenhofer	Bladder cancer 2018	Review
Usaba	Cancer Sci 2019	Diagnosis of BC
Vadasz	Urol Oncol 2018	Diagnosis of BC
Vlachosterigios	World J Urol 2019	Review
Yang	Crit Rev Oncog 2017	Review
Zeuschner	Expert Rev Mol Diagn 2019	Review
Zhan	Mol Cancer 2018	Diagnosis of BC
"bladder cancer" AND "circulating tumor cells": 66 hits		
Excluded: 9x double, 2x other language, 15x review, 1x other tumor type, 7x NMIBC / diagnosis BC, 1x metastatic BC, 4x pre-clinical study, 1x phase I study, 5x case study, 1x comment, 1x meta-analysis, 1x technical protocol, 1x no specific result for peri-operative management, 1x culture of CTCs, 6x outside scope, 2x earliest research bladder cancer		
Abrahamsson	**Urol Oncol 2017**	
Alva	**J Urol 2015**	
Chalfin	**Urology 2018**	
Guzzo	**Urol Oncol 2012**	
Naoe	**Cancer 2007**	
Soave	**Int J Cancer 2017**	
Todenhofer	**J Cancer Res Clin Oncol 2016**	
Winters	**Int J Urol 2015**	
Cegan	*Int J Clin Exp Pathol 2014*	*Outside the scope of this paper*
Leotsakos	*Anticancer Res 2014*	*Earliest research in CTCs bladder cancer*
Lima	*Urol Oncol 2017*	*Outside the scope of this paper*
Osman	*Int J Cancer 2004*	*Earliest research in CTCs bladder cancer*
Pagliarulo	*Clin Genitourin Cancer 2017*	*Outside the scope of this paper*
Retz	*Eur Urol 2001*	*Outside the scope of this paper*

Supplementary Table 1. Overview of all reviewed articles (continued)

Author	Journal	Reason for exclusion
Ribal	*Anticancer Res 2006*	*Outside the scope of this paper*
Soria	*J Urol 2002*	*Outside the scope of this paper*
Anatharaman	BMC Cancer 2016	4 MIBC patients --> case series
Azevedo	Urol Oncol 2018	Review
Balakrishnan	Sci Rep 2019	Case series for bladder cancer
Berrahmoune	J Urol 2009	NMIBC
Bersini	Oncotarget 2018	Pre-clinical study
Bobek	Methods Mol Biol 2018	article encompasses an technical protocol
Busetto	Clin Genitourin Cancer 2017	only T1 tumors included
Cegan	Folia Histochem Cytobiol 2017	Review
Chedgy	Urol Oncol 2016	Review
Cheng	PLoS One 2013	Pre-clinical study
Chi	Ann Oncol 2016	Phase I study, mainly prostate
Engilbertsson	J Urol 2015	Diagnosis of BC --> CTCs during TURB
Fina	Bladder Cancer 2016	Metastatic bladder cancer patients
Gazzaniga	Int J Cancer 2014	only T1 tumors included
Gazzaniga	Ann Oncol 2012	only T1 tumors included
Ju	Cancer Biol Ther 2014	Case study (2 patients)
Karl	Eur J Med Res 2009	5 patients --> case series
Katsumi	Nihon Hinyokika Gakkai 1972	Japanese
Kelloff	Urol Oncol 2015	Review
Kim	J Cancer 2019	Culture of CTCs
Kuppachi	Am J Transplant 2017	Case report
Lianidou	Crit Rev Clin Lab Sci 2014	Review
Liberko	Crit Rev Oncol Hematol 2013	Review
Matsumoto	Cancer Sci 2015	Pre-clinical study
Miremami	Int J Mol Sci 2014	Review
Nagata	Dis Markers 2016	Review
Nandagopal	Bladder Cancer 2016	Review
Nezos	Cancer Treat Rev 2009	Review
Nicolazzo	Oncologist 2019	NMIBC
Nicolazzo	J Cancer Res Clin Oncol 2017	only T1 tumors included
Noguchi	Int J Urol 1997	Prostate cancer
Qi	Tumour Biol 2014	no results for peri-operative patients
Raimondi	Expert Rev Mol Diagn 2014	Review
Roos	Bioanalysis 2010	Review
Roth	Oncotarget 2017	Pre-clinical study
Sanguedolce	Crit Rev Clin Lab Sci 2015	Review
Santoni	Fron Oncol 2018	Review

Supplementary Table 1. Overview of all reviewed articles (continued)

Author	Journal	Reason for exclusion
Schwentner	Curr Opin Urol 2012	Review
Soave	Urol Oncol 2018	Comment
Yang	Sichuan Da Xuq Xuq Bao Yi 2017	Article in Chinese
Zhang	Oncotarget 2017	Meta-analysis
"bladder cancer" and "circulating tumour cells": 10 hits		
Excluded: 1x other language, 2x double, 1x review, 1x case series, 1x NMIBC, 1x pre-clinical paper		
Fina	**Dis Markers 2017**	
Rink	**BJU Int 2011**	
Soave	**BJU Int 2017**	
Bocci	Integr Biol (Camb) 2019	Pre-clinical paper
Gorin	Nat Rev Urol 2017	Review
Gradilone	BJU Int 2010	T1G3 bladder cancer
Kolostova	Can Urol Assoc J 2014	Case series (8 patients)
Schilling	Aktuelle Urol 2011	German
"urothelial carcinoma" and "circulating tumor cells": 20 hits		
Excluded: 14x double, 2x metastatic BC, 2x review		
Flaig	**Urology 2011**	
Rink	**Eur Urol 2012**	
Apolo	Clin Genitourin Cancer 2017	metastatic bladder cancer
Bellmunt	Ann Oncol 2017	metastatic bladder cancer
Rink	Urologe A 2014	Review
Soave	Curr Urol Rep 2015	Review
"urothelial carcinoma" and "liquid biopsy": 16 hits		
Exclude: 13x double, 1x methods, 1x diagnostic paper, 1x review		
Marandino	Expert Rev Anticancer Ther 2019	Review
Mengual	Methods Mol Biol 2018	Methodological paper
Su	Oncotarget 2017	Fish to predict advanced bladder cancer

All papers included for this systematic review. All bold papers are discussed in this review, the italic papers were excluded based on full-text evaluation (n=10) and the rest of the papers were excluded based on title and abstract evaluation (n=127 and doubles).

Chapter 4

Androgen receptor expression in circulating tumor cells of patients with metastatic breast cancer

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ABSTRACT

The androgen receptor (AR) has potential clinical relevance in metastatic breast cancer (mBC) since it might be a treatment target and has been associated with endocrine resistance. A minimal-invasive way to determine AR expression on metastatic tumor cells is by characterization of circulating tumor cells (CTCs). Here, we assessed *AR* mRNA expression in CTCs (CTC-*AR*) and in matched primary tumor samples from mBC patients representing different breast cancer subtypes. In addition, we explored CTC-*AR*-status in relation to outcome on endocrine therapy. *AR,* and 92 *AR* or estrogen receptor (*ER*) related genes, were measured in CellSearch-enriched CTCs from 124 mBC patients and in 52 matched FFPE primary tissues using quantitative reverse-transcriptase PCR. *AR* in CTCs was considered positive if the expression was 1 standard deviation higher than the expression measured in 11 healthy blood donors. A total of 31% of the mBC patients had *AR*-positive (*AR*+) CTCs. 58% of the matched CTC and primary tumor samples were discordant with respect to *AR* status, observing both switches from *AR*+ to *AR*-negative (*AR*-) and vice versa. There was no statistically significant difference in progression-free survival for patients treated with ER-targeting drugs and CTC-*AR*-status (13 *AR*+ / 37 *AR*- cases, p=0.28). Thus, *AR* can be determined in RNA isolated from CTCs, with in our set 31% *AR*-positive samples. Given the discordance between *AR* status in CTC samples and corresponding primary tumors, determination of AR expression in CTCs might be a promising tool to select mBC patients for AR inhibiting agents.

1. INTRODUCTION

The successes of estrogen-receptor (ER) and human epidermal growth factor receptor 2 (HER2)-targeting drugs have substantially increased overall survival (OS) of breast cancer (BC) patients (1,2). This emphasizes the importance of identifying targets for the treatment for breast cancer patients. Alongside these targets, the androgen receptor (AR) is subject of investigation in BC. The AR, which is known as an important target in prostate cancer, is a nuclear transcription factor that is activated by binding of androgens and is a member of the steroid-hormone receptor family (3), like ER and the progesterone-receptor (PR).

The AR-protein is expressed in about 80% of the primary breast cancer tumors. The highest expression levels have been reported in ER-positive tumors (about 75-85% positivity), followed by 60% of the HER2-positive tumors and about 30% of the triple negative breast cancers (TNBC) (4-6). The AR may be of interest in breast cancer for two reasons; 1) several studies have shown that the presence of AR protein in ER-positive BC is associated with endocrine resistance (7-9) and 2) the AR could potentially be a new target for treatment (3, 10-12).

With respect to the latter, AR-targeting drugs that are commonly prescribed in prostate cancer patients such as bicalutamide, abiraterone and enzalutamide, have also been tested in metastatic breast cancer (mBC) patients. All studies performed in mBC so far, included patients based on immunohistochemical (IHC) determination of the AR status on primary or metastatic tumor tissues. These studies have shown that mBC patients can derive clinical benefit from AR-targeted therapies, although the reported outcomes were quite modest with clinical benefit rates (CBR) at 6 months (responses and stable disease lasting longer than 6 months) around 20-30% and response rates of 6-8% (3,10,12-15). Collectively, these data show that AR-targeting drugs are only active in a subset of patients, stressing the need for biomarkers to identify these patients. Additionally, there is a 35% discrepancy rate between AR expression in primary breast cancer and metastatic tissue (16) rendering it questionable whether assessment of AR expression on primary tumor tissue is appropriate to select mBC patients for AR-targeting drugs.

In the most optimal setting, the AR-status of the patient should be assessed directly prior to start of the therapy on metastatic lesions. However, taking biopsies from solid metastases is a cumbersome procedure for patients and is not always possible because of inaccessibility of metastatic lesions. Therefore, a method to determine an up-to-date AR-status in a minimally invasive manner is necessary and could be achieved through the characterization of circulating tumor cells (CTCs) from blood. Importantly, it has already been shown that the characteristics of CTCs resemble that of the metastasis better than that of the primary tumor (17). And since CTCs are easily accessible through a simple venipuncture, this procedure allows for repetition during follow-up to assess changes in a tumor's molecular characteristics over time and during treatment.

In this study, we determined the AR-status of CTCs at the mRNA level in a relatively large series of patients with mBC. We assessed whether the AR-status differed across the different breast cancer subtypes and determined the discrepancy in AR-status between matched primary tumors and CTC samples. Furthermore, the impact of the CTC-*AR*-status on outcome to endocrine therapy was examined in a subset of the patients. Lastly, other relevant genes in the AR- and ER-pathways were investigated to identify genes that correlate with AR expression in mBC.

2. MATERIALS AND METHODS

2.1. Patients

For this study, 140 patients were retrospectively selected from two prospective clinical trials that have been conducted in six hospitals in the Netherlands and Belgium from 2006 till 2015. Both studies enrolled mBC patients who started with a new line of endocrine therapy or chemotherapy (06-248 and 09-405 study) (18-20). Blood for enumeration and molecular characterization of CTCs was collected of all patients before start of this new line of therapy. For characterization, the quantity and quality of the RNA was assessed for every sample (see below for more information). After processing, the epithelial signal of every sample was calculated by summing the ΔC_q signal of 12 genes - which were previously identified to associate with epithelial tumor load (21) (see ***Supplementary Table 1***). Samples with an epithelial signal <-131 were considered ineligible due to no epithelial mRNA present in the sample. For 9 samples, not all 12 genes were determined. Therefore, the epithelial signal was tested in these samples based on the ΔC_q sum of *KRT19* and *EpCAM*, which should be > -15. This -15 cut-off correlated with the -131 of the 12-gene epithelial signal (see ***Supplementary Figure 1***). Of the 140 patients, 12 patients were ineligible due to insufficient quantity or quality of the RNA used for this study and 4 patients had insufficient epithelial signal. In total, 124 patients were included, of which 128 blood samples were available (see ***Table 1*** for more detailed clinical background). The medical ethical commission of the Erasmus MC and the institutional boards of all participating centers approved both studies (MEC-06-248 and MEC-09-405). All patients provided written informed consent. Clinical data was collected from all patients and the ER-, PR- and HER2-status of the primary tumor was collected from the pathology reports. Based on the receptor data of the primary tumor, patients were divided into four categories: 1) ER+ and HER2-, 2) ER+ and HER2+, 3) ER- and HER2+, 4) TNBC. A total of 50 patients received ER-targeted drugs after characterization of the CTCs. This comprised a very heterogeneous group of patients receiving either tamoxifen (n=15), aromatase inhibitors (n=31) or fulvestrant (n=4). These therapies were given in different lines of treatment: 1st line (n=40), 2nd line (n=6) and 3rd line (n=4).

Table 1. Clinical data of all eligible patients (n=124)

	***AR*-negative (n = 85)**	***AR*-positive (n = 39)**	**p-value**
Median age (range)	64 (33-87)	60 (35-83)	
ER			*0.567*
Positive	65 (77%)	33 (85%)	
Negative	18 (21%)	6 (15%)	
Unknown	2	0	
PR			*1.000*
Positive	47 (55%)	23 (59%)	
Negative	33 (39%)	15 (39%)	
Unknown	5	1	
HER2			*1.000*
Positive	19 (22%)	9 (23%)	
Negative	60 (71%)	28 (72%)	
Unknown	6	2	
Neo-adjuvant therapy			
Chemotherapy	3	2	
Endocrine therapy	0	0	
Targeted therapy	0	0	
Adjuvant therapy			*0.333*
Chemotherapy	29	17	
Endocrine therapy	33	22	
Targeted therapy	3	3	
Therapy Inclusion			
Chemotherapy	49	26	
Endocrine therapy	35	12	
Targeted therapy	1	1	

Clinical data of the patients included in the study (n=124) by the AR status of the CTCs. The p-values have been calculated with the Chi-square method. For neo-adjuvant therapy, no p-value is given due to the low numbers in this group.

2.2. Sample processing for gene expression profiling

For the enumeration and characterization of CTCs, respectively 7.5 mL of CellSave and 7.5 mL EDTA blood were processed using the CellSearch system (CellSearch enumeration kit and CellSearch profile kit, Menarini-Silicon Biosystems, Huntington Valley, USA). After enrichment of CTCs, RNA was isolated with the AllPrep DNA/RNA Micro Kit (Qiagen, Germantown, MD, USA) and cDNA was generated, pre-amplified for the targets of interest and real time amplified (RT-qPCR) using Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA) (***Supplementary Table 2a***). A more detailed description how RT-qPCR data were generated has been published previously (21-23).

From 52 patients matched formalin-fixed paraffin embedded (FFPE) tissue from the primary tumor with at least 30% invasive tumor cells was available (median (range); 60% (30%-85%)). RNA from the FFPE samples was isolated with the High-Pure RNA Paraffin Kit (Roche Applied Science, Penzberg, Germany) and the quality and quantity of the RNA was checked with the Nanodrop 1000-v.3.7 (Thermo Scientific, Wilmington, USA), the MultiNA Microchip Electrophoresis system (Shimadzu, Kyoto, Japan), and three reference genes (*GUSB, HMBS* and *HPRT1*) as described before (24).

2.3. Normalization and AR cut-off

Expression levels of the 93 genes in the CTC and tumor samples were normalized by the average C_q value of three reference genes (ΔC_q). If the average C_q value of the reference genes (*GUSB*, *HMBS* and *HPRT1*) was >27.5 in a sample, this sample was considered to have insufficient RNA quantity and/or quality. If the quantity and/or quality of the RNA was insufficient the sample was excluded (n=12, see above). To determine a cut-off for AR and the other genes measured, every individual gene was also measured in blood samples of 11 healthy blood donors (HBDs). To correct for the leukocyte background which is also present in HBD samples, the HBD samples were also enriched by the CellSearch system. The mean ΔC_q value of each gene measured in the HBDs was calculated. If the expression of a specific gene was below the HBD ΔC_q in a CTC or tumor tissue sample, it was considered not detectable. The cut-off for a gene to be considered positive in the CTC or tumor samples was set at one standard deviation higher (+1 SD) than the ΔC_q of the HBDs.

The cut-off for CTC-*AR* at the mRNA level has been compared with the AR-status as determined by IHC on 7 FFPE samples and 12 cell lines.

2.4. Sample processing and AR-IHC

To confirm the validity of the cut-off used to determine the *AR*-status on CTCs, several control experiments were conducted. For this purpose, FFPE tissue of the primary tumor was collected of seven patients. These tumors were stained for AR using standard immunohistochemical techniques with the AR F39.4 protein (dilution of 1:2000), as described before (25) and scored for AR-positivity. The cut-off for AR was set at 10% (nuclear staining), so ≥10% nuclear staining was considered positive and tumors were scored negative if < 10% of tumor cells were stained.

Also 12 different cancer cell lines were added to a cell line microarray (CMA) and this CMA was stained for AR with the same antibody. The cut-off for positivity in the CMA was also set at 10%.

2.5. Gene expression profiles

To analyze downstream markers of AR, a gene panel of 205 genes, based on representation in seven important pathways in breast cancer (the AR-, ER-, FOXO-, HH-, NFKB-, TGFB- and WNT-pathway) (26) was used. Because a background of leukocytes remains present after isolation of CTCs, out of these 205 genes, only those with a higher expression in breast cancer cells than in leukocytes according the SAGE Genie Database of the Cancer Genome Anatomy Project (https://cgap.nci.nih.gov/SAGE/AnatomicViewer) were selected for our CTC *AR* Breast gene expression panel. Therefore, including three reference genes (*GUSB, HMBS, HPRT1*), 96 genes were selected for assay validation. In this set of genes, we selected the CTC specific genes in a subset of samples with > 5 CTCs (n =122). Of the 93 genes (without reference genes), 22 genes, including *AR*, had a significantly higher expression in CTCs than in leukocytes ($p \leq 0.05$, see ***Supplementary Table 2b***). *ESR1* was borderline not significant ($p=0.07$), but since its relevance in the AR- and ER-pathways it was included in our final analysis. So in total, 22 genes were used to investigate the association with *AR* in all the CTC samples.

2.6. Statistical analysis

Differences in *AR* expression levels between the different clinical categories were tested with the chi-square test. This test was also used to compare the clinical data and *AR* expression. The comparison of the *AR* status between matched CTC and FFPE samples was performed with the Wilcoxon signed rank test. For all patients, time from start of treatment till progression (progression-free survival (PFS)) was determined. Patients who did not have progression were censored at the last date of contact (with a maximum of 40 months). Patients who developed a secondary breast cancer during treatment, were censored at the moment of diagnosis of this secondary tumor. One patient was excluded for survival analysis due to the development of an active cholangiocarcinoma during follow up. Survival analysis in the subgroup of patients who received endocrine therapy (n=50) were performed using the Cox proportional hazards model and were visualized with the Kaplan Meier method. We performed this analysis for all patients who were treated with endocrine therapy, but also for patients treated with endocrine therapy only in the first line (n=40). Associations between *AR* and other genes in CTC samples were investigated using the Mann Whitney U test. For the CTC sample analyses the p-value was divided by 22 (since we correlated 22 genes with *AR*). So for genes to be associated with CTC-*AR*-positive or CTC-*AR*-negative samples, the p-value should be $p<0.0023$. Of the 18 genes that were associated with *AR* expression, we reviewed per gene if there was a higher expression in *AR*-positive or *AR*-negative samples by comparing the median ΔC_q values of the *AR*-positive and *AR*-negative patients. All computations were performed using R (version 3.4.1) and all reported *P*-values are two-sided.

3. RESULTS

3.1. Determination of AR cut-off

The cut-off for positivity for mRNA-*AR* was set at -3.03, being 1 SD higher than the ΔC_q of the HBDs. To confirm the validity of this cut-off, this cut-off was tested in 12 cell lines and 7 FFPE samples of which both IHC and mRNA expression data were available. Of the 4 cell lines with *AR* expression below the established cut off, 3 were AR-negative according to IHC staining for AR while in 7 cell lines with positive mRNA-*AR* according to our cut-off, AR protein was indeed detected in all cell lines in more than 10% of the tumor cells. The ZR75.1 cell line was borderline AR-negative on protein level, however in literature this cell line has also been reported to be AR-positive (27). Also in our hands, the AR-status at the mRNA level was inconclusive (in a measurements of two different cultures, one was positive (ΔC_q 0.71) and one was negative (ΔC_q -3.55, data not shown)). All 7 FFPE samples from primary breast cancers were concordant with respect to *AR* status determined at mRNA levels by using our cut-off and AR protein expression determined by IHC (1 positive and 6 negative). This validated cut-off for *AR* was therefore applied to all evaluable CTC and FFPE samples. (For all cut-off validation data see ***Supplementary Table 3***.)

3.2. AR expression in CTCs from different breast cancer subtypes

In total, 124 patients were retrospectively selected for this study. Detailed information on their clinical characteristics is shown in ***Table 1.*** Of the 124 patients, 128 samples were collected. Of the 128 samples, 40 (31%) samples were CTC-*AR*-status positive. There was no difference in prior (neo)adjuvant treatments administered between the *AR*-negative and *AR*-positive groups.

Table 2. Breast cancer subtypes and *AR* expression (128 samples)

Subtype	Description	
ER+ HER2-	ER+, any PR, HER2-	
ER+ HER2+	ER+, any PR, HER2+	
HER2+	ER-, PR-, HER2+	
TNBC	ER-, PR-, HER2-	
Subtype	**Number of patients**	**N (%) AR positivity**
ER+ HER2-	75	27 (36%)
ER+ HER2+	20	5 (25%)
HER2+	8	4 (50%)
TNBC	16	2 (13%)
Total*	119	38 (32%)

A) Breast cancer subtypes determined on the status of the primary tumor. B) Number of patients with AR-positive CTCs in the different breast cancer subtypes. *For 9 of the 128 samples, the pathological subtype was unknown.

When comparing the different breast cancer subtypes in the 128 samples included in the study (see ***Table 2***), no statistical difference was found in the CTC-*AR*-expression status between those subtypes: in the ER+/HER2- group 36% (27/75) of the patients showed CTC-AR positivity, in the ER+/HER2+ group 25% (5/20), in the ER-/HER2+ group 50% (4/8) and in the TNBC group 13% (2/16) (p=0.17). When looking at the ER+ (n=102) and ER- (n=24) subgroups, there was also no difference with respect to *AR*-status (p=0.59).

3.3. AR expression in CTCs vs primary tumors

From 52 patients, a CTC sample and a matched FFPE tissue of the primary tumor was available. In 44 of the 52 primary tumors (85%) positive *AR* expression was detected, while only 16 (31%) of the corresponding CTC samples were *AR*-positive. In 22 of the 52 samples (42%) the *AR*-status was concordant between the primary tumor tissue and the CTC sample (Wilcoxon signed rank p<0.001, see ***Table 3***). In 30 samples (58%) the AR-status between the FFPE sample and corresponding CTC sample was discordant. In the discordant samples, most patients had an *AR*-positive primary tumor but an *AR*-negative CTC sample. One patient with an *AR*-negative primary tumor ($\Delta C_{q<}$ -3.03) had *AR*-positive CTCs (ΔC_q -2.17).

Table 3. AR expression in matched CTC and primary tumor samples (n=52)

	CTC AR+	CTC AR-	*Total*
Primary tumor AR+	15	29	*44*
Primary tumor AR-	1	7	*8*
Total	*16*	*36*	*52*

Correlation between the AR-status of the CTC samples and matched FFPE samples of the primary tumor. Tested with Wilcoxon signed rank test: p<0.001.

3.4. AR expression and response to endocrine therapy

A subset of the mBC patients (n=50) was treated with 1st, 2nd or 3rd-line ER-targeting therapy directly after the CTC sample was obtained. Of these, 13 patients (26%) were *AR*-positive and 37 (74%) *AR*-negative. The median PFS in all ER treated patients was 9 months (range 0-40 months). In *AR*-positive patients, the median PFS was 10 months and in *AR*-negative patients 9 months. When comparing the PFS of the *AR*-positive patients and the *AR*-negative patients in survival analysis, no difference was observed between these groups (HR 1.47, 95% CI 0.74-2.93, p=0.28) (***Figure 1***).

Likewise, in the 40 patients who received first-line ER-targeting therapy, no difference in outcome to ER-targeting therapy by *AR*-status was detected (HR 1.21, 95% CI 0.57-2.57, p=0.63). Of these patients, 12 were *AR*-positive and 28 *AR*-negative. In the *AR*-positive patients the median PFS was 10 months and in the *AR*-negative patients 9 months.

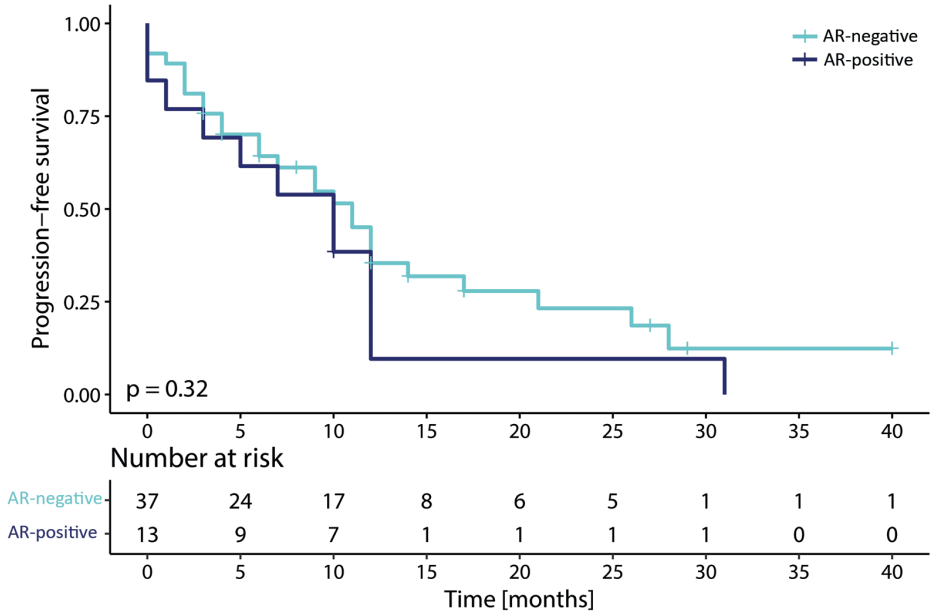

Figure 1. Progression-free survival (PFS) in n=50 endocrine treated patients
PFS as function of AR expression in 50 CTC samples from patients treated with ER-targeting therapy.

3.5. AR-related genes

Assessing the 23 CTC specific genes in all 128 samples, we found 18 genes to be significantly differentially expressed between the *AR*-positive and *AR*-negative CTC samples (Mann-Whitney U: p<0.0023 after correcting for multiple testing): *EPCAM, KRT19, ANGPTL4, CA12, CCND1, CELSR2, ERBB2, FOXM1, FAT1, GLI3, OVOL1, SOX9, TACC2, TBX3, TFF1, TMPRSS2, XBP1* and *ESR1*. All of these genes had higher expression in AR-positive samples *(see* ***Supplementary Table 4***). Looking at the function of the genes higher expressed in *AR*-positive samples (including *AR* itself) using DAVID (28), the function that stands out (after Benjamini correction for multiple testing) is transcription. Hierarchical clustering of the CTC specific genes was also performed (see ***Supplementary Figure 2***). As shown in the cluster, *BNIP3, CELSR2* and *PPARG* appeared inversely related to *AR* expression. Still, only *CELSR2* expression levels showed a significant correlation to AR expression levels (R=0.53, p<0.001). Thus in the CTCs, *BNIP3*, *CELSR2* and *PPARG* are likely together the least related to AR and as a result show up on the opposite side in the clustering.

Of the CTC specific genes, *TMPRSS2* and *TACC2* are known to be involved in the AR-pathway (***Supplementary Table 2***). *TACC2* was expressed in 88% of the AR+ CTC samples and in 69% in the AR- CTC samples. *TMPRSS2* expression was present in 58% of the AR+ CTC samples and in 32% of the AR- CTC samples.

4. DISCUSSION

This study showed that *AR* expression can be detected in 31% of the CTC samples of mBC patients, providing the opportunity to measure the *AR* status in patients in a minimal invasive way right before the start of a new line of therapy. Furthermore, we revealed a substantial discordance (of 58%) in *AR* expression between the primary tumors compared to the CTC samples.

Two other studies also investigated AR expression in CTCs of mBC patients. Both studies were smaller and both used IHC to measure AR-protein expression instead of mRNA. The study of Li *et al*. (15) looked at AR-protein expression in 75 samples with ≥ 3 CellSearch-enriched CTCs. If patients had nuclear fluorescence for AR, they were considered positive. Here, 60 patients (80%) had AR-positive CTCs at baseline. Fujii *et al*. (29) collected whole blood from mBC patients with mostly hormone receptor (HR)+/HER2- primary tumors. They used the Epic Sciences CTC platform to select patient samples with ≥1 CTC on the cytospin. The expression of AR was investigated by IHC staining in 51 patients. Of these patients, 10 patients (20%) had AR expression. The differences in CTC-AR expression are most likely due to the techniques used to determine the AR status. There are several disadvantages of using IHC to determine the AR status on primary tumors, metastatic lesions or CTC samples. First, different antibodies are being used to measure the AR status and different cut-offs to determine AR-protein expression (positive AR expression defined as >0%, ≥1% or ≥10% of the tumor cells being positive) (16). Also, AR-protein expression may be a suboptimal marker for response to AR-targeting therapy since gene expression models seem to predict response to enzalutamide more accurately (30, 31).

Concerning the discrepancy between the AR status of the primary tumor and CTCs, in our study, we found 85% of the primary tumors positive for *AR*, which is concordant with the literature (16, 32). In the study of Bronte *et al*. (16), AR expression was determined on FFPE tissue of the primary tumor and metastatic lesion with IHC. A tissue was positive if it had ≥1% IHC AR staining. In 17 patients with matched primary tumor and metastatic tissues, a 35% discordance rate was observed. A similar discordance rate has been observed between primary tumors and CTCs by Li *et al*. (15). They reported a discordance rate of 32% (24 of the 75 samples) between the AR-status determined on the primary tumor (>10% IHC AR staining) and the CTC sample. There were both losses (11 of 58 patients (19%)) and gains (13 of 17 patients (76%)) of AR over time. Fujii *et al.* (29) reported on a small series of 7 mBC patients with AR+ CTCs. Of these, 4 also had an AR-positive primary tumor (>10% IHC AR staining) and 3 had an AR-negative tumor at diagnosis (≤10% IHC AR staining). In our study, a discrepancy of 58% was found between the primary tumor and the CTC AR-status. The differences in discrepancy percentages are again most likely due to the used techniques for AR determination. However, these

data do suggest that the AR-status of a tumor may change over time and/or under the influence of treatment. This indicates that for identifying metastatic patients eligible for AR-targeting therapy, it is crucial to know the AR-status of the metastatic lesion rather than that of the primary tumor.

In literature, the highest percentages of AR-positive tumors were seen in ER+ primary breast cancers with lower prevalence in tumors belonging to the HER2+ and triple negative tumors, respectively. To explore if the same holds true for *AR* expression in CTCs, we investigated the association of primary tumor subtype and *AR* expression status of CTCs, but observed no statistical difference between the subtypes and *AR* expression in CTCs. More CTCs were positive for *AR* than expected, especially in the HER2+ subtype. Even amongst the TNBC patients, 13% had AR+ CTCs, implying that the CTC-*AR*-status may help guide treatment selection in all molecular subtypes.

To gain insight into genes differentially expressed between *AR*-negative and -positive CTCs, we analyzed the gene expression data of the CTC samples for pathway analysis. Of the 22 CTC specific genes, 18 genes were associated with *AR* expression (*EPCAM, KRT19, ANGPTL4, CA12, CCND1, CELSR2, ERBB2, FAT1, FOXM1, GLI3, OVOL1, SOX9, TACC2, TBX3, TFF1, TMPRSS2, XBP1* and *ESR1)*. Of these genes, *TACC2* and *TMPRSS2* are part of the AR-pathway (33, 34). Both genes had higher expression in the *AR*-positive samples compared to the *AR*-negative samples, which implies that when *AR* is expressed, the AR-pathway is activated. *XBP1*, *ERBB2, CELSR2, ESR1, TFF1* and *CA12* are genes that are known to be regulated by the ER-pathway. All of these genes were also higher expressed in *AR*-positive CTCs. Therefore, it is likely that the ER-pathway is connected to *AR* expression.

A limitation of the approach to determine gene expression in CellSearch-enriched CTCs, is the high leukocyte background still present after the enrichment of CTCs using the CellSearch system. If the expression of a gene is equal to or lower than the expression levels in the leukocytes, this gene cannot be measured in the enriched CTC samples. Therefore, the optimal analysis to gain insight into which genes are activated in *AR*-positive samples should be performed on pure and/or single CTCs. Single CTC analysis does not only eliminate the leukocyte background, but it also provides the opportunity to assess heterogeneity in *AR* expression and its downstream targets within a single patient.

Another limitation of our study is the heterogeneous group of patients which were treated with ER-targeting therapy. Cell line data does suggest that resistance against ER-targeting therapy is associated with both increased androgen and estrogen receptor expression (7). This could mean that patients will have a worse response on ER-targeting therapy if they do express *AR*. Our study is the first to analyze the impact of CTC-AR-expression in mBC patients and outcome to endocrine therapy, but we did not find evidence that patients with AR-positive CTCs have worse outcome to endocrine therapy.

However, since we had a relatively small sample size and heterogeneous group of patients, we cannot draw firm conclusions yet based on this data. So, to ascertain the true clinical relevance of the *AR*-status in patients treated with ER-targeting drugs, this study should be repeated in a larger, homogeneous cohort of patients.

In conclusion, we show that *AR* expression can be detected in CellSearch-enriched CTCs. The presence of *AR* was less prevalent in CTCs than in the corresponding primary tumors and primary tumor *AR*-status is not predictive for that in CTCs. Since the characteristics of metastatic tumor cells are likely of greater value for treatment selection in the metastatic setting than tumor cells of the primary tumor, we expect that establishing the *AR*-status of CTCs will be a valuable, minimally invasive tool, to select mBC patients who may benefit from AR-targeting agents.

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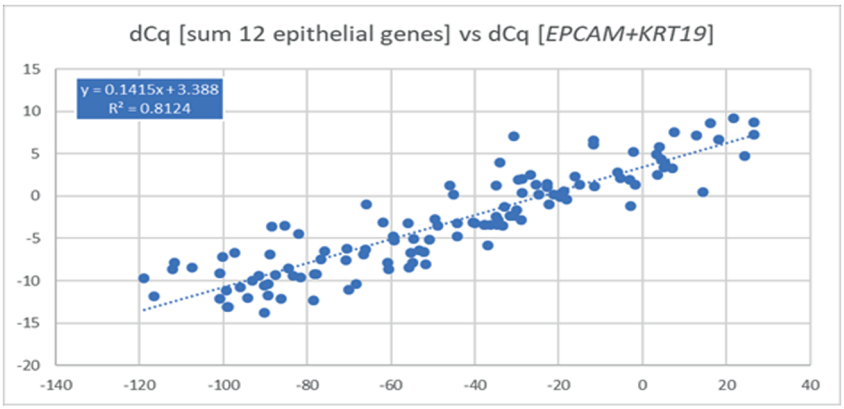
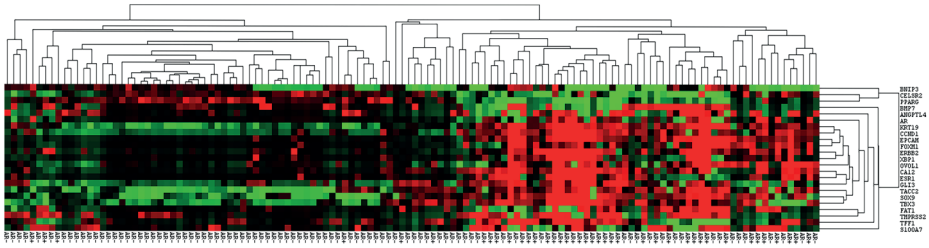
SUPPLEMENTARY MATERIALS



Supplementary Figure 1. Correlation of the epithelial signal methods
Correlation between the two methods used to calculate the epithelial signal. In most samples, the sum of 12 genes was used to determine the epithelial signal, however in 9 samples not all 12 genes were measured. Therefore, EPCAM and KRT19 were used in these samples to determine the epithelial signal. Based on this correlation, a cut-off of -15 was chosen for the EPCAM+KRT19 method.



Supplementary Figure 2. Unsupervised hierarchical cluster of the 23 CTC specific genes
Unsupervised hierarchical clustering of the 23 CTC specific genes in all 128 samples. Expression levels were normalized and the genes were centered on the median. Each row represents a gene, each column corresponds to a sample. The AR status of the sample is shown at the bottom. Red color indicates mRNA expression above the median level, black is the median expression level and the green color an expression level below median of the assay in these 128 samples.

Supplementary Table 1. Genes to determine epithelial signal

Genes			
AGR2	*EPCAM*	*KRT19*	*S100A16*
CLDN3	*FOXA1*	*KRT7*	*SEPP1*
CRABP2	*IGFBP5*	*LAD1*	*SPDEF*

The 12 genes that were used to measure the epithelial signal. Based on the sum of average expression of these genes an epithelial score was calculated in the CTC sample to indicate presence of CTCs. This epithelial signal score should be > -131 for a sample to be evaluable for the detection of AR and AR related genes. If the score is <-131 the sample was excluded.

Supplementary Table 2. Genes measured in CTC samples

A			Pathways				
AR	***ER***	***FOXO***	***HH***	***NFKB***	***TGFB***	***WNT***	***Extra***
ABCC4	CA12	BNIP3	BCL2	MMP9	ANGPTL4	ADRA2C	EPCAM
AR	CELSR2	CAV1	*CCND1*		CDKN2B	BMP7	FASLG
DRG1	COL18A1	*CCND1*	*CCND2*		CTGF	*CCND1*	PTPRC
IGF1	DSCAM	*CCND2*	FOXA2		GADD45A	COL18A1	SLC7A5
KLK3	EBAG9	CCNG2	FOXF1		HMGA2	DKK1	MAPT
LRIG1	ERBB2	ESR1	FOXL1		ID1	EPHB2	APOBEC3B
NKX3_1	ESR1	FBXO32	FOXM1		IL11	FAT1	SSTR2
NTS	GREB1	GADD45A	FST		MMP2	FZD7	GRPR
PMEPA1	KRT19	INSR	GLI1		MMP9	KIAA1199	
PTPN1	PGR	NOS3	GLI3		NKX2_5	LECT2	
TACC2	TFF1	POMC	H19		OVOL1	LEF1	
TMPRSS2	WISP2	PPARGC1A	JAG2		PDGFB	NKD1	
UGT2B15	XBP1	TNFSF10	MYLK		PTHLH	PPARG	
			NKX2_2		SMAD4	SOX9	
			PTCH2		SMAD5	TBX3	
			S100A7		SMAD6	TCF7L2	
			TSC22D1		SMAD7		
					SNAI1		
					SNAI2		

B		CTC specific genes		
CELSR2	*BNIP3*	*CCND1*	*KRT19*	*AR*
EPCAM	*BMP7*	*CA12*	*SOX9*	*TACC2*
ANGPTL4	*ERBB2*	*OVOL1*	*TFF1*	*TBX3*
FOXM1	*FAT1*	*PPARG*	*S100A7*	*GLI3*
XBP1	*TMPRSS2*	*ESR1*		

A) Panel of genes involved in AR- and ER-related pathways. Only genes with low expression level in leukocytes were measured in the CTCs. The genes that were significantly higher expressed in CTC samples than in healthy blood donors were included in the gene panel for further analyses. This resulted in 23 CTC-specific genes (B). CCND1, ESR1, CCND2, GADD45A, MMP9 and COL18A1 are added to multiple pathways, therefore 100 genes in total are sown, but 93 unique ones.

Supplementary Table 3. Cut off AR gene celline and FFPE data

A	Cell line	ΔCq AR spiked in HBD	ΔCq AR not spiked; CellSearch enriched	HBD corrected cut off for mRNA (-3.03)	Final score mRNA data	Vectra average positivity (%)	Average Quantity MT (%)	Literature and/or IHC final score
	BT20	-15.00		below cut off	*negative*	0.83	0.00	*negative*
	PC3	-15.00	-10.90	below cut off	*negative*			*negative*
	MCF7	-4.71		below cut off	*negative*	10.43	36.67	positive
	SUM149	-3.22		below cut off	*negative*	1.33	0.00	*negative*
	ZR75.1	0.71	0.77	above cut off	*positive*	1.34	0.00	??
	MM453	-0.12	1.92	above cut off	*positive*	29.46	53.33	*positive*
	CAMA1	0.46	0.84	above cut off	*positive*	21.61	60	*positive*
	22RV1	60	1.76	above cut off	*positive*			*positive*
	MM415	0.89	1.58	above cut off	*positive*	7.93	20	*positive*
	LNCaP	1.89	2.37	above cut off	*positive*			*positive*
	SUM185PE	1.9	3.41	above cut off	*positive*	46.37	86.67	*positive*
	VCaP	4.44	5.11	above cut off	*positive*			*positive*

B	Tumor	AR IHC	AR IHC duplicate	% tumor cells	ΔCq AR mRNA	mRNA AR score
	1	negative	negative	35	-4.21	negative
	2	negative	negative	35	-5.30	negative
	3	negative	negative	<30	-4.92	negative
	4	positive	positive	30	1.12	positive
	5	negative		<30	-3.99	negative
	6	negative	negative	<30	-3.54	negative
	7	negative		30-50	-15.00	negative

(**A**) Comparison of the mRNA level (ΔCq) and the protein level (IHC) of AR, determined in 12 cell lines.
(**B**) Protein level (IHC) and mRNA level (ΔCq) of AR, determines in 7 primary tumors.

Supplementary Table 4. Comparison of *AR* with other CTC specific genes

Gene name	p-value	Median in AR-	Median in AR+
ANGPTL4	0.0004833	-12.47	-8.2
EPCAM	1.38E-11	-3.32	-0.71
KRT19	2.59E-12	-1.98	2.69
BMP7	*0.1417*		
BNIP3	*0.9873*		
CA12	6.17E-11	-5.79	-1.38
CCND1	1.23E-10	-4.68	-0.36
CELSR2	8.85E-05	-11.67	-9.69
ERBB2	1.71E-12	-5.45	-0.92
ESR1	2.21E-07	-2.06	-1
FAT1	3.93E-07	-7.24	-4.06
FOXM1	3.29E-09	-2.84	-1.32
GLI3	8.78E-07	-8.35	-3.44
OVOL1	9.63E-09	-10.19	-5.96
PPARG	*0.0337*		
S100A7	*0.004766*		
SOX9	6.05E-09	-8.19	-2.57
TACC2	9.71E-09	-6.17	-1.63
TBX3	8.30E-08	-7.86	-3.01
TFF1	7.04E-06	-13.91	-7.28
TMPRSS2	6.14E-05	-15	-6.27
XBP1	4.64E-11	-1.89	-0.85

Comparison of AR with other CTC specific genes, determined with Mann-Whitney U test. P-values were corrected for multiple testing, so samples with $p<0.0023$ are differentially expressed between the AR-positive and AR-negative samples. Of the genes that are differentially expressed, the median value of each gene in the AR-positive and AR-negative samples was calculated to determine if the genes had higher expression in the AR-positive or AR-negative samples.

Chapter 5

Circulating Tumor Cell Enumeration and Characterization in Metastatic Castration-Resistant Prostate Cancer Patients Treated with Cabazitaxel

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ABSTRACT

Background: Markers identifying which patients with metastatic, castration-resistant prostate cancer (mCRPC) will benefit from cabazitaxel therapy are currently lacking. Therefore, the aim of this study was to identify markers associated with outcome to cabazitaxel therapy based on counts and gene expression profiles of circulating tumor cells (CTCs).

Methods: From 120 mCRPC patients, CellSearch enriched CTCs were obtained at baseline and after 6 weeks of cabazitaxel therapy. Furthermore, 91 genes associated with prostate cancer were measured in mRNA of these CTCs.

Results: In 114 mCRPC patients with an evaluable CTC count, the CTC count was independently associated with poor progression-free survival (PFS) and overall survival (OS) in multivariable analysis with other commonly used variables associated with outcome in mCRPC (age, prostate specific antigen (PSA), alkaline phosphatase, lactate dehydrogenase (LDH), albumin, hemoglobin), together with alkaline phosphatase and hemoglobin. A five-gene expression profile was generated to predict for outcome to cabazitaxel therapy. However, even though this signature was associated with OS in univariate analysis, this was not the case in the multivariate analysis for OS nor for PFS.

Conclusion: The established five-gene expression profile in CTCs was not independently associated with PFS nor OS. However, along with alkaline phosphatase and hemoglobin, CTC count is independently associated with PFS and OS in mCRPC patients who are treated with cabazitaxel.

1. INTRODUCTION

The number of treatment options for metastatic castration-resistant prostate cancer (mCRPC) have increased rapidly in the last decade. Multiple treatment options are now available for mCRPC patients, including the chemotherapeutic agents docetaxel and cabazitaxel, the endocrine therapies abiraterone and enzalutamide, and options such as Radium-223 (1–7). Therefore, biomarkers able to predict outcome to a particular treatment are necessary to select the right drug for the right patient at the right time. As the optimal treatment sequencing is currently unclear, one of the agents that would benefit from biomarkers for predicting which patients will benefit from that therapy is cabazitaxel, which is commonly given after progression to docetaxel in mCRPC patients.

The enumeration and characterization of circulating tumor cells (CTCs) hold great promise as predictive biomarkers for guiding optimal treatment sequencing. CTCs are cells that are shed from the tumor and/or metastatic lesions and can be isolated from the peripheral blood. In prostate cancer, the enumeration of CTCs is a strong independent prognostic factor for progression-free survival (PFS) and overall survival (OS) both before and during therapy, as shown in multiple studies (8–11). However, the prognostic value for patients treated with cabazitaxel has only been analyzed in a small series of 56 patients (12).

Besides the enumeration of CTCs, characterization of these CTCs can help in predicting therapy response. One of the biomarkers that may carry predictive value is the androgen receptor (AR) splice variant 7 (AR-V7). Prior research has shown that AR-V7-positive patients benefit less from endocrine treatment than those who are AR-V7-negative (13), but that the AR-V7 status does not influence response to chemotherapy (14,15).

The advantage of CTCs is that they can be obtained in a minimally-invasive manner and mRNA of these cells can be isolated and analyzed. Therefore, gene expression profiles containing multiple genes can be measured in CTCs, which might be helpful in predicting outcome to therapies.

Here, we aimed to identify markers associated with outcome to cabazitaxel therapy. To this end, we enumerated CTCs to confirm the prognostic value of CTC counts in mCRPC patients treated with cabazitaxel. Furthermore, we aimed to identify a gene expression profile determined in CTCs that is associated with outcome in mCRPC patients treated with cabazitaxel therapy.

2. RESULTS

2.1. Patient Characteristics

As shown in ***Table 1***, the median age of all 120 evaluable patients was 69 years (range 49–82 years). Only patients with a WHO performance score of 0 or 1 were included in the study. All patients had been previously treated with docetaxel. Only one patient received another line of chemotherapy besides docetaxel in a trial. In total, 36% of the patients had received anti-AR treatment (mainly abiraterone 23/43, 53%) for mCRPC before enrollment. Of the 114 patients with a known baseline CTC enumeration, 42 received anti-AR therapy and 72 did not. Of the patients who received anti-AR therapy, 14 (33%) patients had < 5 CTCs and 28 (67%) had ≥ 5 CTCs. In the 72 patients without endocrine pretreatment, 23 (32%) had < 5 CTCs and 49 (68%) had ≥ 5 CTCs ($p = 1.00$).

2.2. Circulating Tumor Cells at Baseline

In six of the 120 patients a baseline CTC enumeration could not be performed—three patients missed the baseline blood draw for enumeration and characterization of CTCs, one patient only missed the baseline blood draw for enumeration of CTCs, and two patients did not have enough blood in the CellSave tube to perform a reliable CTC enumeration. Therefore, in 114 patients a baseline CTC enumeration was performed. The median baseline CTC count was 15.5 (range 0–1025). In total, 37 patients had < 5 CTCs at baseline and the remaining 77 had ≥ 5 CTCs. When looking at the patient characteristics, only WHO performance status, LDH and alkaline phosphatase were significantly different between the patients with < 5 and ≥ 5 CTCs ($p = 0.045$, $p = 0.001$ and $p < 0.001$, respectively) (see ***Table 1***).

Patients with <5 CTCs before start of cabazitaxel therapy had a significantly better PFS and OS as compared to patients with ≥ 5 CTCs at baseline (both $p < 0.001$) (***Figure 1***). The median PFS in the entire cohort was 5.3 months. Patients with < 5 CTCs had a median PFS of 8.0 months, while this was 4.4 months for patients with ≥ 5 CTCs ($p < 0.001$). The median OS was 11.8 months in all patients ($n = 114$), 18.9 months in the patients with < 5 CTCs, and 7.9 months for patients with ≥ 5 CTCs ($p < 0.001$).

2.3. Circulating Tumor Cell Dynamics at Baseline and Follow-Up

A matched baseline and follow-up (at 6 weeks) CTC enumeration was available for 95 patients. These patients were divided into four groups: group 1 contained patients with < 5 CTCs at baseline who remained < 5 CTCs during follow-up ($n = 24$); group 2 had ≥ 5 CTCs at baseline but had < 5 CTCs during therapy ($n = 19$); group 3 had < 5 CTCs at baseline and ≥ 5 CTCs during therapy ($n = 5$); and group 4 were patients who had ≥ 5 CTCs both at baseline and during therapy ($n = 47$).

Table 1. Patient characteristics

Category	N	%	< 5 CTC	≥ 5 CTC	*p*-value
Age					0.968
< 65 years	31	26	10	19	
≥ 65 years	89	74	27	58	
WHO performance score					0.045
0	61	51	24	33	
1	59	49	13	44	
Type of castration					1.000
Surgical	15	13	5	10	
LHRH agonist	105	88	32	67	
Prior chemotherapy lines					
1 (only docetaxel)	119	99	37	76	
2	1	1	0	1	
Prior antiandrogen therapy for mCRPC*					0.326
Abiraterone	23	19	10	12	
Enzalutamide	9	8	2	7	
Orteronel	12	10	3	9	
None	77	64	23	49	
Baseline chemistry	Median	Range	Median (range)	Median (range)	
LDH U/L (*n* = 118)	312	141–1843	237.5 (151–531)	353 (141–1843)	0.001
ALP U/L (*n* = 119)	128	39–909	105 (43–409)	174 (39–909)	<0.001
PSA µg/L (*n* = 120)	152	4.5–5300	120 (45–2000)	175 (7–5300)	0.519
CTC count (*n* = 114)	15.5	0–1025	1 (0–4)	51 (5–1025)	

Patients characteristics of the 120 patients included in this study. Note: CTC = circulating tumor cells, LHRH agonist = luteinizing hormone-releasing hormone agonist; mCRPC = metastatic castration-resistant prostate cancer; ALP = alkaline phosphatase; LDH = lactate dehydrogenase; PSA = prostate specific antigen. * One patient received enzalutamide and orteronel sequentially. CTC counts are from baseline CTC enumeration.

Median PFS for group 1 was 8.7 months, while it was 6.4 months for group 2, 7.4 months for group 3, and 3.5 months for group 4. There was a significant difference between group 4 and group 1 ($p < 0.001$), group 2 ($p < 0.001$), and group 3 ($p = 0.032$). Between the other groups, no significant difference was found. Median OS for group 1 was 19.0 months, while it was 12.8 months for group 2, 23.0 months for group 3, and 6.9 months for group 4. For OS, there was also a difference between group 4 and group 1 ($p < 0.001$), group 2 ($p = 0.003$), and group 3 ($p = 0.003$). Also, a difference between group 1 and 2 was found ($p = 0.016$). In ***Figure 2***, a Kaplan–Meier plot for PFS and OS of the different groups of patients is shown.

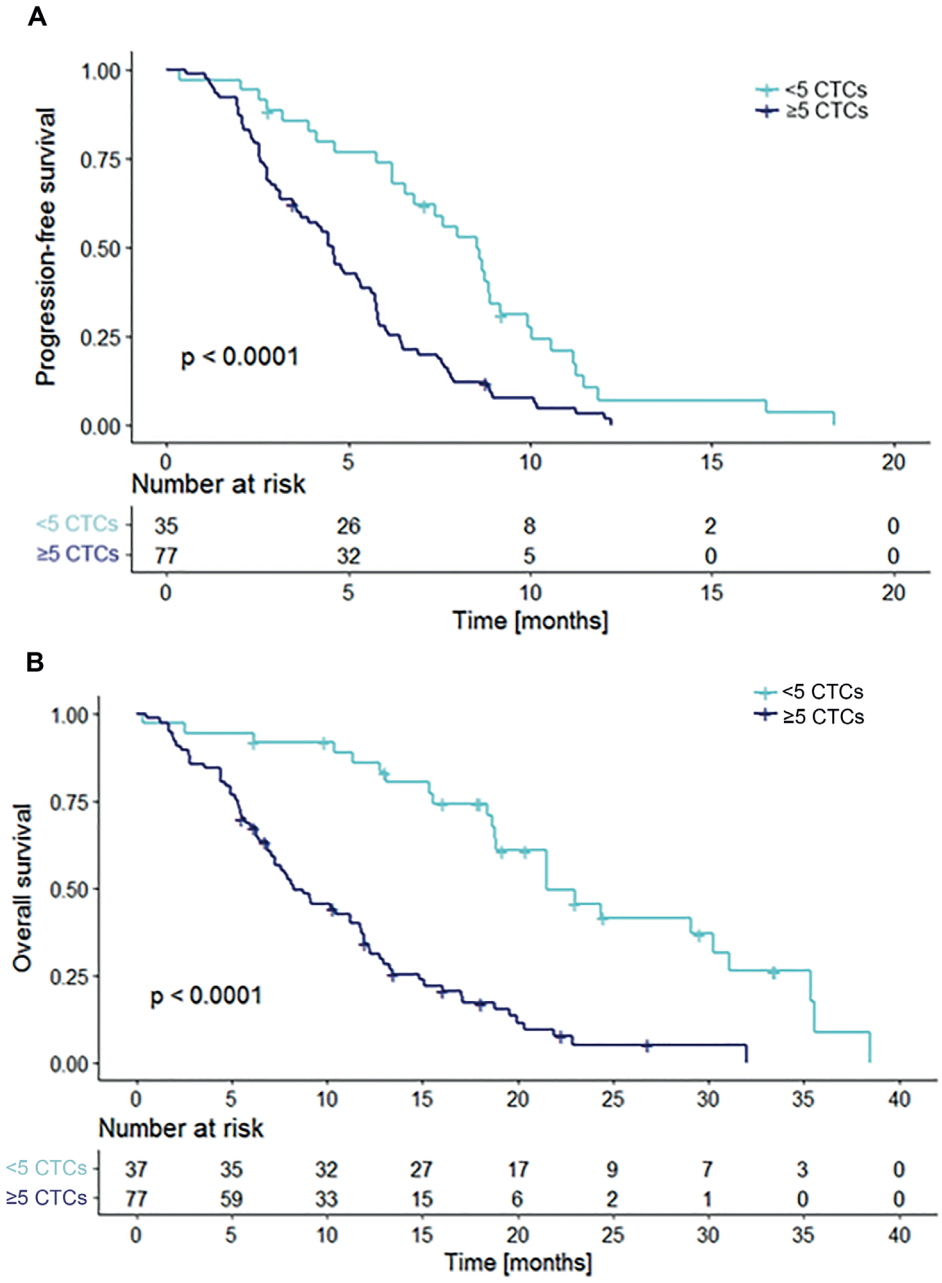

Figure 1. PFS and OS in relation to dichotomized CTC count at baseline.
Kaplan Meier curves of (**A**) progression-free survival (PFS) and (**B**) overall survival (OS) in relation to circulating tumor cell (CTC) count at baseline. CTC counts are divided into two categories of < 5 CTCs and ≥ 5 CTCs.

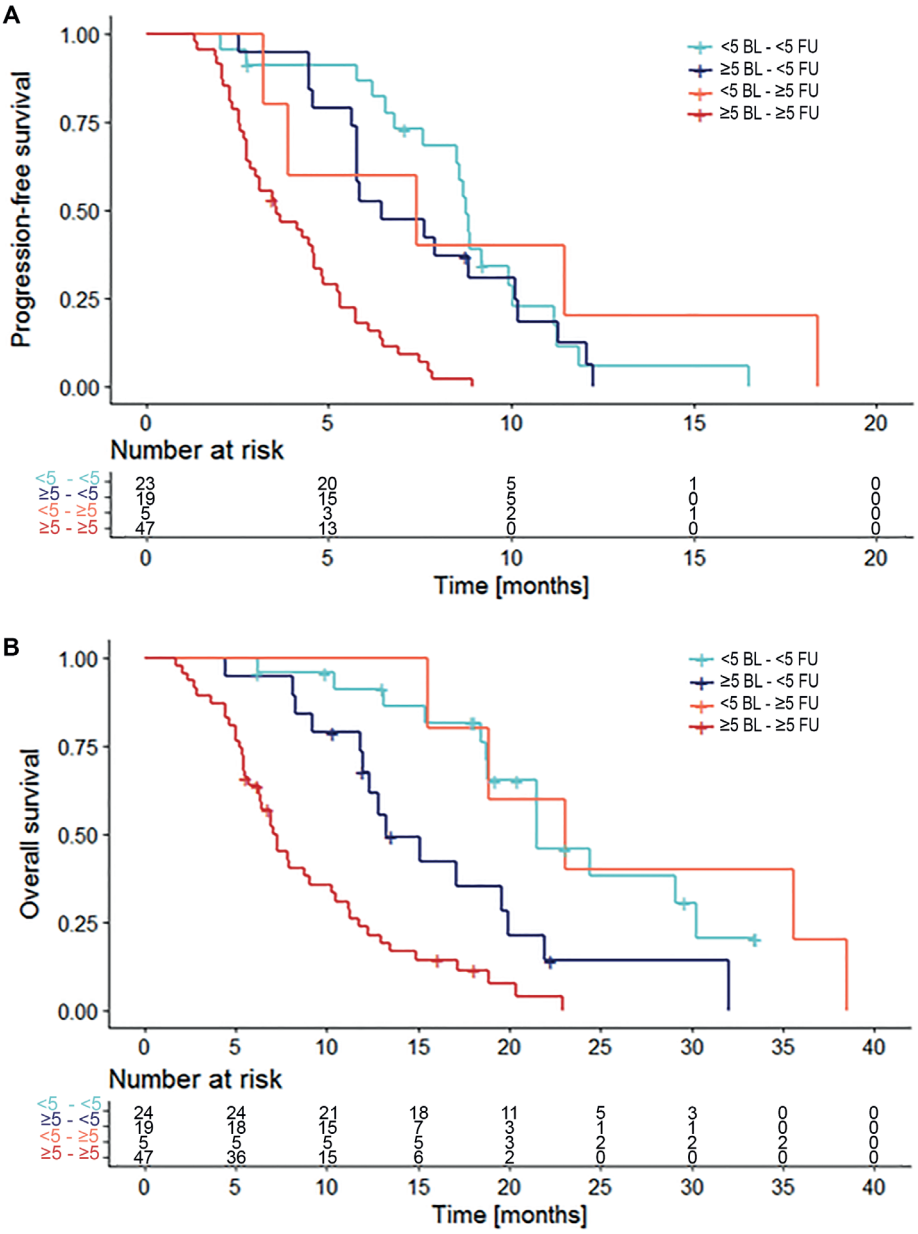

Figure 2. PFS and OS in relation to CTC groups.
Kaplan Meier curves of (**A**) progression-free survival (PFS) and (**B**) overall survival (OS) in relation to circulating tumor cell (CTC) count. CTC counts are divided into four categories: 1) <5 CTCs at baseline and <5 CTCs during treatment, 2) ≥5 CTCs at baseline and <5 CTCs during treatment, 3) <5 CTCs at baseline and ≥5 CTCs during treatment and 4) ≥5 CTCs at baseline and ≥5 CTCs during treatment.

2.4. Gene Expression Profiles of CTC-Specific Genes

To evaluate the predictive value of gene expression levels for therapy outcome for patients treated with cabazitaxel, 91 genes were measured (see ***Supplementary Table 1a***). Of these genes, 34 had a significantly higher expression in CTC samples compared to samples from healthy blood donors (HBDs) (with a false discovery rate (FDR) of 5%). These genes are listed in ***Supplementary Table 1b***. Hierarchical clustering of the 34 CTC-specific genes in all baseline samples with available gene expression data ($n = 63$) divided the samples into two groups (***Supplementary Figure 1***). The median CTC count in the first group was 19, while this was 154 in the second group ($p < 0.001$). However, when these two subgroups were visualized in a Kaplan–Meier plot for PFS and OS and tested with the Log-Rank test, there was no difference in PFS or OS between the groups (see ***Supplementary Figure 2***).

2.5. Gene Expression Profiles in Relation to Outcome to Cabazitaxel Therapy

The 34 CTC-specific genes were also analyzed to determine a gene expression panel that predicts outcome to cabazitaxel therapy. In the 63 baseline samples, five genes were identified with the highest discriminatory power to predict PFS on cabazitaxel therapy. These genes were *AGR2, FOXA1, DKK1, FAT1* and *TMPRSS2*. These were also the genes that were significantly associated with prolonged PFS in univariate Cox regression analysis (see ***Supplementary Table 2***). The patients were divided into two groups using a median split of the predicted risk score, with 32 patients having a high predicted risk score and 31 patients having a low predicted risk score. However, these groups did not show statistically different survival curves for PFS ($p = 0.27$). When we used this estimated risk score for OS, there was a difference in outcome between the high and low risk groups ($p = 0.039$). ***Supplementary Figure 3*** shows the Kaplan Meier curves of both PFS and OS in relation to the high and low risk groups.

2.6. Cox Regression Analysis

As the primary objective of this study was to identify the most powerful markers for outcome to cabazitaxel therapy, all available variables (baseline CTC count, age, PSA, alkaline phosphatase, lactate dehydrogenase (LDH), albumin, hemoglobin and the 5-gene profile) were analyzed in uni- and multivariate Cox regression models for both PFS and OS in the 63 patients for which all of these data were available. For PFS, univariate Cox regression analyses showed a significant association with both CTC count and hemoglobin. In multivariate regression analysis, both CTC count (HR 1.001, 95% CI 1.00–1.00, $p = 0.023$) and hemoglobin (HR 0.731, 95% CI 0.56–0.95, $p = 0.020$) remained independent prognostic factors for PFS (see ***Table 2A***). For OS, five variables were significant in univariate Cox Regression analysis: the 5-gene profile, CTC count, alkaline phosphatase, LDH and hemoglobin. In multivariate analysis, only CTC count (HR 1.002,

95% CI 1.00–1.00, $p = 0.009$) and hemoglobin (HR 0.642, 95% CI 0.48–0.85, $p = 0.002$) remained significant independent factors for OS (see ***Table 2B***).

When looking at the baseline variables without the 5-gene expression profile, 114 patients had all of these data available. Baseline CTC count, alkaline phosphatase, LDH and hemoglobin were significant in univariate analysis for PFS. In multivariate analysis, CTC count (HR 1.002, 95% CI 1.00–1.00, $p < 0.001$), alkaline phosphatase (HR 1.002, 95% CI 1.00–1.00, $p = 0.014$), and hemoglobin (HR 0.749, 95% CI 0.61–0.92, $p = 0.005$) remained independent prognostic factors for PFS (see ***Table 3A***). For OS, CTC count, alkaline phosphatase, LDH, albumin and hemoglobin were significant in univariate analysis. In multivariate analysis, only CTC count (HR 1.002, 95% CI 1.00–1.00, $p < 0.001$), alkaline phosphatase (HR 1.002, 95% CI 1.00–1.00, $p = 0.006$), and hemoglobin (HR 0.591, 95% CI 0.47–0.74, $p < 0.001$) remained independent prognostic factors (see ***Table 3B***).

Table 2. Cox Regression analysis ($n = 63$)

(A)	Univariate analysis			Multivariate Analysis		
Variable	HR	95% CI	*p*-value	HR	95% CI	*p*-value
5-gene profile	1.325	0.80–2.20	0.279			
CTC count	1.001	1.00–1.00	0.011	1.001	1.00–1.00	0.023
Age	0.988	0.95–1.02	0.508			
PSA	1.000	1.00–1.00	0.777			
ALP	1.001	1.00–1.00	0.101			
LDH	1.000	0.99–1.00	0.248			
Albumin	0.954	0.91–1.00	0.070			
Hemoglobin	0.709	0.54–0.93	0.011	0.731	0.56–0.95	0.020

(B)	Univariate Analysis			Multivariate Analysis		
Variable	HR	95% CI	*p*-value	HR	95%CI	*p*-value
5-gene profile	1.743	1.02–2.98	0.042			
CTC count	1.002	1.00–1.00	0.006	1.002	1.00–1.00	0.009
Age	1.015	0.98–1.05	0.418			
PSA	1.000	1.00–1.00	0.090			
ALP	1.001	1.00–1.00	0.038			
LDH	1.001	1.00–1.00	0.006			
Albumin	0.949	0.90–1.00	0.056			
Hemoglobin	0.631	0.48–0.84	0.002	0.642	0.48–0.85	0.002

Cox regression analysis in 63 patients with baseline gene expression data available for (A) progression-free survival and (B) overall survival. The 5-gene profile was dichotomized to high and low predicted risk, while the other variables are continuous. Note: ALP = alkaline phosphatase; HR = hazard ratio; LDH = lactate dehydrogenase; PSA = prostate specific antigen. CTC counts are from baseline CTC enumeration.

Table 3. Cox Regression analysis (*n* = 114)

(A)	Univariate Analysis			Multivariate Analysis		
Variable	HR	95% CI	*p*-value	HR	95%CI	*p*-value
CTC count	1.002	1.00–1.00	<0.001	1.002	1.00–1.00	<0.001
Age	0.989	0.96–1.02	0.453			
PSA	1.000	1.00–1.00	0.732			
ALP	1.002	1.00–1.00	<0.001	1.002	1.00–1.00	0.014
LDH	1.001	1.00–1.00	0.007			
Albumin	0.983	0.94–1.03	0.490			
Hemoglobin	0.694	0.57–0.85	<0.001	0.749	0.61–0.92	0.005

(B)	Univariate Analysis			Multivariate Analysis		
Variable	HR	95% CI	*p*-value	HR	95%CI	*p*-value
CTC count	1.003	1.00–1.00	<0.001	1.002	1.00–1.00	<0.001
Age	1.017	0.99–1.05	0.276			
PSA	1.000	1.00–1.00	0.112			
ALP	1.002	1.00–1.00	<0.001	1.002	1.00–1.00	0.006
LDH	1.002	1.00–1.00	<0.001			
Albumin	0.952	0.91–0.99	0.044			
Hemoglobin	0.551	0.44–0.68	<0.001	0.591	0.47–0.74	<0.001

Cox regression analysis in 114 patients with baseline CTC enumeration available for (A) progression-free survival and (B) overall survival. All variables are continuous in this regression analysis. Note: ALP = alkaline phosphatase; HR = hazard ratio; LDH = lactate dehydrogenase; PSA = prostate specific antigen. CTC counts are from baseline CTC enumeration.

3. DISCUSSION

Our data confirms the strong prognostic value of CTCs in patients treated with cabazitaxel. We found the CTC count to be independently associated with outcome in mCRPC patients who were treated with cabazitaxel in the second line of chemotherapy, besides the well-known prognostic parameters hemoglobin and alkaline phosphatase. To the best of our knowledge, this is the largest study investigating the association between CTC numbers and outcome to cabazitaxel treatment. Climent et al. have assessed baseline CTC numbers in 56 patients receiving cabazitaxel therapy. They also found an association between CTC count and PFS, however they did not find a statistically significant association between CTC count and OS (12).

Conversions between the favorable group (< 5 CTCs) and unfavorable group (≥ 5 CTCs) have been described in mCRPC patients, however not yet in a large set of cabazitaxel treated patients. Literature has shown for both metastatic breast and prostate cancer that patients who are in the favorable group at baseline and follow-up have the best

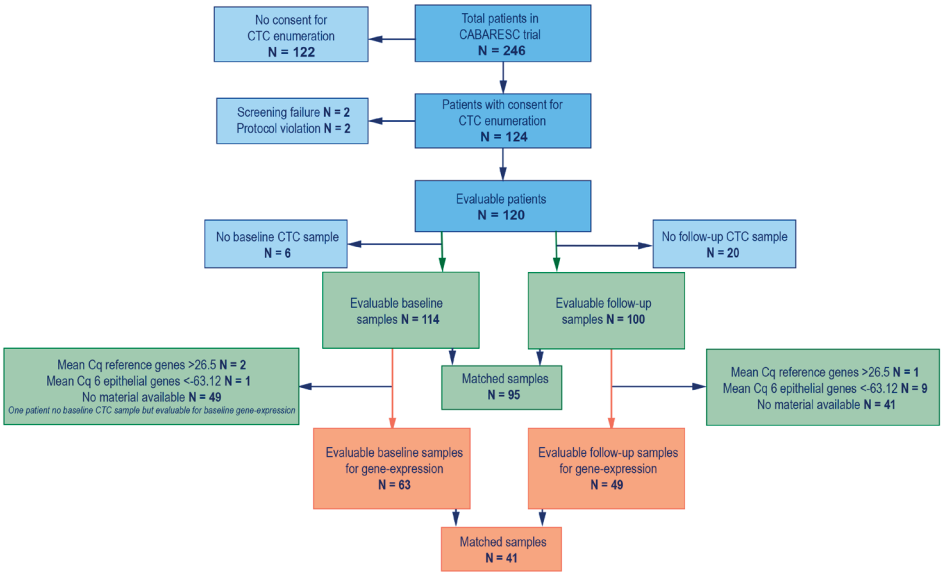

Figure 3. Patient inclusion.
Flowchart of patient inclusion. In blue all patients who are included in the CABARESC study and the side CTC enumeration study. In green the patients included for this manuscript with available CTC enumeration. In Orange the patients included for this manuscript with available gene expression data.

prognosis, while patients who are in the unfavorable group at baseline and follow-up have the worst prognosis (8,16). In these studies, mCRPC patients who switched from ≥ 5 CTCs at baseline to < 5 CTCs at follow-up had a better prognosis than patients who had < 5 CTCs at baseline but increased to ≥ 5 CTCs at follow-up. In the current study, we looked at baseline CTC count and CTC count after six weeks of therapy instead of after therapy failure, which was done in the previous described study (8). We chose the six-week time point as our aim was to detect early progression in our patients. This would allow patients to switch therapies in an early stage of therapy. Our data showed that after dividing our patients into four groups depending on CTC count before and during therapy, patients with an unfavorable CTC count at baseline and during treatment indeed had the worst prognoses. However, the difference between the other 3 groups was less clear. The relatively small sample size of these subgroups (especially group 3 (< 5 CTCs to ≥ 5 CTCs) contained only 5 patients) limits the power of this analysis, so based on our data no firm conclusion can be drawn for CTC conversions during therapy. Preferably, this analysis should be repeated with more patients and with CTC count assessed at progression of disease.

In multivariable analysis for both PFS and OS, CTC count, alkaline phosphatase and hemoglobin were independent prognostic factors. Hemoglobin and alkaline phosphatase are known prognostic factors in mCRPC (17). Patients with lower hemoglobin levels

(< 12 g/dL) have shorter OS, while for CTC count and alkaline phosphatase the relation is inversed. Alkaline phosphatase originates mainly from bone and liver, with 40–50% coming from bone in adults (18). Since 90% of the mCRPC patients have radiologic evidence of bone metastasis (3), alkaline phosphatase is an important biomarker in prostate cancer. For cabazitaxel, no direct effect on alkaline phosphatase is known, only indirect when the tumor burden decreases, alkaline phosphatase levels also decrease (19). Also for hemoglobin no direct effect of cabazitaxel is known. Both alkaline phosphatase and hemoglobin can be influenced by tumor burden, but also by a patient's underlying condition. CTCs are the only factor that reflect the tumor burden directly. However, all of these markers together can help physicians in deciding which patients should receive cabazitaxel treatment at that moment.

In addition to traditional clinical parameters and CTC counts, we also assessed the clinical value of a CTC derived gene expression profile to gain more insight into the biology of the tumors. Therefore, 91 genes with proven involvement in prostate cancer were measured in mRNA from the CTC enriched samples. Of these genes, 34 genes had higher expression in the CTCs than in the leukocyte background and were used for further analysis. Using these 34 genes to cluster the 63 samples for which baseline gene expression data were available, the samples were clearly divided into two groups, which showed a significant difference in CTC count. The cluster could be biased by CTC count, however, we do observe clear variation in gene expression beyond sheer CTC count, indicating that the differences are probably not just driven by numbers of CTCs.

The five genes from the gene expression signature with the highest discriminatory power to predict PFS on cabazitaxel were *AGR2, FOXA1, DKK1, FAT1* and *TMPRSS2*. All genes used for the gene expression panel were selected based on expression and clinical relevance in prostate cancer. Looking at these five genes, *AGR2* is a CTC-specific gene in metastatic breast and colorectal cancer that has been associated with poor outcome in prostate cancer patients (20–23). *FOXA1* is a key component of the AR transcriptional complex and overexpression leads to enrichment of the PTEN and WNT signaling pathway (24). *DKK1* is a WNT signaling inhibitor and has been associated with poor survival in prostate cancer patients (25). *FAT1* is a tumor suppressor gene that affects the WNT signaling pathway (26) and *TMPRSS2* is an AR-regulated gene that is involved in a gene fusion (*TMPRSS2-ERG*) in prostate cancer (27). Prostate cancers with a positive *TMPRSS2-ERG* fusion show increased WNT signaling (28). In summary, these genes are especially involved in the AR and WNT signaling pathways. While the role of the AR pathway has been well-established in prostate cancer, the role of the WNT signaling pathway is less clear. However, changes in the WNT signaling pathway have been described in prostate cancer, especially in the development of mCRPC. The genes presented here emphasize the need to investigate the role of the WNT pathway in mCRPC and eventually look at the possibility of using WNT modulators in mCRPC patients (29,30).

The gene expression signature of five genes to predict outcome on cabazitaxel therapy showed no significant difference in PFS. Reviewing the Kaplan–Meier curve, a difference in the first 7 months of treatment is visible. However, after that moment, the number of patients is too small to make firm conclusions. For OS, there was a significant difference based on this 5-gene profile, however, in multivariate analysis this was not an independent prognostic factor. Literature shows several studies with gene expression profiles determined in peripheral blood (31–36) or on CTCs (37–43) that are associated with outcome in patients with metastatic prostate cancer. Especially the studies of Wang et al. (36), Olmos et al. (34), and Ross and al. (35), in which gene expression profiles were established in whole blood of mCRPC patients, showed prognostic value of the established gene profiles in an independent training and validation set. However, all of the studies mentioned above still lack prospective validation. Also, none of these studies assessed a gene expression profile that purely predicts response to cabazitaxel. Despite the lack of clinical value of the approach we described here, we still feel that assessing the clinical value of gene expression profiles established in CTCs is worth pursuing. CTCs, as enumerated by the CellSearch system, which probably does not isolate the CTCs with a more mesenchymal phenotype (44), do have strong prognostic power, as shown here and in many other studies, and therefore represent a biologically relevant population of cells. A limitation of CTC isolation with the CellSearch system is the background of leukocytes present after enrichment of CTCs, however, there is less background present than after isolation of peripheral blood mononuclear cells (PBMCs). This background severely limits the number of genes that can be evaluated, since only genes can be evaluated that are solely expressed in CTCs and not in leukocytes. Using techniques that enable the isolation of pure fractions of CTCs would increase the number of genes that can be evaluated for prognostic and predictive relevance and also gives the opportunity to assess heterogeneity between CTCs within one patient, and therefore deserves further study.

A limitation of this study was the number of patients that were available for analysis. For CTC comparison analysis, 95 matched CTC samples were available. However, when dividing these patients into different subgroups, some subgroups were too small to make firm conclusions. Also, for baseline gene expression samples only 63 samples were available, of which almost all patients had ≥ 5 CTCs, which made it difficult to make comparisons in this group. Furthermore, this group mostly contained patients with worse prognoses based on their CTC count.

4. MATERIALS AND METHODS

4.1. Patients

The mCRPC patients starting with a new line of cabazitaxel treatment were selected from a prospective multicenter trial (CABARESC trial, NTR2991). In this study, mCRPC patients who progressed after docetaxel treatment for CRPC were included and randomized to receive 25 mg/m^2 of cabazitaxel with or without budesonide treatment (45). Treatment with cabazitaxel continued until progression of disease or unacceptable toxicity with a maximum of ten cycles. The primary objective of CABARESC was to evaluate the effects of budesonide on cabazitaxel-induced diarrhea (45). As a side study, blood was drawn for CTC enumeration and characterization in this trial. If a patient provided consent to participate in this side study, blood was drawn before the start of cabazitaxel treatment and before the third cycle of cabazitaxel. In total, 246 mCRPC patients were included, of whom 124 provided consent for CTC enumeration and characterization. Of these 124 patients, four patients were excluded due to protocol violation ($n = 2$) and screen failure ($n = 2$). Therefore, a total of 120 patients were available for this study (see ***Figure 3***). The study was approved by the Erasmus MC and local Institutional Review Boards (METC 11–324). All patients provided written informed consent. Clinical data were collected from all patients.

4.2. CTC Enumeration and mRNA Isolation

For CTC enumeration 7.5 mL blood was collected in a CellSave tube and for characterization 7.5 mL blood was collected in an EDTA tube before start and during cabazitaxel treatment. Blood was processed with the CellSearch system (CellSearch enumeration kit and CellSearch profile kit, Menarini-Silicon Biosystems, Huntington Valley, PA, USA). For enumeration, blood was processed within 96 hours, while for characterization blood was processed within 24 hours. After CTC enrichment, mRNA was isolated with the AllPrep DNA/RNA Micro Kit (Qiagen, Germantown, MD, USA). Thereafter, cDNA was generated, which was pre-amplified for the targets of interest and real time amplified (RT-qPCR) using Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA). For a more detailed description, see previously published papers (22,46).

A baseline CTC sample was collected in 114 of the 120 patients. For six patients a baseline CellSave tube was not drawn, and therefore CTCs could not be enumerated. For 100 patients a follow-up sample was collected after six weeks. For the gene expression assays, material from 66 baseline samples and 59 follow-up samples was available. However, three baseline samples and ten follow-up samples were excluded due to insufficient cDNA quality or quantity (see below). Therefore, 63 baseline samples and 49 follow-up samples were included for gene expression analysis, which contained 41 matched samples (see ***Figure 3***).

4.3. Sample Processing and Normalization

A gene expression profile of 147 genes was developed based on expression and clinical relevance in prostate cancer, as derived from literature. Since a background of leukocytes remains after CTC isolation with the CellSearch System, only genes with higher expression in prostate cancer than in leukocytes were selected. Therefore, all genes were reviewed for expression in leukocytes and in prostate cancer (SAGE Genie Database of the Cancer Genome Anatomy Project (http://cgap.nci.nih.gov/SAGE/AnatomicViewer)). The 91 genes with the lowest leukocyte expression or highest upregulation in prostate cancer (> 10×) were selected. Besides these 91 genes, three reference genes (*GUSB*, *HMBS*, and *HPRT1*) were added to determine the quality of the sample, along with a negative control (H_2O) and a marker for leukocyte contamination (PTPRC, Protein Tyrosine Phosphatase Receptor type C). Samples with an average reference gene signal of $\Delta C_q > 26.5$ were considered to be of insufficient cDNA quantity and/or quality, and were therefore excluded ($n = 3$).

For each of the 91 genes, the ΔC_q of 15 CellSearch enriched healthy blood donor (HBD) samples was determined. A CTC sample was considered positive for a given gene if the C_q value was at least one standard deviation higher (+1SD) than the mean ΔC_q of the 15 HBDs. If the expression of a gene was below this cut-off, it was considered not detectable and given the value of the mean ΔC_q of the 15 HBDs.

To further correct for the leukocyte background and ensure the presence of a CTC-derived signal, an epithelial profile was calculated for every sample. Previously, a 12-gene signal was identified that was associated with epithelial tumor load in breast cancer patients (22). The gene expression profile used here for prostate cancer contained 6 of these 12 genes. Therefore, we correlated these 6 genes (*AGR2, FOXA1, EPCAM, IGFBP5, LAD1* and *S100A16*) from our prostate panel with the 12 genes from the epithelial signal, which was measured in CTCs of 910 breast cancer patient samples and 20 HBD samples. For these 930 CTC samples, the ΔC_q sum of these 12 genes resulted in a cut-off of -131 to identify the presence of at least one CTC in a sample. After correction for the slightly different HBD-corrected cut-off in this prostate cancer panel than in the breast cancer panel, the cut-off used here based on the ΔC_q of six genes to identify a positive epithelial signal, and therefore presence of at least one CTC, was -63.12 (see ***Supplementary Figure 4***). Based on this epithelial cut-off, 10 samples (1 baseline and 9 follow-up samples) were excluded for supposedly not containing any CTC-derived signal. Therefore, a total of 63 baseline samples and 49 follow-up samples were available for comparison of the gene expression profiles at baseline and after 6 weeks of therapy.

4.4. Statistical Analysis

Patient characteristics were compared between those with < 5 CTCs and those with ≥ 5 CTCs by means of chi-square tests for categorical variables and the Mann–Whitney U

test for continuous variables. The cut off of 5 CTCs was chosen as this has been proven to be prognostic in patients with mCRPC in several independent studies (8,47). Progression free survival (PFS) was defined as time between start of treatment and progression of disease, and overall survival (OS) was defined as time from start of treatment until death by any cause. CTC count was determined both at baseline and follow-up. Besides studying the effect of baseline CTC count as a marker for the survival outcomes, the four possible groups of CTC count development (i.e., CTC count remaining smaller than 5 or ≥ 5, CTC count increasing from < 5 to ≥ 5, or the other way around) were also studied. Furthermore, a gene expression profile was developed and related to survival outcomes. For all these variables the relation with PFS and OS was studied by means of log-rank tests and visualized with Kaplan–Meier plots. Furthermore, univariate and multivariate Cox proportional hazards analyses were performed to quantify the relationships further. For the multivariate analyses, a stepwise backward procedure was followed including all available variables, and a threshold of $p < 0.05$ was applied for the selection of variables to remain in the model.

In order to select the genes from the gene expression panel that were CTC-specific, we compared expression levels per gene in the 112 patient CTC samples and in 15 HBD samples with the Mann–Whitney U test. To correct for multiple testing, a FDR of 5% was applied. Gene expression data of the 63 baseline samples were compared with the Survival Risk Prediction (SRP) tool from Biometric Research Branch ArrayTools (BRB-ArrayTools, http://linus.nci.nih.gov/BRB-ArrayTools.html), using a *p*-value of < 0.05. For hierarchical clustering, both the genes and the samples were normalized, the genes were centered, and average uncentered clustering was performed using Cluster 3.0 and visualized with Java Treeview version 1.6. All computations were performed using R (version 3.4.1) and all reported *p*-values are two-sided (unless stated otherwise).

5. CONCLUSION

These data show that characterization of CTCs as of yet did not hold sufficient power to distinguish outcome to cabazitaxel treatment. However, our data confirms the strong, independent prognostic value of the CTCs for both PFS and OS in mCRPC patients who are treated with cabazitaxel in the second line, together with alkaline phosphatase and hemoglobin.

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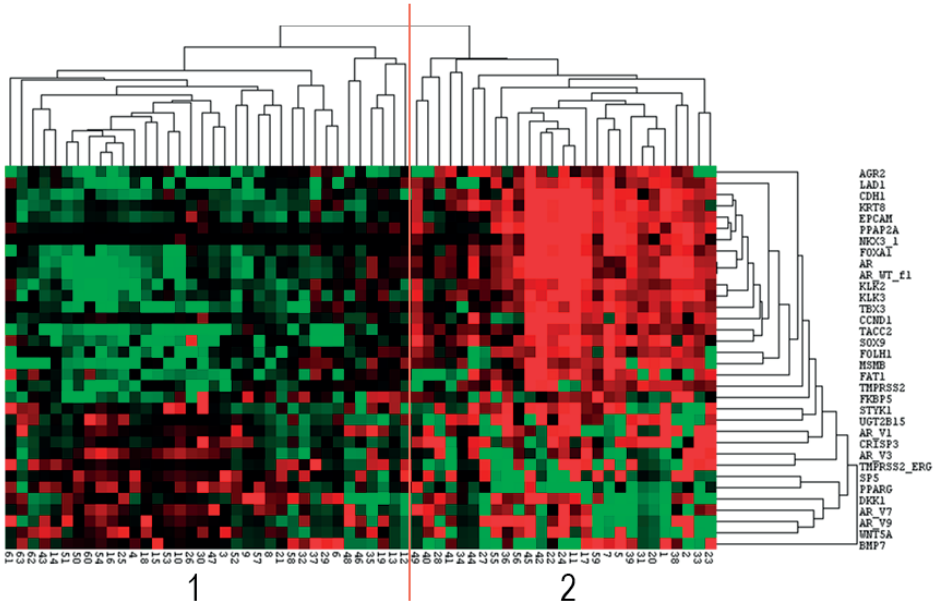
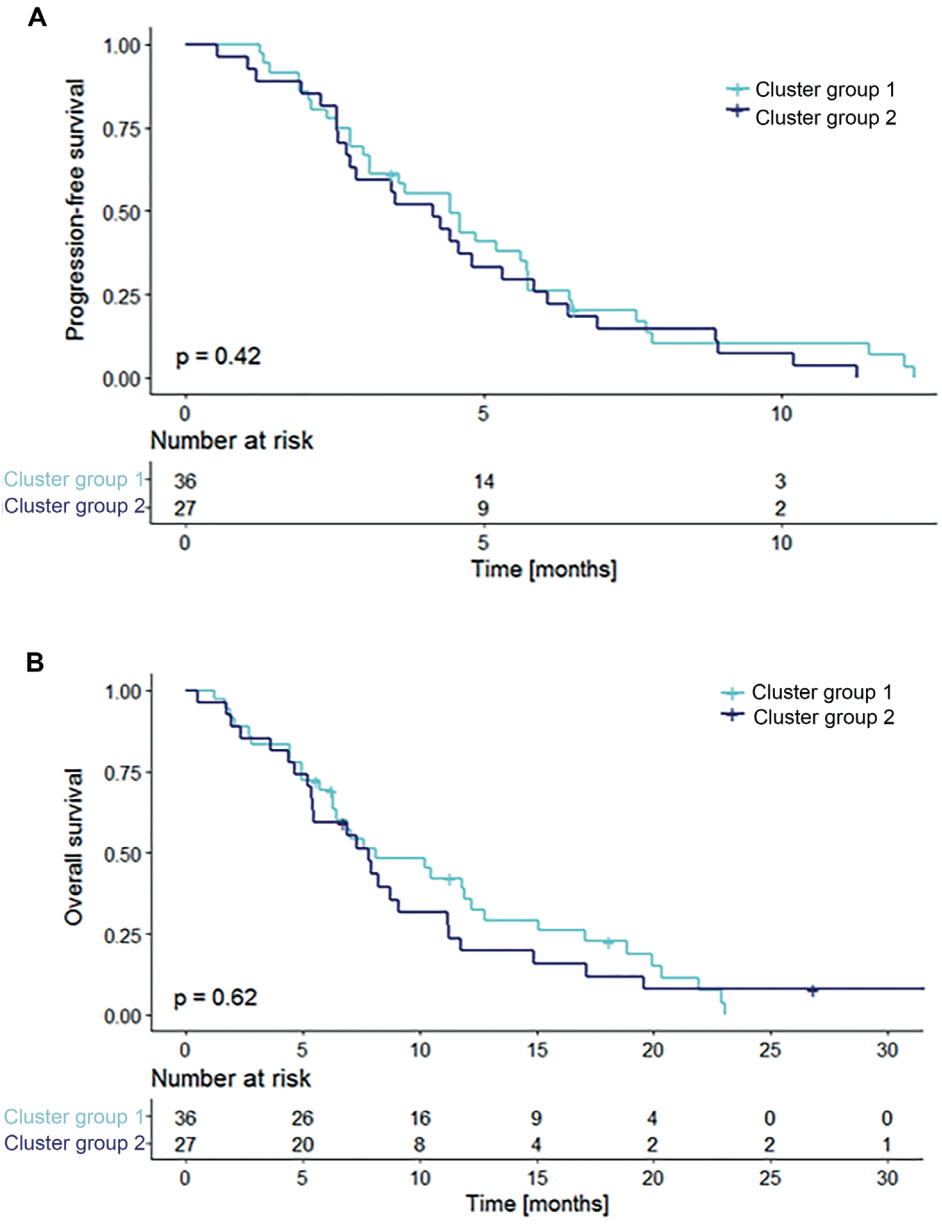
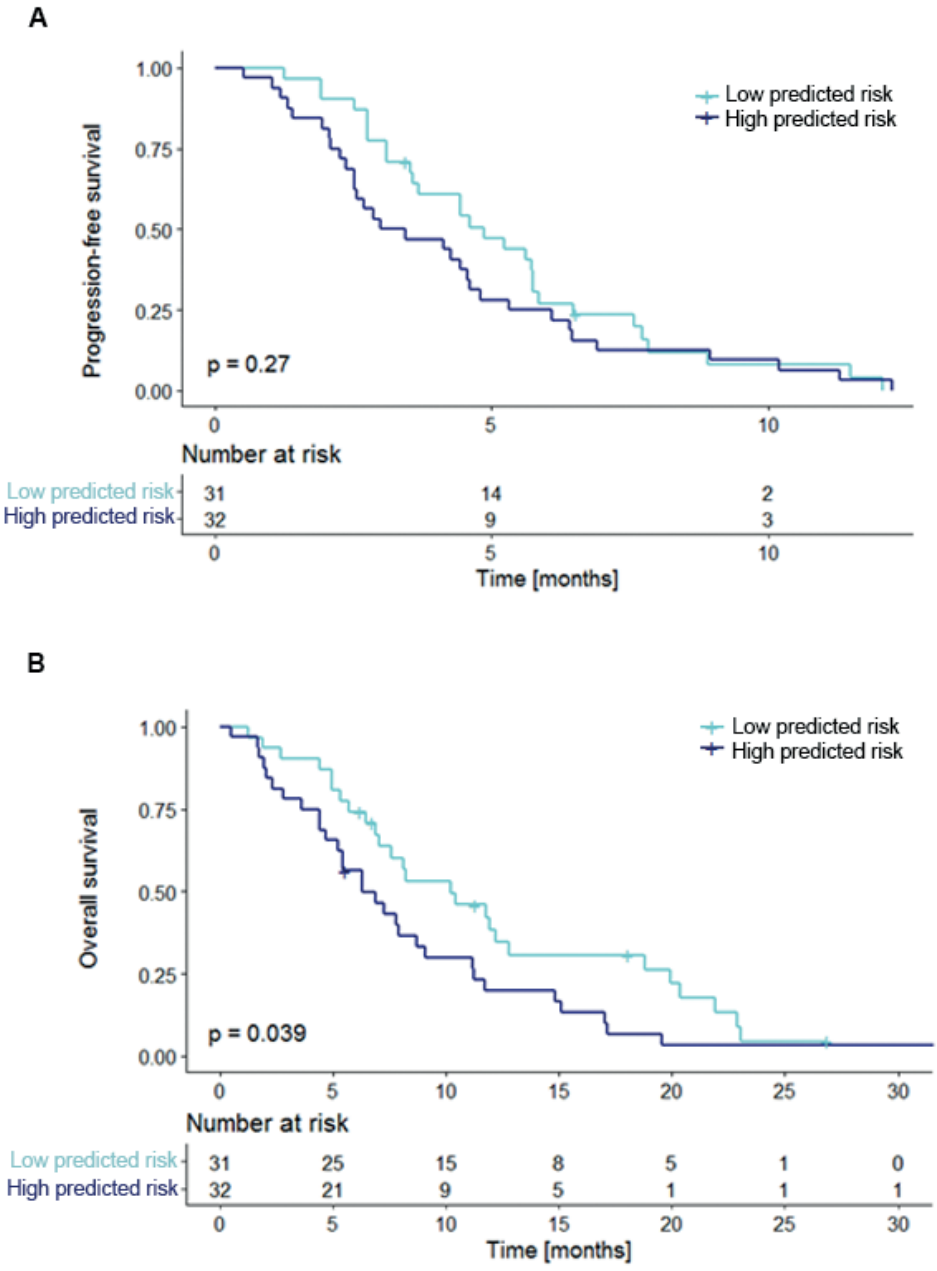
SUPPLEMENTARY MATERIALS



Supplementary Figure 1. Clustering of gene expression data
Hierarchical clustering of the 34 CTC-specific genes in the 63 mCRPC patients. Red indicates above median and green below median expression level of a gene over all samples.

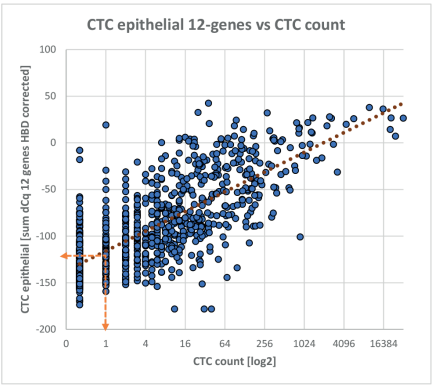


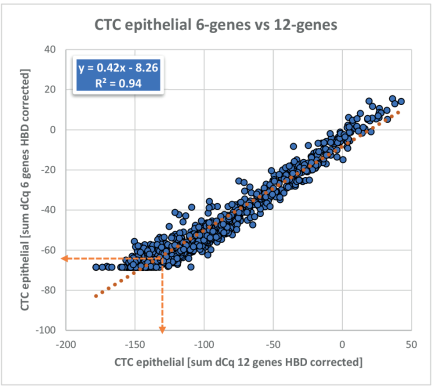
Supplementary Figure 2. PFS and OS in relation to the clustering groups
Kaplan-Meier curves of (A) progression-free survival (PFS) and (B) overall survival (OS) in relation to the groups divided by the cluster that was created in Supplementary Figure 1.



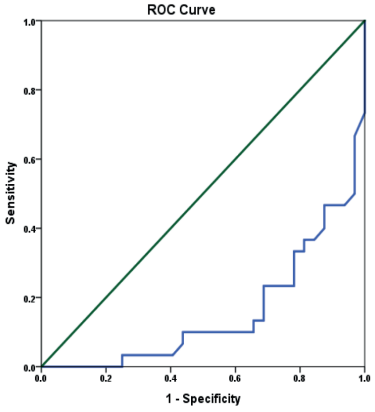
Supplementary Figure 3. PFS and OS in the 5-gene expression profile at baseline
Kaplan-Meier curves of (**A**) progression-free survival (PFS) and (**B**) overall survival (OS) in relation to the 5-gene expression profile. The groups are divided into two categories based on their low (1) and high (2) predicted risk based on this 5-gene profile by BRB Array tools.

A)





B)



Area Under the Curve for de average dCq of 6 epithelial genes

Test Result Variable(s): CTC count

		Confidence Interval	
Std. Error[a]	Asymptotic Sig.[b]	Lower Bound	Upper Bound
0.049	3.0E-06	0.060	0.250

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Supplementary Figure 4. Cut off epithelial profile

Pearson correlation between the epithelial profile that has been determined on 12 genes in metastatic breast cancer patients and the epithelial profile used in this metastatic prostate cancer group based on 6 out of those 12 genes (A). In B) a receiving operating characteristic (ROC) curve with an area under the curve (AUC) value of this threshold is shown.

Supplementary Table 1. Genes used for gene expression profile

A	All 96 genes in the gene expression panel (including 3 reference genes)			
ABCC4	*CDK12*	*FZD7*	*NTS*	*STYK1*
ACPP	*CDK4*	*GUCY1A3*	*PCGEM1*	*TACC2*
ADRA2C	*CEACAM5*	*HNF1A*	*PIK3CA*	*TBX3*
AGR2	*COL18A1*	*IGFBP4*	*PIK3CB*	*TCF7L2*
AKR1C3	*CREB3L4*	*IGFBP5*	*PLAU*	*TMPRSS2*
APC	*CRISP3*	*ITGA6*	*PMEPA1*	*TMPRSS2_ERG*
APP	*CYP11A1*	*KIT*	*PPAP2A*	*UGT2B15*
AR	*CYP17A1*	*KLK2*	*PPARG*	*WNT11*
AR_V1	*DDC*	*KLK3*	*PRKACB*	*WNT4*
AR_V3	*DHCR24*	*KRT8*	*PTEN*	*WNT5A*
AR_V7	*DKK1*	*LAD1*	*PTPN1*	*ZBTB16*
AR_V9	*DRG1*	*LEF1*	*RAD51B*	
AR_WT_fl	*EAF2*	*LRIG1*	*RAD51C*	Reference genes
AXIN2	*ELL2*	*MALAT1*	*RB1*	*GUSB*
BMP7	*EPCAM*	*MED12*	*S100A16*	*HMBS*
BRCA2	*EZH2*	*MKI67*	*SLC45A3*	*HPRT1*
CAV1	*FAT1*	*MSMB*	*SLCO1B3*	
CCND1	*FKBP5*	*MYC*	*SOX9*	
CDH1	*FOLH1*	*NDRG1*	*SP5*	
CDH10	*FOXA1*	*NKX3_1*	*SRD5A2*	

B	CTC-specific genes			
AGR2	*BMP7*	*FKBP5*	*MSMB*	*TACC2*
AR	*CCND1*	*FOLH1*	*NKX3_1*	*TBX3*
AR-V1	*CDH1*	*FOXA1*	*PPAP2A*	*TMPRSS2*
AR-V3	*CRISP3*	*KLK2*	*PPARG*	*TMPRSS2_ERG*
AR-V7	*DKK1*	*KLK3*	*SOX9*	*UGT2B15*
AR-V9	*EPCAM*	*KRT8*	*SP5*	*WNT5A*
AR_WT_fl	*FAT1*	*LAD1*	*STYK1*	

(A) All 96 genes that were measured in the samples with available RNA. (B) The 34 genes with higher expression in CTC samples compared to the background of leukocytes.

Supplementary Table 2. Univariate regression analysis of PFS

Univariate analysis PFS			
Gene	**Exp(B)**	**95% CI**	**sig**
AGR2	1.087	1.02-1.16	0.008
AR	1.069	1.0-1.15	0.059
AR_V1	1.012	0.96-1.07	0.653
AR_V3	1.034	0.96-1.12	0.393
AR_V7	1.013	0.93-1.10	0.768
AR_V9	0.996	0.94-1.06	0.905
AR_WT_fl	1.066	1.00-1.14	0.071
BMP7	1.042	0.99-1.10	0.141
CCND1	1.031	0.92-1.16	0.607
CDH1	1.037	0.96-1.13	0.391
CRISP3	0.962	0.86-1.08	0.498
DKK1	1.072	1.01-1.14	0.022
EPCAM	1.056	0.94-1.18	0.341
FAT1	1.068	1.01-1.13	0.030
FKBP5	1.004	0.84-1.21	0.969
FOLH1	1.047	1.00-1.10	0.074
FOXA1	1.078	1.01-1.15	0.024
KLK2	1.055	0.99-1.13	0.115
KLK3	1.034	0.97-1.10	0.310
KRT8	1.065	0.96-1.18	0.223
LAD1	1.036	0.99-1.09	0.146
MSMB	1.028	0.98-1.08	0.261
NKX3_1	1.012	0.78-1.31	0.928
PPAP2A	1.058	0.93-1.20	0.376
PPARG	0.985	0.85-1.14	0.832
SOX9	1.030	0.98-1.09	0.283
SP5	1.041	0.99-1.10	0.115
STYK1	1.013	0.95-1.08	0.699
TACC2	1.040	0.99-1.09	0.129
TBX3	1.050	0.99-1.11	0.092
TMPRSS2	1.058	1.00-1.12	0.037
TMPRSS2_ERG	1.026	0.98-1.08	0.318
UGT2B15	1.052	0.98-1.13	0.173
WNT5A	1.033	0.96-1.12	0.407

Univariate Cox Regression analysis of all 34 genes in comparison with PFS in 63 patients with available gene expression data.

Chapter 6

Prospective evaluation of a circulating tumor cell sensitivity profile to predict response to cisplatin chemotherapy in metastatic breast cancer patients

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** These authors contributed equally*

Submitted

ABSTRACT

Background: Cisplatin (cDDP) has regained interest for metastatic breast cancer (MBC) patients, given the platinum sensitivity in subtypes and better manageable toxicity. Here, the primary aim was to determine whether molecular characteristics of circulating tumor cells (CTCs) could identify patients responding to cDDP and to describe the outcomes to cDDP monotherapy in a large group of MBC patients pretreated with anthracycline- and taxane-based treatments.

Methods: Based on cell line data, a CTC-cDDP-sensitivity profile was generated. Applying an A'Herns single-stage phase II design, further investigation was considered worthwhile if 5/10 patients with a favorable profile responded to cDDP. Patients received 70mg/m^2 cDDP every three weeks, CTCs were enumerated and CTC-cDDP-sensitivity profile was determined. In total, 65 heavily pretreated mBC patients (77% received ≥2 lines of previous chemotherapy for MBC) were eligible for the per-protocol analysis. Primary endpoint was response rate, secondary endpoints included best observed response, progression-free survival (PFS) and overall survival (OS).

Results: The best observed response during cDDP therapy was a partial response in 7% and stable disease in 56% of the patients. None of the patients with a favorable CTC-cDDP-sensitivity profile had a response. The median baseline CTC count was 8 (range 0-3254). Patients with <5 CTCs had a better PFS and OS than patients with ≥5 CTCs (median PFS 4.5 (95%CI 2.38-6.62) vs. 2.1 months ((95%CI 1.34-2.80)(p=0.009)) and median OS 13.1 (95%CI 9.89-16.33) vs. 5.6 months ((95%CI 3.60-7.64)(p=0.003)). No other factors than CTC count were associated with outcome to cDDP therapy, including triple-negative breast cancer versus ER+ tumors.

Conclusions: The CTC-cDDP-sensitivity profile was unable to select patients responding to cDDP monotherapy. In an unselected group of heavily pretreated MBC patients, cDDP yields outcomes comparable to other chemotherapeutic regimens for heavily pretreated MBC patients. CTC count was the only factor associated with outcome in these patients.

1. INTRODUCTION

For patients with metastatic breast cancer (MBC), several systemic therapies are available, aiming to prolong survival with an acceptable quality of life. Despite the fact that only for eribulin evidence exists for superiority over other regimens from randomized trials (1, 2), multiple agents are used in anthracycline- and taxane-pretreated patients.

Agents that are increasing used are platinum derivatives. One of these derivatives is cisplatin (cis-diamminedichloroplatinum (II), cDDP), an alkylating agent clinically available since the 1970s that is still being used in a wide range of tumor types. Most studies evaluating the effect of cDDP monotherapy in MBC are from the 1980s. Small phase-II studies reported response rates (RR) of 47-54% in previously untreated patients (3, 4) and of 15-21% in heavily pretreated patients (5, 6). Although the outcomes for cDDP in the first line are comparable with other chemotherapeutic agents applied in MBC, its side-effects prevented implementation into the clinical practice. However, the use of cDDP regained interest since its main toxicities, in particular nausea/vomiting and nephrotoxicity, can be handled much better nowadays. Also, there is improved insight into the tumor biology, which suggests subtypes of patients exist with tumors displaying a high sensitivity to platinum-based therapies (7-9).

Therefore, a method to select patients who will benefit from cDDP therapy is highly needed. Molecular characteristics of tumor cells can be associated with outcome to certain agents. Most molecular characterization is performed on primary tumor material. However, since the characteristics of the primary breast tumor and metastatic lesions can change over time and under treatment pressure (10), metastatic tumor cells should be explored for characteristics predicting outcome. However, obtaining tissue from metastatic lesions is an invasive and often painful procedure and sometimes impossible because of inaccessible lesions. Circulating tumor cells (CTCs), which can be repeatedly isolated from peripheral blood, represent an attractive alternative. Besides CTC enumeration, which is a proven prognostic marker in MBC (11-13), characterization of these CTCs is also possible (14-17). The characteristics of CTCs resemble the characteristics of the metastatic lesions better than that of the primary tumor (18). Therefore, characterization of these CTCs can be a promising tool to select patients who are sensitive to cDDP therapy.

The primary aims of this study were to determine whether a CTC gene expression profile based on cell lines enabled the identification of patients responding to cDDP and to describe the outcomes to cDDP monotherapy in a large group of MBC patients pretreated with anthracycline- and taxane-based treatments. A secondary aim was to identify other clinical and molecular characteristics which can be used to select patients benefiting from cDDP.

2. METHODS

2.1. Cell line data

Breast cancer cell line cells were cultured in their respective growth media until near confluence before being plated in a 96-wells plate or added to 7.5mL blood of a healthy donor. The identity of all 17 cell lines used in this study were routinely validated by short tandem repeat (STR) analyses (PowerPlex 16 system, Promega, Madison, WI, USA). For determining the IC50 cisplatin sensitivity, cells were plated at a density of 1,000 to 10,000 cells per well in complete growth medium in the absence or presence of increasing concentrations of cisplatin (3x10-11 to 1x10-5 M). Cisplatin was dissolved in phosphatate-buffered saline and four days later cells were analyzed with the Sulforhodamine B (SRB) assay to quantify the percentage of cells remaining. IC50-values were calculated based on these data. Cell lines were classified based on their IC50 as cDDP sensitive (+2 standard deviation (SD) from the median) or resistant (-2 SD). Based on this classification, there were five cDDP resistant cell lines (T47D, SUM185, MM-453, CAMA-1 and BT-474) and eight sensitive cell lines (MM-468, SUM149, SUM52, SUM229, BT20, HCC-1937, UACC893 and SKBR-3, see ***Supplementary Figure 1***). To evaluate the mRNA expression profiles, 50 cells of each cell line were spiked into 7.5mL EDTA blood of a healthy donor and enriched by CellSearch as described below. For both the cisplatin IC50 determination and the generation of the cisplatin sensitivity profiles, cell lines were analyzed in at least two independent experiments.

2.2. CTC-cDDP-sensitivity profile on cell lines

To identify a CTC mRNA profile associated with outcome to cDDP, the gene expression data of our previously described panel of 93 genes (17) (as described below) were analyzed in the eight sensitive versus five resistant cell lines with the Diagonal Linear Discrimination Analysis (DLDA) Class Prediction tool (v4.4.1) of Biometric Research Branch ArrayTools (BRB-ArrayTools, http://linus.nci.nih.gov/BRB-ArrayTools.html) using $p<0.05$. The DLDA-predictor model in combination with a leave-one-out cross-validation method to compute the miss-classification rate was applied to identify a set of genes significantly differently expressed between the sensitive and resistant breast cancer cell lines to generate the CTC-sensitivity profile.

2.3. Patient data

The CTC-cDDP study (Dutch Trial Register NTR4046) was a prospective international multicenter trial in the Netherlands and Belgium. In this study, 72 MBC patients who had at least been pre-treated with anthracycline- and taxanes-based chemotherapy and were deemed fit enough for cDDP therapy by their treating physicians were included. For the complete in- and exclusion criteria see ***Supplementary Table 1***. A flowchart of

the included patients is shown in ***Figure 1***. The dose of cDDP therapy was 70 mg/m2 every three weeks and treatment continued until progression of disease, unacceptable toxicity or if patients wished to stop, with a maximum of six cycles. Treatment delay up to two weeks and dose reductions were permitted. Blood was drawn for CTC enumeration and characterization before start of cDDP therapy. Toxicity was recorded according to the National Cancer Institute Common Toxicity Criteria (CTCAE) version 4.0. Computed Tomography (CT)-scans were performed at baseline and after the second, fourth and sixth cycle and were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (19). Treatment responses according to RECIST were assessed by the radiologist of the hospital and verified by one of the authors (I.K./N.B). The study was approved by the Medical Research Ethics Committee of the Erasmus MC and local Institutional Review Boards (METC 13-007). All patients provided written informed consent.

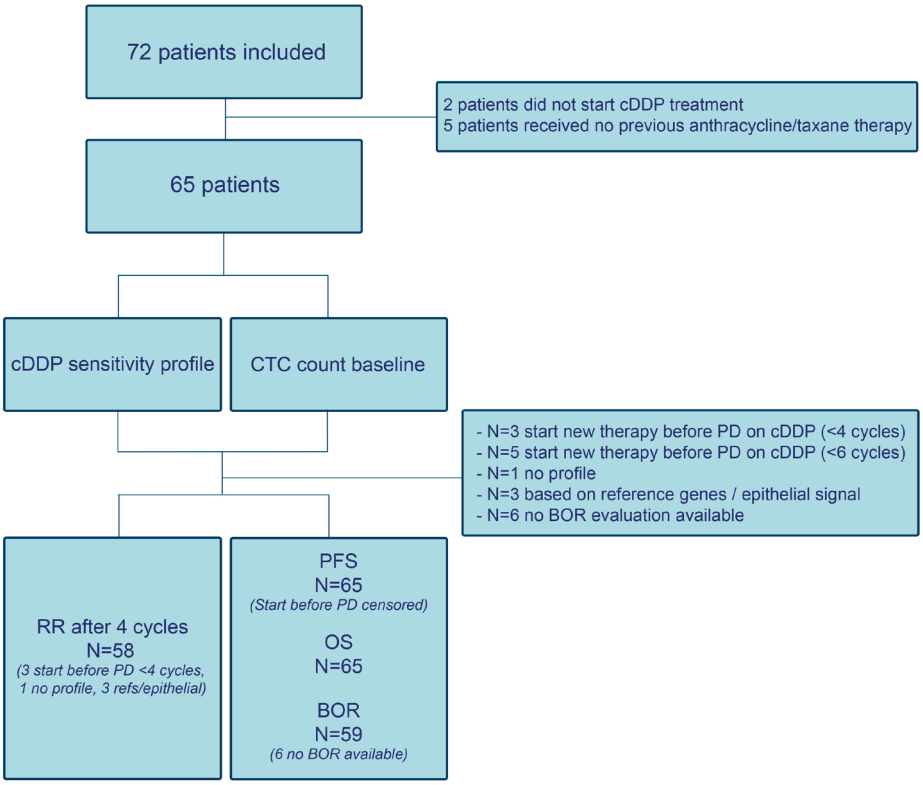

Figure 1. Flow chart
Flow chart of all patients included in the study. RR = response rate, PFS = progression-free survival, BOR = best observed response, OS = overall response, PD = progressive disease, cDDP = cisplatin.

2.4. CTC enumeration and mRNA isolation

Two tubes of blood were collected from all patients before start of cDDP treatment: 7.5mL of CellSave blood for CTC enumeration and 7.5mL EDTA blood for CTC characterization. Both tubes were processed with the CellSearch system (CellSearch enumeration kit and CellSearch profile kit; Menarini-Silicon Biosystems, Huntington Valley, PA, USA). CellSave blood was processed within 96 hours and EDTA blood within 24 hours. For CTC characterization, a detailed description has been published previously (17, 20). In short, mRNA was isolated with the AllPrep DNA/RNA Micro Kit (Qiagen, Germantown, MD, USA). Thereafter, cDNA was generated and pre-amplified for the targets of interest, and real time amplified by quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR) using Taqman Gene Expression Assays (Applied Biosystems, Carlsbad, CA, USA).

2.5. Sample processing and normalization

To establish the quality of the mRNA samples of the 17 cell lines spiked into blood as well as the 70 patient mRNA samples, three reference genes (GUSB, HMBS and HPRT1) were added to the previously described 93-gene breast cancer profile (17). If the average reference signal of a sample was ΔCq >26.5, it was considered to be of insufficient cDNA quantity and/or quality and therefore excluded (n=2). Furthermore, to ensure that the expression of the genes was CTC-specific, the 12-gene epithelial profile that was established before (17), was applied to these samples. This epithelial profile has been selected from CTC samples of 910 breast cancer samples and 20 samples from healthy blood donor (HBD) to guide the selection of samples with adequate, CTC-driven RNA signal. A cut-off of -131 ΔCq (sum of the 12 genes) was applied to select samples with at least one CTC. Samples with an epithelial cut-off below -131 were therefore excluded (n=1).

Of the 93 genes, 55 genes are known to have a higher expression in the CTC samples than in the contaminating leukocyte background that is present after isolation of CTCs with the CellSearch system (17). In the cDDP-treated patients with sufficient cDNA quantity and quality, the 93 genes were measured and the CTC-sensitivity profile determined, as was generated based on the cell line data.

2.6. Statistical analysis

The sample size for this study was based on the response to cisplatin in CTC-cDDP sensitive patients. Since RRs of 15-21% have been reported in unselected, heavily pretreated patients, a RR of 20% in the CTC-cDDP sensitive patients was deemed too low to justify further exploration (p0) in a phase III trial. A RR of approximately 60% in MBC patients with ≥5 CTCs and a favorable cDDP-sensitivity profile was considered high enough to justify further testing (p1). Applying an A'Herns single-stage phase II design to the cohort of patients with ≥5 CTCs, sufficient reference signal (ΔCq >26.5), an epithelial

profile >-131, and a favorable cDDP-sensitivity profile, with p0 = 20%; p1 = 60%, α = 0.05 and β = 0.20, implied that ≥5 out of 10 evaluable patients should achieve a response to warrant further testing. Therefore, inclusion continued until 10 evaluable patients with ≥5 CTCs and a favorable cDDP-sensitivity profile were included.

The primary endpoint of this study was the RR per RECIST of patients with a favorable CTC-cDDP profile after four cycles of cDDP. All patients who had received at least one cycle of cDDP treatment were considered for the primary objective. Patients with progressive disease (PD) at the evaluation following two cycles of cDDP were considered having PD at the primary endpoint. Patients who went off study due to toxicity before the assessment following four cycles were considered ineligible for the primary endpoint and patients who went off study prior to this assessment for reasons other than toxicity were considered as having PD. The only exception were patients who switched therapy without objectified PD on cDDP therapy. These were excluded for the primary endpoint if the new therapy was started before the fourth cycle and censored at the moment of start of the new therapy for the secondary endpoints.

Secondary endpoints included progression-free survival (PFS) and overall survival (OS). PFS was defined as the time between start of treatment and progression of disease. OS was defined as time between the start of treatment till death of any cause. We also objectified the best observed response on cDDP therapy for all patients as secondary endpoint, which is the best response during therapy recorded from the start of the study treatment until disease progression or stop of treatment (according to RECIST). This was determined as complete response (CR), partial response (PR; confirmed or unconfirmed if this was the last response measurement), stable disease (SD) longer than six weeks or progressive disease (PD). All analyses were carried out in the per-protocol population.

Survival analysis were studied with the log-rank test and visualized with Kaplan Meier plots. Furthermore, univariate and multivariate Cox proportional hazards analyses were performed. For multivariate analyses, only the significant variables (P<0.05) from univariate analyses were added to the model. All computations were performed using R (version 3.4.1) and all reported p-values are two-sided.

3. RESULTS

3.1. Preclinical cell line model

To evaluate the gene expression profiles of cell line cells with a known cisplatin sensitivity, 50 cells per cell line were spiked into EDTA blood of a healthy blood donor prior to CellSearch enrichment, RNA isolation and RT-qPCR analysis. The DLDA test resulted in the following formula to identify resistant cells based on the expression levels of 9 genes: -1.3201*KRT7-0.4157*KRT17+0.5381*ERBB3-0.488*PTRF+0.4452*TFF1+0.4281*TFF3-

0.4613*EGFR+0.37*TNRC9-1.0933*IGFBP3. Using optimal binning, a threshold of 7.9 was calculated to identify cisplatin resistant cells. Results were validated in an independent spike-in experiment encompassing the same cell line cells. The sensitivities and specificities for the discovery and validation experiments are given in ***Supplementary Table 2*** and the distribution of the cell line cells after applying our 7.9 cut-off in ***Supplementary Figure 2***. To ensure that the created CTC-sensitivity profile could also be detected in patient samples, we retrospectively looked into our CTC mRNA profiling data from previously published studies (17, 21, 22). Based on these data (n=432), the profile could be measured in around 35% of the patients with ≥5 CTCs present.

3.2. Patient and cycle characteristics

In total, 72 patients signed informed consent for this study. Two patients did not start cDDP therapy due to rapid deteriorating clinical condition; five patients did not previously receive anthracycline and/or taxanes therapy. Consequently, per-protocol analysis was performed on 65 patients. Of these, 72% had ER (estrogen receptor)-positive breast cancer, the others had triple-negative breast cancer (TNBC). Most patients (77%) had already received ≥2 lines of chemotherapy for metastatic disease. Full patient characteristics are presented in ***Table 1***. The median number of cDDP cycles these patients received was three (range 1-6). In total, 14 patients (22%) completed all six cycles of cDDP. There were nine patients who had to stop treatment due to toxicity and six patients who wanted to stop treatment in general, not due to objectified toxicity.

3.3. Response to cDDP in patients with favorable cDDP-sensitivity profile

The primary aim of this study was to determine if the CTC-sensitivity profile we determined in cell lines could predict the RR after four cycles of cDDP therapy. Seven patients could not be evaluated for the primary objective: in four patients the sensitivity profile could not be determined (lack of mRNA quality (n=3) or EDTA blood had not arrived <24 hours (n=1)), and three patients received a new therapy before they had progression on cDDP therapy. Of the 58 eligible patients, ten patients had ≥5 CTCs and a favorable CTC-sensitivity profile. None of these patients had a response after four cycles of cDDP therapy. Median PFS in these patients was 2.0 months (95%CI 0.47 -3.47) and median OS 3.1 months (95%CI 0.66-5.52). The best observed response was SD in 50% (5/10) of the patients.

Median PFS in all 58 patients was 2.5 months (95%CI 1.84-3.16) and median OS 6.9 months (95%CI 3.80-9.94). The CTC-sensitivity profile in relation to PFS and OS is shown in ***Supplementary figure 3***.

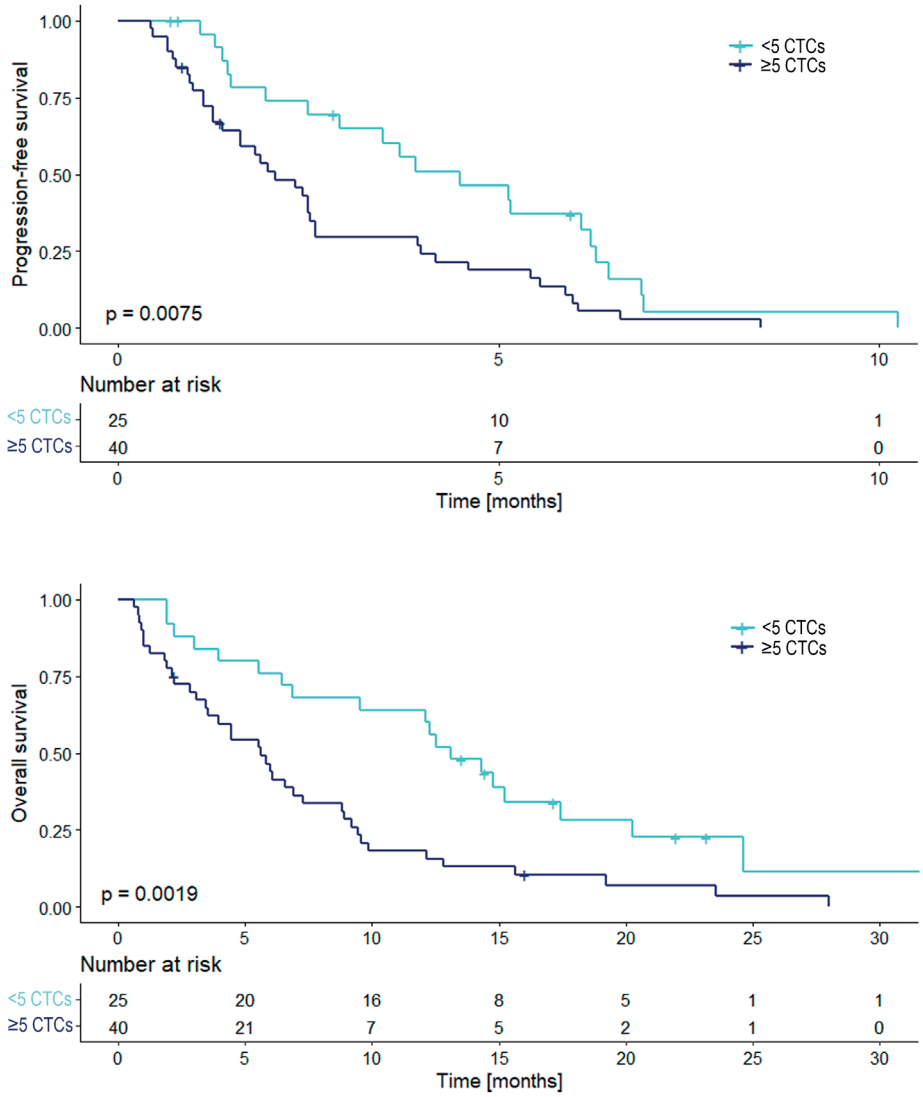

Figure 2. PFS and OS in relation to the CTC count (n=65)
Kaplan Meier curves of (**A**) progression-free survival (PFS) and (**B**) overall survival (OS) in relation to CTC count at baseline. CTC counts are divided into two categories of < 5 CTCs and ≥ 5 CTCs.

3.4. Outcomes in the full cohort

Six patients were non-evaluable for the best observed response, as they had to stop cDDP treatment due to toxicity, leaving 59 evaluable patients. The best observed response was a PR 7%, while 56% had SD and 32% experienced PD (5% not evaluable). Median PFS and OS for all cDDP-treated 65 patients was 2.5 months (95%CI 2.21-2.79) and 6.9 months (95%CI 4.08-9.78), respectively.

Table 1. Patient characteristics (*n*=65)

	N	%		N	%
Age			**BRCA mutation**		
≤ 40	6	9.2	Positive	8	12.3
41-55	24	36.9	Negative	16	24.6
> 55	35	53.9	Unknown	41	63.1
WHO performance status			**Previous (neo)adjuvant chemotherapy**		
0	16	24.6	Yes	45	69.2
1	46	70.8	None	20	30.8
2	3	4.6			
			Previous adjuvant endocrine therapy		
Menopausal status			Yes	30	46.2
Premenopausal	10	15.4	None	35	53.8
Perimenopausal	9	13.9			
Postmenopausal	45	69.2	**Number of previous palliative chemotherapy agents**		
Unknown	1	1.5	0	5	7.7
			1	10	15.4
BR grade			2	18	27.7
1	0	0.0	3	17	26.2
2	16	24.6	4	10	15.4
3	27	41.5	5	3	4.6
Unknown	22	33.9	6	2	3.1
ER status			**Number of previous palliative endocrine agents**		
Positive	47	72.3	0	27	41.5
Negative	18	27.7	1	11	16.9
			2	14	21.5
PR status			3	5	7.7
Positive	33	50.8	4	5	7.7
Negative	32	49.2	5	2	3.1
			6	1	1.5
HER2 status					
Positive	2	3.1	**PARP-inhibitor received previously**		
Negative	62	95.4	Yes	5	7.7
Unknown	1	1.5	None	60	92.3
Subtype					
ER+/HER2-	44	67.7			
ER+/HER2+	2	3.1			
Triple negative	18	27.7			
Unknown	1	1.5			

Patient characteristics for all 65 patients. ER = estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor 2.

The median number of CTCs at baseline was 8 (range 0-3254) in all 65 patients. The patients were divided into two groups: <5 CTCs (n=25) and ≥5 CTCs (n=40). Comparing these two groups showed that patients with <5 CTCs had a significantly longer PFS and OS than patients with ≥5 CTCs (HR 2.10, 95%CI 1.21-3.65, p=0.009 and HR 2.38, 95%CI 1.36-4.18, p=0.003 respectively; ***Figure 2***).

3.5. Evaluation of other prognostic factors

Established prognostic factors (CTC count (<5 CTCs/≥5 CTCs), subtype, BRCA-status, BR (Bloom-Richardson) grade, previous lines of palliative chemotherapy, previous lines of palliative endocrine therapy, presence of visceral metastasis, WHO status and age) were compared in relation to PFS and OS. Only a CTC count of ≥5 was associated with a shorter PFS in univariate analysis (HR 2.10, 95%CI 1.21-3.65, p=0.009, see ***Table 2a***). Therefore, no multivariate regression analysis could be performed. For OS, in univariate analysis CTC count and the previous lines of palliative chemotherapies were associated with outcome. When adding these variables to the multivariate analysis, both were independent prognostic factors for OS (CTC count ≥5 (HR 2.22, 95%CI 1.26-3.90, p=0.006) and higher number of palliative chemotherapies (HR 1.96, 95%CI 1.12-3.44, p=0.019)) (see ***Table 2b***).

As shown in the univariate analysis, there was no difference in PFS (p=0.373) nor OS (p=0.928) between the patients with TNBC and ER+ primary breast cancer in relation to cDDP therapy. Median PFS in the ER+ patients (n=47) was 2.5 months (95%CI 1.83-3.17) and median OS 7.3 months (95%CI 3.68-10.90). TNBC patients (n=18) had a median PFS of 2.9 months (95%CI 1.66-4.18) and OS of 6.1 months (95%CI 5.12-7.04) (***Supplementary Figure 4***). The BRCA status was known in 24 patients. Between BRCA-positive (n=8) and BRCA-negative (n=16) patients, no difference was found in PFS (p=0.119) and OS (p=0.200). Median PFS in the 8 patients with a known BRCA-mutation was 4.5 months (95%CI 0.00-10.47) and median OS was 9.6 months (95%CI 0.00-20.98). For the 16 patients without BRCA-mutation, median PFS was 2.6 months (95%CI 2.46-2.74) and median OS 6.6 months (95%CI 5.26-7.94).

3.6. Toxicity of cDDP therapy

All serious adverse events (SAEs) and all adverse events (AEs) of grade ≥3 were reported in all patients who received ≥1cycle of cDDP (n=65). In total, 119 SAEs were reported; in 27 patients, no SAEs were reported. The following SAEs were reported five times or more: nausea, dyspnea, acute kidney failure, anemia and hypercalcemia. A line listing of all SAEs is shown in ***Supplementary Table 3***. Of the 119 reported SAEs, only 12 of the SAEs (10%) were grade 3 or higher and related to cDDP treatment.

Table 2. Univariate and multivariate Cox Regression analysis

(A)	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	p-value	HR	95%CI	p-value
CTC count	**2.098**	**1.21-3.65**	**0.009**	**2.098**	**1.21-3.65**	**0.009**
Subtype	0.765	0.42-1.38	0.373			
BRCA	0.464	0.18-1.22	0.119			
BR grade	0.677	0.35-1.32	0.251			
Palliative chemo	0.949	0.57-1.60	0.844			
Palliative endo	0.820	0.49-1.39	0.458			
Visceral metastases	0.846	0.36-1.99	0.700			
WHO	0.998	0.56-1.78	0.995			
Age	1.022	0.99-1.05	0.145			

(B)	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	p-value	HR	95%CI	p-value
CTC count	**2.381**	**1.36-4.18**	**0.003**	**2.219**	**1.26-3.90**	**0.006**
Subtype	0.974	0.54-1.74	0.928			
BRCA	0.512	0.18-1.43	0.200			
BR grade	0.568	0.29-1.11	0.100			
Palliative chemo	**2.139**	**1.22-3.75**	**0.008**	**1.958**	**1.12-3.44**	**0.019**
Palliative endo	0.800	0.47-1.36	0.411			
Visceral metastases	1.522	0.65-3.59	0.338			
WHO	0.955	0.51-1.79	0.885			
Age	1.026	1.00-1.06	0.060			

Univariate and multivariate Cox regression analysis (n=65). (**A**) shows all variables in relation to PFS and (**B**) in relation to OS. CTC count was analyzed as dichotomized variable (<5 CTCs / ≥5 CTCs) and age as continuous variable. For subtype patients were divided in ER+ versus TNBC and for BR (Bloom-Richardson) grade all patients were grade 2 or 3. Palliative chemotherapy was divided in 0-2 and 3-6 lines of chemotherapy for advanced breast cancer. Palliative endocrine therapies were divided in 0-1 and 2-6 lines of endocrine therapies for advanced breast cancer. WHO stands for WHO performance status and was divided in WHO 0 or WHO 1-2. HR = hazard ratio.

4. DISCUSSION

The data presented here shows that the CTC-cDDP-sensitivity profile was unable to select patients who will respond to cDDP treatment. None of the patients with a favorable profile had a response to cDDP therapy. The CTC-cDDP-sensitivity profile was generated based on 17 breast cancer cell lines which were thought to represent the clinical breast cancer subtypes. However, it could be that these cell lines were not representative enough. Also, since breast cancer is a heterogeneous disease, it can be difficult to generate a profile that predicts response for all breast cancer subtypes. Furthermore,

only 55 genes in the measured CTC mRNA profile were CTC-specific. This selection of genes might have been too limited for accurate prediction of cDDP sensitivity, or relevant genes related to cDDP sensitivity might have been excluded from the mRNA profile because their expression in CTCs does not significantly exceed their expression in leukocytes. Measuring gene expression in single or a collection of pure CTCs (23) could give a more comprehensive and reliable sensitivity profile.

Despite the failure to meet the primary endpoint, to the best of our knowledge, this is the largest group of MBC patients treated with cDDP monotherapy thus far. A few studies have investigated cDDP monotherapy for breast cancer in the neoadjuvant or metastatic setting, but these were all smaller (3-7, 24-28). In these studies, a variety of RRs have been reported. In patients who received prior treatment for metastatic disease (patients were treated with cDDP in the second to fifth line of therapy), average RRs were 9% (range 0-21%) (5, 6, 26-28), which is comparable with the 7% PR as best observed response in our study.

In our study, 33 (56%) patients had SD as best observed response. The median PFS of all patients was 2.5 months and the median OS 6.9 months, which is as expected in this heavily pretreated group of patients. Data from Cortes and colleagues is closest to our cohort of patients for comparing outcome to cDDP to other treatments given in this setting (2). They investigated eribulin treatment (n=503) versus treatment of choice of the treating physician (TPC) in heavily pretreated patients with locally recurrent or metastatic breast cancer. This TPC (n=247) consisted of 25% vinorelbine, 19% gemcitabine, 18% capecitabine, 15% taxanes, 10% anthracyclines and 10% other chemotherapies. In the eribulin group RRs of 12% were found and in the TPC group of 5%. Stable disease was found in 44% of the eribulin group and in 45% of the TPC group. Median PFS for eribulin was 3.7 months and the median PFS in the TPC group was 2.2 months (2). So, comparing this to our data, similar RRs were found for cDDP treatment in heavily pretreated patients.

In the search for markers which predict response to cDDP therapy, impact of the tumor subtypes on outcome was assessed in exploratory analysis. As commonly done, these subtypes were determined on primary tumor tissue. It should be kept in mind that during the course of disease and under treatment pressure the molecular characteristics determining the subtypes can change. To the best of our knowledge, we are the first to measure CTCs in heavily pretreated MBC patients who received cDDP therapy. In accordance with data from MBC patients who were not heavily pretreated (13), CTCs were an independent prognostic marker for both PFS and OS in our set of MBC patients receiving cDDP. While literature shows that patients with TNBC and/or a BRCA1 mutation may have a better response to platinum treatment with RRs up to 80% (24, 25, 29), our data did not show an improved PFS or OS in TNBC patients nor in BRCA-positive patients. However, for the majority (41 out of 65 (63%)) of the patients the BRCA status

was unknown, resulting in a very low power to detect an effect. For future research, it would be interesting to investigate in a set of BRCA mutation carriers whether a gene expression profile in CTCs can discriminate patients with a good from those with a poor outcome. And also, it would be interesting to look at homologous recombination deficiency (HRD) since HRD can identify TNBC tumors that are more likely to respond to platinum-containing therapies (30).

As mentioned before, toxicity might be one of the reasons that cDDP is not widely considered as a treatment option in MBC. Treatment with cDDP in this study seemed to be tolerable with 9% (6/65) of the patients discontinuing cDDP treatment due to objectified toxicity and 10% of the patients experiencing grade 3-4 toxicity related to the cDDP treatment.

5. CONCLUSIONS

In conclusion, the CTC-cDDP-sensitivity profile derived from breast cancer cell lines was unable to select patients responding to cDDP therapy. In an unselected group of heavily pretreated MBC patients, cDDP monotherapy yields outcomes comparable to the outcomes achieved with other regimens which are used in this setting. Furthermore, the prognostic value of CTC enumeration was also found in cDDP-treated MBC patients. Further studies are needed to identify biomarkers which can be used in the clinic to specifically select patients for platinum-compounds.

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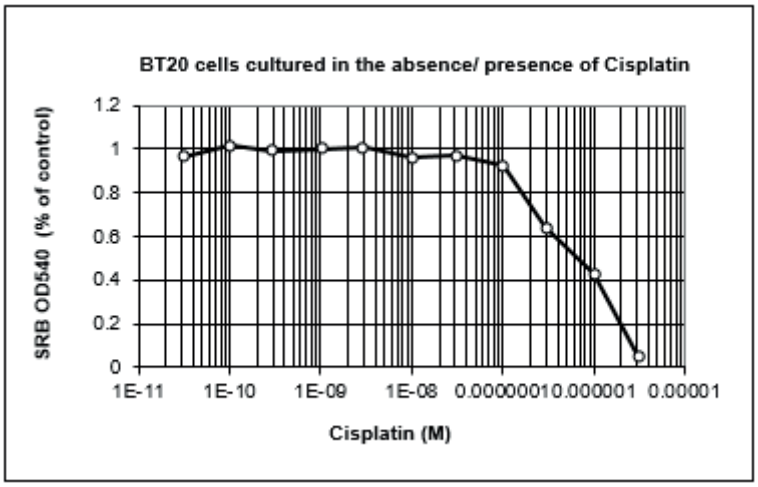
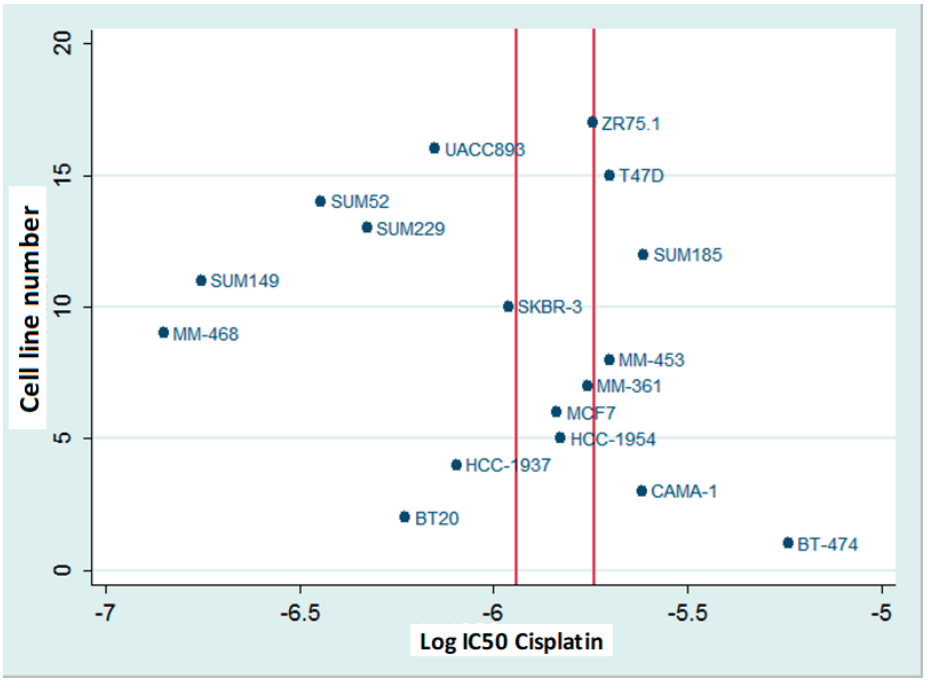
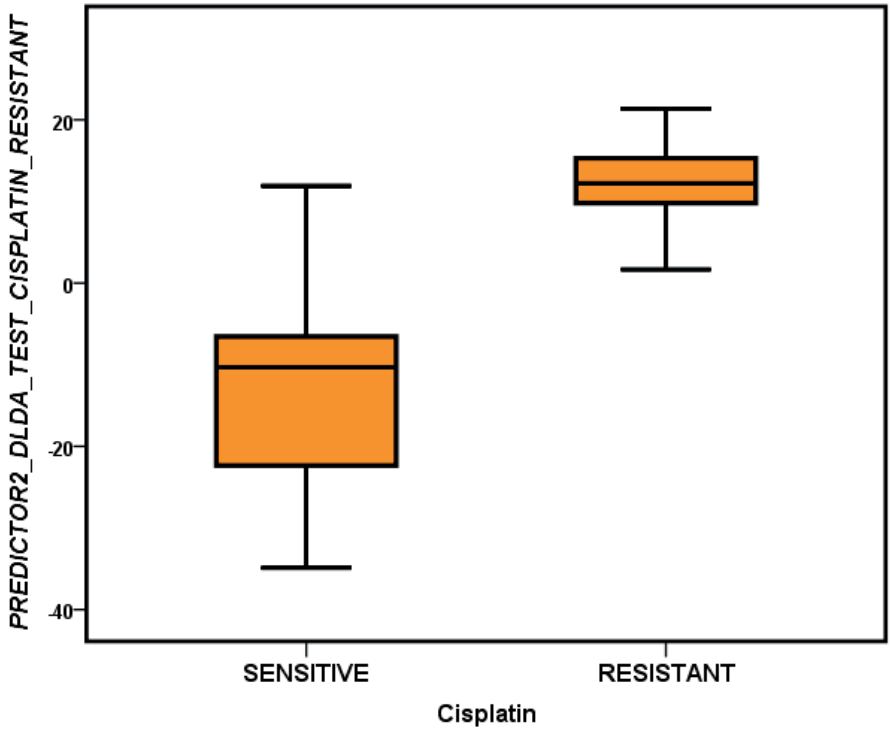
SUPPLEMENTARY MATERIALS

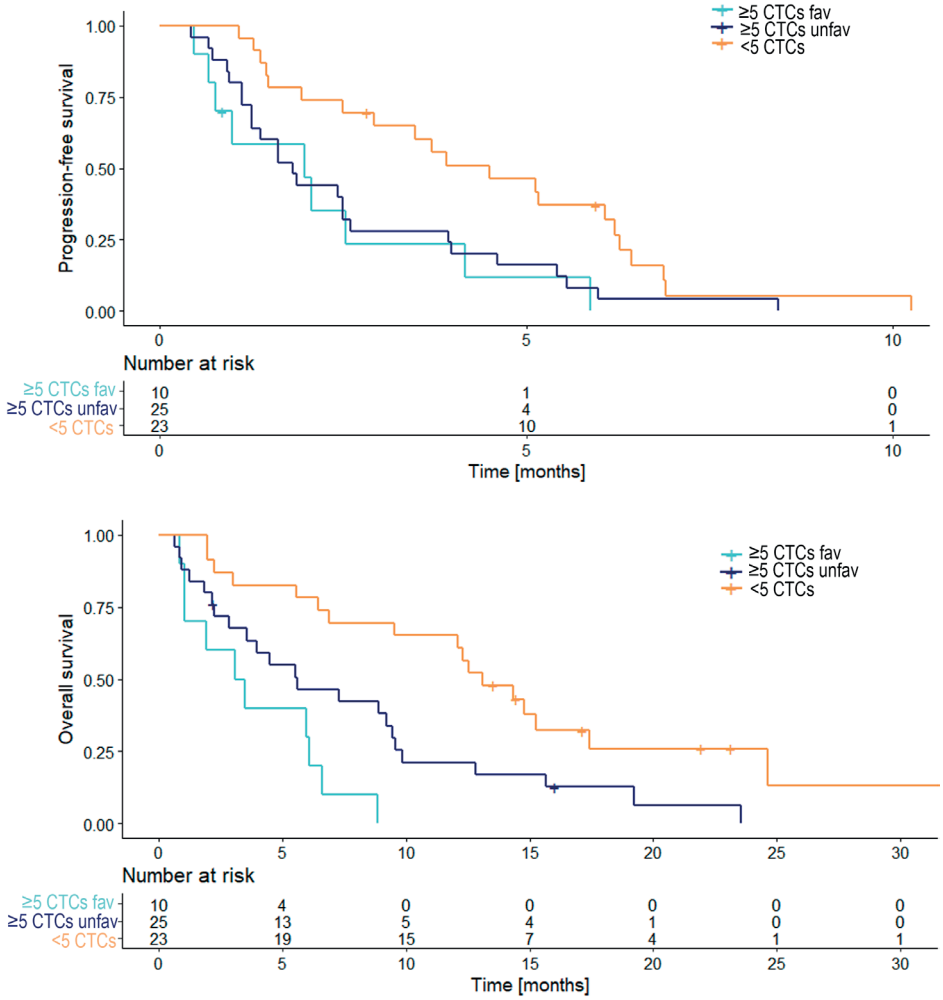




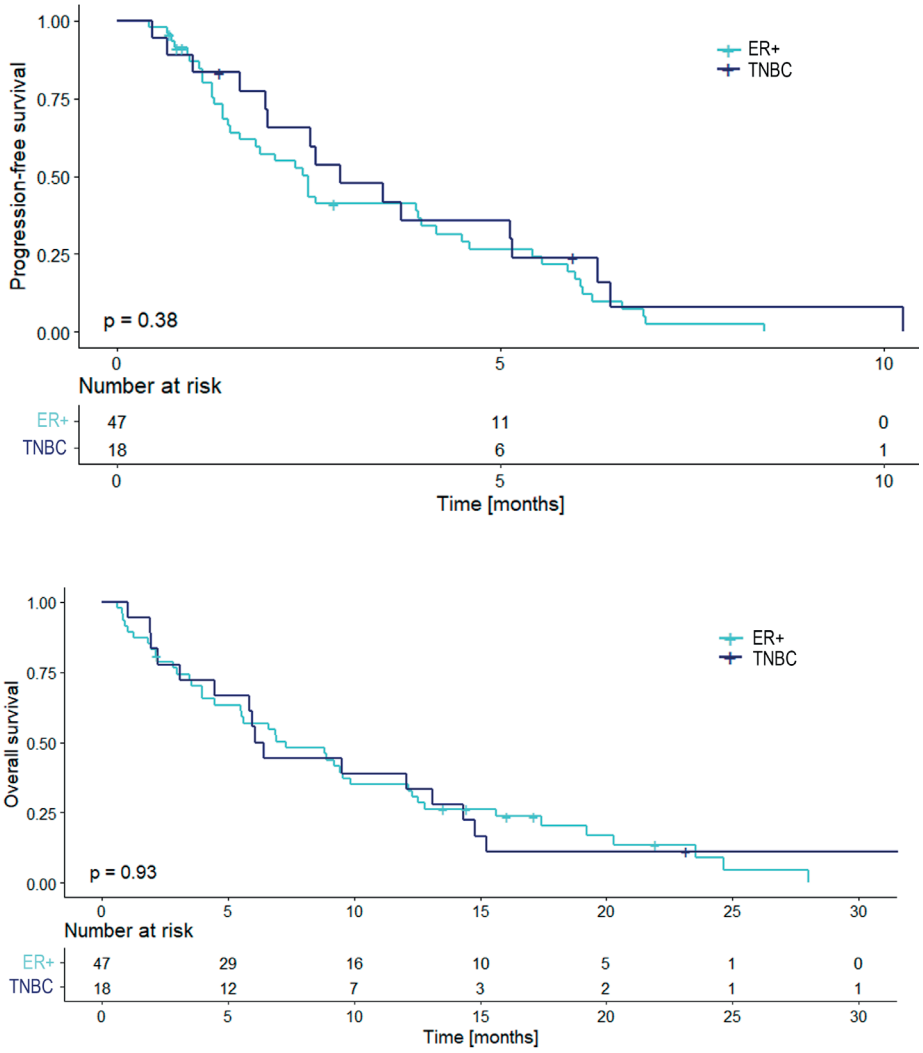
Supplementary Figure 1. Cisplatin sensitivity of cultured cell line cells
Cells were cultured in their respective growth medium in the absence and presence of increasing concentrations of cisplatin. (**A**) an example of estimating cisplatin sensitivity in BT20 cells. (**B**) the resulting data of all cell lines summarized. For this, the mean IC50 data of two independent cell culture experiments was used. Cell lines outside the median ± 2SD, depicted by the red lines, were considered to be sensitive (at the left side) or insensitive/resistant (right side). The cell line cells between the lines with inconclusive data were not further explored in our spike-in experiments.



Supplementary Figure 2. Distribution of the cell lines
Distribution of the sensitive and resistant cell lines based on the predictor generated with the DLDA test.



Supplementary Figure 3. PFS and OS in relation to the CTC-sensitivity profile (*n*=58)
Kaplan Meier curves of (**A**) progression-free survival (PFS) and (**B**) overall survival (OS) in relation to the CTC-cDDP-sensitivity profile. Patients with ≥5 CTCs were divided into the favorable and unfavorable group. Furthermore, patients with <5 CTCs are depicted.



Supplementary Figure 4. PFS and OS in relation to the breast cancer subtypes (*n*=65)
Kaplan Meier curves of (**A**) progression-free survival (PFS) and (**B**) overall survival (OS) in relation to the breast cancer subtypes. In light blue the ER-positive patients are depicted, while in dark blue patients with triple negative breast cancer are shown.

Supplementary Table 1. In- and exclusion criteria

Population
Female patients with metastatic breast cancer who have been pretreated with at least anthracycline- and taxane-based chemotherapy in the adjuvant and/or metastatic setting
Inclusion criteria
Measurable disease according to RECIST 1.1
Age ≥ 18 years
WHO performance status ≤2
Adequate haematological functions defined as ANC ≥ 1.0 x 109/L, platelets ≥ 100 x 109/L
Adequate renal function defined as creatinin clearance ≥ 60 mL/min (Cockcroft Gault)
Patients with reproductive potential must use a reliable method of contraception
Written informed consent
Exclusion criteria
Other anticancer chemotherapy, use of biological response modifiers, or immunotherapy within two weeks prior to treatment start
Hormonal antitumor treatment within one week prior to treatment start
Hearing loss of at least Common Terminology Criteria for Adverse Events (CTCAE) grade 2
Neuropathy of at least CTCAE grade 2
Pregnant or lactating patients
Serious illness or medical unstable condition prohibiting adequate treatment and follow-up
Symptomatic CNS metastases
History of psychiatric disorder that would prohibit the understanding and giving of informed consent or adequate follow-up

Supplementary Table 2. Sensitivity and specificity discovery and validation CTC-cDDP-sensitivity profile

PREDICTOR_DLDA_TEST CISPLATIN	DISCOVERY	VALIDATION
Sensitivity	100.00%	85.71%
Specificity	100.00%	85.71%
Positive predictive value	100.00%	85.71%
Negative predictive value	100.00%	85.71%
Disease prevalence	38.46%	50.00%

Supplementary Table 3. Line listing of all SAEs

Patient	Description	Cycle	Worst grade	SAE	SUSAR	Related
P01	No SAE's					
P02	Congestive heart failure	1	3	Yes	No	No
P03	Pneumonia	2	3	Yes	No	No
	Post obstruction pneumonia	3	3	Yes	No	No
P04	No SAE's					
P05	Increased Gamma-GT	3	3	Yes	No	No
P06	No SAE's					
P07	No SAE's					
P08	No SAE's					
P09	**Acute kidney failure**	**2**	**2**	**Yes**	**No**	**Yes**
	Urinary tract infection	2	2	Yes	No	No
P10	**Anorexia**	**2**	**2**	**Yes**	**No**	**Yes**
	Hypoglycemia	2	4	Yes	No	No
	Nausea	**2**	**2**	**Yes**	**No**	**Yes**
	Pain arm with sensory disorder	2	2	Yes	No	No
	Weight loss	**2**	**2**	**Yes**	**No**	**Yes**
P11	Muscle weakness right arm	Baseline	3	Yes	No	NA
	Nausea	3	3	Yes	No	No
	Vomiting	3	3	Yes	No	No
P12	No SAE's					
P13	No SAE's					
P14	Anemia	1	3	Yes	No	No
	Pleuritic pain	1	3	Yes	No	No
	Nausea	1	2	Yes	No	No
	Febrile neutropenia	2	3	Yes	No	No
	Thrombocytopenia	2	4	Yes	No	No
	Nausea	2	2	Yes	No	No
	Hepatic failure	2	3	Yes	No	No
P15	No SAE's					
P16	Hypokalemia	1	3	Yes	No	No
	Sepsis	1	4	Yes	No	No
	Dyspnea	1	3	Yes	No	No
	Thromboembolic event	1	2	Yes	No	No
P17	Nausea	6	3	Yes	No	No
P18	No SAE's					
P19	Diarrhea	1	2	Yes	No	No
	Nausea	1	2	Yes	No	No
P20	No SAE's					

Supplementary Table 3. Line listing of all SAEs (continued)

Patient	Description	Cycle	Worst grade	SAE	SUSAR	Related
P21	Dyspnea	1	3	Yes	No	No
	Anemia	1	2	Yes	No	No
P22	Back pain	1	3	Yes	No	No
	Hypotension	1	2	Yes	No	No
	Nausea	**1**	**2**	**Yes**	**No**	**Yes**
	Hypotension	2	1	Yes	No	No
	Nausea	**2**	**1**	**Yes**	**No**	**Yes**
P23	Fatigue	1	3	Yes	No	No
	Dyspnea	1	3	Yes	No	No
P24	Abdominal pain	1	2	Yes	No	No
	Agitation	1	2	Yes	No	No
	Fecal incontinence	1	1	Yes	No	No
	Fever	1	1	Yes	No	No
	Pain thorax	1	2	Yes	No	No
	Urine incontinence	1	2	Yes	No	No
P25	No SAE's					
P26	No SAE's					
P27	No SAE's					
P28	No SAE's					
P29	Dehydration	4	4	Yes	No	No
P30	Acute kidney failure	4	1	Yes	No	No
	Fever	4	1	Yes	No	No
	Hypercalcemia	4	1	Yes	No	No
P31	Hyponatremia	1	3	Yes	No	No
	Acute kidney failure	1	1	Yes	No	No
P32	No SAE's					
P33	Abdominal pain	5	3	Yes	No	No
	Hypokalemia	**5**	**3**	**Yes**	**No**	**Yes**
	Hypomagnesemia	**5**	**1**	**Yes**	**No**	**Yes**
P34	Thromboembolic event	3	3	Yes	No	No
P35	No SAE's					
P36	Hyperglycaemia	1	3	Yes	No	No
	Acute kidney failure	3	2	Yes	No	No
	Acute kidney failure	4	2	Yes	No	No
	Nausea	4	2	Yes	No	No
	Vomiting	4	2	Yes	No	No
P37	Fever	Baseline	1	Yes	NA	NA
	Nausea	Baseline	2	Yes	NA	NA

Supplementary Table 3. Line listing of all SAEs (continued)

Patient	Description	Cycle	Worst grade	SAE	SUSAR	Related
	Hypercalcemia	Baseline	2	Yes	NA	NA
	Acute kidney failure	Baseline	1	Yes	NA	NA
P38	Back pain	1	3	Yes	No	No
	Radicular pain left leg	1	2	Yes	No	No
	Nausea	1	2	Yes	No	No
P39	**Acute kidney failure**	**2**	**2**	**Yes**	**No**	**Yes**
	Anemia	2	3	Yes	No	No
	Bleeding lower tr. digestivus	2	4	Yes	No	No
	Hypocalcemia	**2**	**3**	**Yes**	**No**	**Yes**
	Hypokalemia	**2**	**3**	**Yes**	**No**	**Yes**
	Hypotension	2	4	Yes	No	No
	Reversible posterior leukoencephalopathy syndrome	**2**	**4**	**Yes**	**No**	**Yes**
	Thrombocytopenia	2	4	Yes	No	No
	Mucositis	**2**	**3**	**Yes**	**No**	**Yes**
P40	Hypercalcaemia	Baseline	3	Yes	NA	NA
	Impending femur fracture	Baseline	3	Yes	NA	NA
	Back pain	Baseline	2	Yes	NA	NA
	Hypercalcaemia	1	3	Yes	No	No
	Nausea	1	3	Yes	No	No
	Hypercalcaemia	2	3	Yes	No	No
	Nausea	2	3	Yes	No	No
P41	No SAE's					
P42	Posterior vitreous detachment	1	1	Yes	No	No
P43	Anemia	Baseline	3	Yes	NA	NA
	Radicular back pain	Baseline	3	Yes	NA	NA
	Anemia	1	3	Yes	No	No
	Dyspnea	1	3	Yes	No	No
	Pleural effusion	1	3	Yes	No	No
P44	Congestive heart failure	1	3	Yes	No	No
	Acute kidney failure	**1**	**1**	**Yes**	**No**	**Yes**
	Hypokalemia	1	1	Yes	No	No
P45	Cough	Baseline	2	Yes	NA	NA
	Hoarseness	Baseline	2	Yes	NA	NA
	Pain lymph node metastasis	Baseline	2	Yes	NA	NA
	Constipation	1	2	Yes	No	No
P46	**Hyponatremia**	**1**	**3**	**Yes**	**No**	**Yes**
	Vomiting	**1**	**2**	**Yes**	**No**	**Yes**

Supplementary Table 3. Line listing of all SAEs (continued)

Patient	Description	Cycle	Worst grade	SAE	SUSAR	Related
P47	Urinary tract infection	1	3	Yes	No	No
	Hyponatremia	1	3	Yes	No	No
	Thrombocytopenia	**1**	**3**	**Yes**	**No**	**Yes**
	Acute kidney failure	1	1	Yes	No	No
	Dizziness	**2**	**2**	**Yes**	**No**	**Yes**
P48	No SAE's					
P49	Pleural effusion	Baseline	3	Yes	NA	NA
	Palpitations	1	1	Yes	No	No
	Nausea	**1**	**3**	**Yes**	**No**	**Yes**
	Anorexia	**1**	**3**	**Yes**	**No**	**Yes**
	Fatigue	**1**	**3**	**Yes**	**No**	**Yes**
P50	No SAE's					
P51	Abdominal pain	1	2	Yes	No	No
P52	**Hearing loss**	**4**	**2**	**Yes**	**No**	**Yes**
P53	No SAE's					
P54	Hematuria	Baseline	3	Yes	NA	NA
	Acute kidney failure	**1**	**4**	**Yes**	**No**	**Yes**
	Ascites	1	3	Yes	No	No
P55	Thromboembolic event	1	2	Yes	No	No
P56	**Nausea**	**2**	**3**	**Yes**	**No**	**Yes**
	Pleural effusion	2	2	Yes	No	No
	Ascites	2	2	Yes	No	No
P57	No SAE's					
P58	No SAE's					
P59	Anemia	Baseline	2	Yes	NA	NA
	Leg pain	2	2	Yes	No	No
P60	No SAE's					
P61	No SAE's					
P62	No SAE's					
P63	No SAE's					
P64	No SAE's					
P65	Dyspnea	1	2	Yes	No	No
	Cough	1	2	Yes	No	No

Line listing of all found SAEs (serious adverse events) in the participating patients. Patient numbers are assigned randomly. Only the bold indicated SAEs are related to cDDP therapy. NA = not applicable (baseline SAEs cannot be a SUSAR (suspected unexpected serious adverse reaction) or related with cDDP since these are present before the start of therapy).

Chapter 7

General discussion and future perspectives

INTRODUCTION

The focus of this thesis was on characterization of tumor cells and exploring the opportunities that liquid biopsies can offer, as well as the limitations of their use. For characterization of tumor cells and evaluation of prognostic and predictive markers, a biopsy of primary or metastatic tissue is the golden standard. However taking a biopsy of particularly metastatic tissues can be a cumbersome procedure with the risk of infection or bleeding. In addition, not all tumor sites are amenable for a tissue biopsy. Liquid biopsies are minimally invasive, can be used to obtain an up-to-date status of tumor characteristics, and may allow real-time monitoring of tumor burden during the course of the disease. The latter is frankly impossible using tumor biopsies. Since a progressing tumor or a tumor under treatment pressure due to (epi)genetic heterogeneity can evolve, it is important to establish an up-to-date and, preferably, complete picture of the tumor characteristics before a subsequent personalized treatment decision is made. Therefore, liquid biopsies are a promising tool to apply in cancer management, which includes determination of heterogeneity, prediction of efficacy or (early) relapse during treatment, determination of the disease burden, determination of prognosis, identification of (genetic) targets for therapy and guidance for treatment selection. Furthermore, numerous studies have been performed to investigate if liquid biopsies can be used in early detection or as diagnostic tool, however, sensitivity still remains an issue, especially for detection of early stage tumors (49, 50). However, when comparing enumeration of circulating tumor cells (CTCs) to radiological imaging, CTCs inform about disease status at an earlier time point and are a more robust predictor of survival status (51). Moreover, adding CTC counts to current clinicopathological predictive models in metastatic breast cancer (mBC) patients, CTCs add prognostic impact while established serum derived tumor makers fail to do so (18). Nonetheless, CTC numbers in particular and liquid biopsies in general are only sporadically used in clinical practice. So, while most of the research performed seems promising for the application of liquid biopsies, their clinical implementation is still ahead of us, since there are no studies that show the clinical utility of liquid biopsies. Here, the opportunities and limitations of liquid biopsies will be discussed based on the chapters of this thesis.

ENUMERATION OF CIRCULATING TUMOR CELLS

In metastatic breast cancer (mBC) patients, CTCs are found in 60-70% of the patients (17, 52) and in mCRPC this is in up to 90% of the patients (53). The median number of CTCs detected are 3-5 in mBC and 7-9 in mCRPC (18, 52, 54, 55). In the non-metastatic setting, these numbers are much lower (52), probably since there is less tumor burden in these

patients. Since not in all patients CellSearch CTCs are found, and the number of CTCs found is low, improvement of CTC detection is a constant topic of investigation.

Circulating tumor cells and EMT

Epithelial tumor tissues giving rise to CTCs are hypothesized to undergo epithelial-to-mesenchymal transition (EMT) during the process of dissemination from primary or metastatic sites to gain migratory and invasive properties. During this process, epithelial features are downregulated while mesenchymal characteristics are gained (56). When looking into the characteristics of CTCs, enrichment of cells with mesenchymal markers has been reported. Also, biphenotypic cells, which are rare in the primary breast tumor, are reported to be enriched in CTCs of breast cancer patients (57-59). Since EpCAM is an epithelial marker and the CellSearch system is dependent on EpCAM for the isolation of CTCs, one of the fields of interest for increasing CTC detection is to find new or additional markers for CTC detection and isolation (60, 61). Using EpCAM for detection of CTCs especially hampers the isolation of normal-like/claudin-low breast cancer CTCs, which are considered to be more aggressive (46). In most cases, breast cancer cell lines that lack EpCAM expression do express CD146 (47). Furthermore, CD146 is especially known for its role in EMT, tumor aggressiveness and resistance against tamoxifen therapy. As is shown in **chapter 2** of this thesis, CD146 is present in 11% of the primary breast cancer tissues and could therefore be an interesting marker for CTC detection and/or isolation. In primary breast cancer patients, CTC detection increased from 18% to 25% at baseline and from 16% to 30% after one cycle of neo-adjuvant chemotherapy (NAC) with the addition of CD146 as marker for CTC detection to EpCAM (62). However, the results from **chapter 2** suggested there was no inverse relationship between CD146 and EpCAM expression in any of the primary tumors of all investigated breast cancer subtypes. Thus, while in cell lines the association between CD146 and EMT is clear, in primary breast cancers it is not clear-cut that CD146 expression is indicative for EMT. So, the question remains what the prognostic value is of CTCs detected with other markers than EpCAM. Furthermore, there are also observations that it is not necessary for cells to undergo EMT to gain metastatic capabilities (63). Although the median number of EpCAM-positive CTCs detected with the CellSearch system remains low, these CTCs are proven prognostic in multiple independent studies (see also **chapter 5** and **chapter 6** of this thesis) and seem a biologically relevant population of cells. Therefore, the question remains if EpCAM-negative or EpCAM low CTCs can be detected and what their clinical value is.

Circulating tumor cells and EpCAM expression

Since EpCAM seems to be heterogeneously expressed in breast cancer (64), there are studies that looked into EpCAM-negative or EpCAM-low CTCs. For instance Terstappen et al. of the University of Twente (65) discovered a way to investigate the blood waste

that was discarded after depletion of EpCAM CTCs by the CellSearch system. This blood, depleted of regular CTCs, was filtrated on a microsieve, stained with CD45 and a pan-CK cocktail and visualized with a fluorescent microscope. EpCAM^{low} cells were scored when they were DAPI-positive, CK-positive and CD45-negative. First, they analyzed 108 mCRPC patients and 22 mBC patients samples for the presence of EpCAM^{high} and EpCAM^{low} CTCs. Of these, 53% of the mCRPC patients and 32% of the mBC patients had ≥5 EpCAM^{high} CTCs. However, in 28% of the mCRPC and in 36% of the mBC patients ≥5 EpCAM^{low} CTCs were present. While patients with ≥5 EpCAM^{high} CTCs as expected showed shorter overall survival (OS) than those with <5 EpCAM^{high} CTCs, the presence of EpCAM^{low} CTCs showed no association with OS of these patients (66). They also performed this filtration on EpCAM CTC-depleted blood of 97 NSCLC (non-small cell lung cancer) patients. Of these, 21% had ≥2 EpCAM^{high} CTCs and 15% ≥2 EpCAM^{low} CTCs. Also in these patients, the presence of EpCAM^{high} CTCs was associated with poor OS, but the presence of EpCAM^{low} CTCs was not (67). As discussed in **chapter 3**, not all non-metastatic bladder cancer tissues express EpCAM (68), therefore, there have been attempts to isolate CTCs in bladder cancer patients with other systems than the CellSearch. Indeed, there are studies that find CTCs with other phenotypic definitions than CellSearch (69). However, when EpCAM is excluded, specificity may be compromised since other cells, such as circulating endothelial cells, may also express CK18 and are therefore also captures by a pan-CK marker (47). The research group of the University of Twente investigated if the EpCAM^{low}/CK+ CTCs they found are truly cancer cells. In one patient, they performed fluorescent in situ hybridization (FISH) to determine the ALK-status of the captured cells. Indeed, next to chromosomal aberrations, ALK-rearrangement were observed in 43% of the EpCAM^{low} CTCs (70). Interestingly, the ALK-rearrangement that was found in the EpCAM^{low} CTCs was not present in the primary tumor, but was found when the patient had progressive disease. So it would be interesting, for example in bladder cancer, to genetically characterize EpCAM^{high} and EpCAM^{low} CTCs to explain the difference with regard to their prognostic value.

Isolation of circulating tumor cells with different techniques

Considering the low numbers of CTCs found with the CellSearch system and its EpCAM dependency, many other techniques have been developed to isolate CTCs from the blood. Up till now, the CellSearch System remains the only system that is cleared by the FDA for CTC enumeration in metastatic breast, prostate and colorectal cancer and it has been used in numerous clinical studies. Also, acceptable reproducibility (high inter- and intra-assay concordance) was shown for the CellSearch system (71, 72). However, as shown in **chapter 3** and as briefly mentioned in the previous paragraph, other techniques to isolate CTCs from blood have been used in clinical studies, although most of them use different definitions to describe the phenotype of CTCs. Until today,

prognostic value of CTC numbers detected by assays other than CellSearch remained anecdotal and has never been rigorously validated as compared to the CellSearch System. Systems that isolate CTCs can roughly be divided into four categories: 1) assays similar to the CellSearch system, like the AdnaTest, that are based on immunomagnetic beads; 2) assays that depend on physical properties (size, elasticity, density) for example the Parsortix assay (73); 3) assays that discriminate on functional properties, like EPISPOT (74), and, 4) assays dependent on microdevices / microfluidic platforms, like the CTC chip, which may include different antibodies to detect CTCs (75, 76). There is even a technology that isolates CTCs in vivo using a needle coated with antibodies punctured directly into the vein of a patient. With this CellCollector (GILUPI) cells are lysed immediately after capture, which can prevent errors in downstream analysis since the nucleic acids are stabilized this way before being transported to the lab for readout. However, only few studies have been conducted with this technique and by lysing the cells the morphological characteristics of the cells cannot be captured.

Multiple studies have compared systems that isolate CTCs with the CellSearch system. As an example, here the AdnaTest is discussed to clarify the differences compared to the CellSearch system. The AdnaTest is frequently used in various clinical studies, for example in studies to determine AR-V7 on CTCs (40). The AdnaTest uses immunomagnetic enrichment of tumor cells via epithelial and tumor-associated antigens. After isolation of the labeled cells, these are lysed and mRNA expression is investigated for epithelial markers, which are different for the different cancer types. However, there are some disadvantages to the AdnaTest: first, the AdnaTest is not approved by the FDA, which hinders the use of this test in clinical studies and in clinical practice. Second, CTCs cannot be studied morphologically since CTCs are lysed immediately after capture to allow molecular research, which is an essential part of this assay. For the breast cancer AdnaTest, anti-EpCAM and anti-MUC1 are used to isolate the cells. After isolation and lysing, HER2, MUC1 and EpCAM are determined on mRNA with real time PCR. A test is performed in duplo and considered positive if one of the tumor-associated transcripts and a fragment of the control gene (Actin) are detected in both samples.

Several studies comparing CellSearch to the AdnaTest have been performed. For mBC, a direct comparison of the AdnaTest and CellSearch was performed in three studies. In the first study the comparison was performed in a heterogeneous group of mBC patients (n=76) and healthy volunteers (n=20) (77). For CellSearch, 59% of the mBC patients showed ≥1 CTC. However, in this study a sample was considered positive when ≥2 CTCs were found, which led to 36% CTC-positive samples. The AdnaTest was positive for 22% of the samples (positive in both samples). Direct comparison showed a significant difference in positivity between the two assays (McNemar $p = 0.013$). The second study showed in 55 mBC patients that the CellSearch was positive (≥2 CTCs) in 47% patients and in 36% ≥5 CTCs were found. The AdnaTest was positive in 53% of the patients. With

a cut-off of ≥2 CTCs, a positive agreement of 73% was found, while for ≥5 CTCs the positive agreement was 69% (78). The last and largest study in mBC patients compared the AdnaTest and CellSearch in 245 patients (79). For this study, a CTC sample was considered positive when ≥5 CTCs were detected with CellSearch. With the CellSearch assay 50% of the patients were CTC-positive and with the AdnaTest 40% of the patients had a positive result in both samples. A concordance of 64% between the assays was found. The differences in AdnaTest positivity can possibly be explained by the number of HER2-positive tumors in the different studies or a difference in cut-offs used for AdnaTest positivity. Only the last study investigated the prognostic impact of the AdnaTest and CellSearch. For CellSearch, CTC enumeration was an independent prognostic factor for OS, but not for PFS. The authors conclude that the lack of association with PFS was explained by the different definitions used for disease progression between the different participating centers, which makes the PFS data less reliable. For the AdnaTest, no prognostic impact was found which could probably have to do with the marker choice in the AdnaTest. For instance, MUC1 may also be expressed by activated leukocytes which can generate false-positive results. Therefore, sensitivity and specificity issues need to be resolved for the AdnaTest before potential prognostic impact can be proven.

CHARACTERIZATION OF CIRCULATING TUMOR CELLS

Besides the value of CTC enumeration as prognostic marker, characterization of CTCs can be an important tool to guide treatment in cancer patients.

Heterogeneity primary tumor vs. metastasis

As mentioned, characterization of CTCs offers a chance to obtain an up-to-date status before the start of a new treatment. Since the characteristics of tumor cells can change over time, under treatment pressure or during progression, most optimal treatment should be based on characteristics of that moment, and not on that of the primary tumor in case of metastatic disease. Research has shown that the characteristics of CTCs resemble that of the metastasis more than that of the primary tumor (41, 80). For breast cancer, therapies against the estrogen receptor (ER) or human epidermal growth factor receptor 2 (HER2) are administered based on the characteristics of the primary tumor in most cases. However, it is known that the ER-status and HER2-status can change over time (81-83). So taking a biopsy of metastatic tissue is necessary before the start of a new treatment, however, CTCs can also play a role in this characterization. When comparing gene expression levels in CTCs with expression levels in primary breast cancers, molecular differences between the two were frequently found (32, 80). To investigate the clinical implications of the change in receptor status over time, the CareMore studies

were designed: the CareMore-AI and the CareMore-Trastuzumab. Since there is evidence that cases with a HER2-negative primary tumor can still respond to trastuzumab therapy, one group of patients who might benefit from adding HER2-targeted therapy to chemotherapy are patients with initially a HER2-negative primary tumor but at presentation of metastasis displaying HER2-positive CTCs (84, 85). The CareMore-Trastuzumab trial investigates if this is indeed the case. Similarly, patients with a ER+/HER2+ primary tumors do worse on endocrine therapy than patients with an ER+/HER2- primary tumor (86). Therefore, in the CareMore-AI trial it is investigated if mBC cases with a HER2-negative primary tumor but with HER2-positive CTCs have less benefit from endocrine therapy with aromatase inhibitors (AIs). The results from these two studies, investigating the impact of tumor heterogeneity as judged by biomarker research on CTCs on ER- or HER2-targeted treatment, are expected in 2021.

The therapies against ER and HER2 have been very successful in breast cancer patients. However, also with these therapies primary or secondary resistance is known. Therefore, new therapies against BC are a constant subject of investigation. Another promising therapy target for BC is the androgen receptor (AR), which is well known from prostate cancer. However, earlier studies targeting the AR in mBC patients showed quite modest outcome to these therapies (87-92), with data suggesting AR targeting therapies may only being active in subsets of patients. Also, AR-status was shown to be discrepant in 35% of the matched primary and metastatic breast cancer tissues (93). Based on the data presented before, in **chapter 4** a study was conducted in which the AR-status was determined on the mRNA level in CTCs of mBC patients. The study showed that AR can be measured in CTCs of mBC patients and that the AR-status between the primary tumor and matched CTCs can be discrepant. This emphasizes the need to determine an up-to-date AR-status before the start of AR-targeted therapies, for which CTCs can be a valuable, minimally invasive tool.

Characterization of tumor cells as predictive marker

Besides characterizing CTCs as target for therapy or for determination of primary tumor discrepancy, it is also possible to analyze gene expression profiles in CTCs. The best examples for implementation of gene expression profiles in breast cancer diagnosis are prognostic signatures such as Mammaprint and Oncotype DX, as a result of successful prospective validation in the MINDACT trial (94) and the TAILORx trial respectively (95). Although these clinically relevant gene expression-profiling assays are employed on tumor tissues, gene expression profiles can also be determined in CTCs. Our research group established an assay to profile mRNA expression of 96 genes specific for breast cancer in CTCs. Of these 96 genes, 65 genes were determined as being CTC-specific, which means the expression of these genes is significantly higher in breast cancer derived CTCs enriched fractions after CellSearch than in cell fractions mostly comprised of

leukocytes similarly enriched from healthy blood donors. Using this 96-gene expression panel, a CTC derived gene expression profile of 16 genes was identified that provided additional prognostic value to CTC count in mBC patients. However, this profile could not be validated in an independent cohort of patients (37). Similarly, amongst the expression of these 96 genes, an expression profile of eight genes in CTCs predicted response to first-line AI treatment in mBC patients (96). However, also this profile could not be validated. These results highlight the potential of molecular characterization of CTCs, however, also emphasize that further optimization is needed to generate clinical impact for CTC derived gene expression profiles.

In **chapter 6** of this thesis, the primary aim was to determine if characterization of CTCs could identify heavily pretreated mBC patients who would respond to cisplatin (cDDP) therapy. For this purpose, a CTC-sensitivity profile was based on the expression of the above mentioned 96 (breast cancer-specific) genes in CTCs. However, in this case the predictive gene expression signature used was based on differential expression of these 96 genes between cell lines sensitive and resistant to cDDP. Based on these data, an upfront-defined sensitivity profile was measured in CTCs of patients before the start of cDDP therapy. However, while CTC count was an independent prognostic factor in this set of heavily pretreated mBC patients, the CTC-cDDP-sensitivity profile was unable to select patients responding to cDDP. In **chapter 5**, CTCs were used to predict response to cabazitaxel therapy in mCRPC patients. Besides enumeration of CTCs, a prostate cancer-specific 94-gene expression panel was measured in mCRPC samples of patients before the start of cabazitaxel treatment. Of these genes, a 5-gene profile was generated to predict for outcome to cabazitaxel. In this study, CTC count was an independent prognostic factor for both PFS and OS in mCRPC, together with alkaline phosphatase and hemoglobin. Although the 5-gene expression profile was associated with OS in univariable analysis, it was not an independent prognostic factor for OS nor PFS in these patients.

So while molecular characterization on CTCs can be obtained, most of the profiles that were generated could not be validated in independent cohorts or lacked prospective validation. However, since CTCs determined with the CellSearch System are proven prognostic and characterization of these cells can give valuable information about these cells, it is still worth further investigating gene expression profiles on CTCs. However, there are several technical difficulties discussed below that should be overcome to enhance the predictive power of gene expression profiles.

Technical limitations

The largest limitation of CTC isolation using the CellSearch System is the background of leukocytes that is present after CTC enrichment. Since most genes are also expressed in leukocytes, the first limitation is the number of genes that can be tumor-specifically

measured in the CTC fraction. Another limitation is the low numbers of CTCs that are usually present in one tube of blood. Therefore, the input of the cancer cells is generally low, especially in contrast to the leukocyte background. Therefore, sensitive techniques are necessary to measure gene expression in mRNA from a few CTCs. Most current techniques therefore require pre-amplification of the mRNA, which can induce mistakes or noise. On top of that, it is known that cancer is a very heterogeneous disease, which can further hamper the measurement of cancer-specific gene expression profiles. Even among the CTCs of one patient, gene expression can be different between CTCs, further dampening tumor specific signals. The solution for all these limitations would be to increase the number of CTCs by performing procedures like diagnostic leukapheresis and/or by performing molecular characterization on pure and/or single CTCs of patients.

CFDNA VS. CTCS

Both the detection and clinical utility of cfDNA and CTCs have improved the last years. Although clinical implementation of liquid biopsies are still rare, there are some assays that are used in the clinic. Here, an example of clinical utility for both liquid biopsies will be given, after which a short comparison of the similarities and differences between these liquid biopsies is provided.

An example of the clinical use of cfDNA is the determination of EGFR (epidermal growth factor receptor) mutations in patients with lung cancer. Patients with an actionable EGFR mutation are more prone to benefit from tyrosine kinase inhibitor (TKI) therapy. In 2016 the Food and Drug Administration (FDA) approved the first liquid biopsy assay (Cobas® EGFR Mutation Test v2, Roche Molecular Systems) which detects EGFR mutations in patients with non-small cell lung cancer (NSCLC) (97). When an actionable mutation is detected in ctDNA, targeted treatment with TKIs can be administered. However, when an actionable mutation is not detected in ctDNA, a secondary more sensitive ctDNA technique is needed or a tumor biopsy can be taken to be subjected to NGS for EGFR mutation detection (98). Another application of ctDNA in these patients is monitoring of the EGFR mutations during therapy. But also for monitoring during disease, a negative result should be considered inconclusive and requires further assessment (50, 98). However, in patients in which mutations are found by using cfDNA, a tumor biopsy is not necessary and treatment can be adjusted according to the detected mutation. A more recent example is the FDA approval (2019) of a PIK3CA ctDNA test (*therascreen*® PIK3CA RGQ PCR Kit) in patients with advanced or mBC. For mBC patients with a PIK3CA mutation, targeted treatment with alpelisib (a PI3Kα-specific inhibitor) can be administered, since alpelisib is also FDA approved since 2019.

A promising assay that can be determined on RNA from CTCs are AR splice variants. Especially AR splice variant 7 (AR-V7) has been investigated in metastatic castration-resistant prostate cancer (mCRPC). Research has shown that AR-V7-positive patients are less likely to benefit from endocrine treatment than AR-V7-negative patients (40, 99). In additional research, which investigated AR-V7 in mCRPC patients receiving chemotherapy, no influence of AR-V7 status on response was recorded (38, 39). However, studies on the one hand question whether the predictive ability of AR splice variants goes beyond factors related to tumor burden (CTC count, ctDNA fraction) or on the other suggest it being inferior to the predictive power of inactivating mutations in TP53 (100, 101). So, while AR-V7 detection in CTCs has been thoroughly validated (102) its clinical use is still subject of further investigation, and as a result not widely implemented into the clinical practice.

Both cfDNA and CTCs are minimally invasive, since only a blood draw is needed to collect these liquid biopsies. Therefore, they can be repeated serially and may thus provide real-time information about the tumor. As a result, both cfDNA and CTCs can give diagnostic and prognostic information about the cancer genotype. However, there are also differences between these liquid biopsies. The biggest difference is obviously that CTCs are intact tumor cells while cfDNA only contains DNA from most likely apoptotic (tumor) cells. Therefore, characterization of CTCs can take place at RNA, DNA and protein level. While for cfDNA 'only' quantification and analysis of DNA that is largely fragmented can be performed. Also, since CTCs are intact cells, morphologic and functional characteristics can be investigated next to their enumeration. The presence of CTCs in blood indicates that dissemination of tumor cells might occur or is taken place, while a rise in ctDNA concentrations only represents tumor activity, but not necessarily the circulation of tumor cells. Also, since CTCs are often viable tumor cells, they can be used to derive in *ex-vivo* models (103, 104).

While DNA can be isolated from CTCs, analysis of this CTC-DNA has limitations. With newer, more specific techniques like digital PCR (dPCR), mutations in CTC-DNA can be detected, however, research shows that for mutations cfDNA is the better substrate to investigate. An example are mutations in the *ESR1* gene in breast cancer patients, that can be detected in both CTCs and cfDNA, however, cfDNA has a higher sensitivity than CTCs for the detection of such mutations (105). For now, this is probably due to the background of leukocytes that is present after CellSearch enrichment of CTCs, and the low number of CTCs found by processing a tube of blood. What is also different is that for cfDNA isolation 2 mL of plasma is necessary while for CTC detection and isolation 10 mL of blood is required. Thus, cfDNA is easier to quantify than CTCs. However, for CTCs the FDA approved CellSearch is available, while for cfDNA there is no standardized method yet.

Probably since the tumor load is higher in metastatic setting, studies with liquid biopsies are mostly conducted in patients with advanced disease. However, also for the early stage patients, liquid biopsies are a subject of investigation. Although the sensitivity of detection of CTCs and cfDNA in this early stage is still low, there are studies that show that liquid biopsies can also be useful in patients with localized breast cancer (106, 107). There is even data suggesting liquid biopsies can be used in diagnosis of cancer, although sensitivity and false positive patients are still an issue (44, 108). The use of liquid biopsies in early stage cancer is most promising for cfDNA. It is very difficult to detect CTCs in this stage and cfDNA concentrations are almost similar in patients with early stage cancer and healthy individuals. However, the analysis of somatic mutations can be a promising tool in cancer diagnosis even in close to normal concentrations of cfDNA, while for CTC analysis these cells need to be isolated from the blood first (44).

Since a biopsy of a tumor or metastasis only gives information about that part of the tumor, liquid biopsies can give more information about tumor heterogeneity in general. At this moment, cfDNA is most suitable to investigate tumor heterogeneity and obtain the mutational landscape and heterogeneity therein of the tumor. Also, by generating an individual mutational profile of a tumor using cfDNA, this profile can be used to detect minimal residual disease during follow up. However, in case large numbers of pure CTCs can be retrieved, CTCs are even more suited to investigate tumor heterogeneity. Particularly, since CTCs are intact cells with their genome intact as well, while cfDNA only contains fragments of a cancer genome. With the advent of single cell sequencing, copy number alterations (CNAs) as well somatic variants and thus heterogeneity can be assigned to individual CTCs.

So, both CTCs and cfDNA have their own capabilities and advantages. It depends on the question which liquid biopsy is the better substrate. Also, several studies have shown that cfDNA and CTCs can complement each other. For instance, ESR1-mutations are most often more sensitively detected in cfDNA, while there were also mutations that were only found in the CTC samples (105). So for some research or clinical questions it could also be the best option to use information from multiple types of liquid biopsies.

In **chapter 3**, a systematic review about the role of liquid biopsies in muscle-invasive bladder cancer (MIBC) is described. Since platinum-based neo-adjuvant chemotherapy (NAC) is recommended in these patients, but the overall survival benefit is modest and toxicity can be significant, biomarkers to select the right patients for NAC are to be searched for. The review describes the role CTCs and cfDNA in selecting the patients in which NAC could be recommended. It was found that the presence of ≥1 CellSearch CTC before radical cystectomy is a marker for poor recurrence-free and overall survival. However, for cfDNA it is known that it can be detected in the blood of MIBC patients, but only small studies have been conducted and the results vary. More research should be done to establish the role of cfDNA in MIBC patients. Since there are only few hotspot

mutations, this research should likely use (patient-specific) next-generation sequencing panels, containing a select set of genes. Another option could be to look at CNAs in ctDNA with shallow whole-genome sequencing (sWGS). This review shows that liquid biopsies hold promise in selecting MIBC patients for perioperative chemotherapy, however, more large prospective studies investigating the true clinical applicability for treatment decision-making are needed.

FUTURE PERSPECTIVES

As mentioned both cfDNA and CTCs are promising tools to use in the clinical setting, but there are still some limitations that hinder the use of liquid biopsies in the clinic. To overcome some limitations, new technologies are subject of investigation.

The first limitation is the background of leukocytes that is present after isolation of CTCs with the CellSearch system, which hinders the characterization of CTCs. One of the possibilities is to characterize the CTCs with the use of padlock probes (PLP). The in situ PLP-assay can for example be used to visualize AR-V7 on a cellular level (109). However, this technical challenging technique, with sensitivity and specificity issues, can only be used to look at a limited number of markers of interest at the same time. Therefore, technologies that allow for single CTC isolation, like the DEPArray (110) and the VyCAP single cell system (111), are promising tools to overcome this limitation. Several studies have shown that it is feasible to perform DNA and RNA analysis and look at DNA methylation on single CTCs (112-116). Moreover, it has even been shown that it is feasible to investigate the genome and transcriptome of one CTC (117, 118). These techniques give insight into the genomic and transcriptomic landscape of CTCs, and therefore, the tumors of the cancer patients they are derived from. Through the analysis of multiple single CTCs within one patient, information on CTC heterogeneity, and, therefore, within one tumor can be assed, which gives even more information about the tumor landscape.

Since for the characterization of CTCs, those CTCs need to be isolated first, another limitation is the number of CTCs found in patients and the number of patients in which CTCs are present at all. To increase those numbers, a possible option is to increase the blood volume that is analyzed. Several studies have already been conducted in which leukapheresis is performed on cancer patients (119-121). With the use of a diagnostic leukapheresis (DLA), 2.5-5.0 liters of blood can be processed for CTC isolation. This clinically safe method could increase the number of CTCs available for characterization, as well as the number of patients in whom CTC characterization can be performed. Whether this technique could be of clinical importance, is for example to be shown in a new study protocol (the SYBILLA trial) in primary ER+ breast cancer patients who have been treated with five years of adjuvant endocrine therapy. Since more than 50% of the recurrences

in breast cancer patients occur after five years of adjuvant endocrine therapy, tools to determine which patients should receive prolonged adjuvant endocrine treatment are necessary. In this study, ER+ lymph node positive primary breast cancer patients who received five years of adjuvant endocrine treatment will undergo DLA for CTC enumeration. The hypothesis is that CTC enumeration using DLA is a promising technique to determine which patients should receive prolonged adjuvant treatment.

Lastly, the use of CTCs in selecting patients for immunotherapy will be briefly described. Since it is known that tumors use the activation of PD-1/PD-L1 signaling as an immunosuppressive mechanism, multiple different immune checkpoint inhibitors have been developed (122). However, these promising therapies are expensive, and therefore selecting patients who will respond to these treatments is necessary. Since PD-L1 expression can also be determined on CTCs, CTCs can possibly play a role in this urge by selecting patients for immunotherapy or predicting progression at an early stage (123-125).

The abovementioned techniques are time consuming, expensive and still technically challenging. Since single CTCs only provide low RNA or DNA input, and therefore reliable and unbiased amplification of this material is needed, the next few years will show if these promising techniques can indeed be implemented in the clinic and therefore help in providing personalized medicine to patients.

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Chapter 8

Summary / Nederlandse samenvatting

Summary

The chapters of this thesis emphasize the importance of characterization of tumor cells or tumor DNA and the role that liquid biopsies can play for this characterization.

In **chapter 2** the characterization of tumor cells is described by determination of CD146 on primary tumor tissues. Since not in every patient circulating tumor cells (CTCs) can be detected, the question remains whether there are no CTCs in these patients or whether they cannot be detected. Based on cell line gene expression data, CD146 was linked to epithelial-to-mesenchymal transition (EMT) and considered a possible candidate to be used as a new marker for CTC isolation. Furthermore, in literature, CD146 was correlated with poor prognosis and cancer aggressiveness, but thus far, no data is available in patients who did not receive adjuvant systemic therapy. Also, from cell line data it has been derived that CD146 may be linked to tamoxifen resistance. Therefore, based on these observations, CD146 could be a useful marker for predicting disease progression and response to endocrine treatment. However, since most research was performed on cell line data, the primary aim here was to investigate the association of CD146 expression with clinical outcome in breast cancer. Therefore, primary breast cancer tissues from 1342 patients were stained immunohistochemically for CD146. Of these tissues, 11% showed CD146 expression and expression of CD146 was most prevalent in the triple negative tumors (64%). In patients with lymph node negative disease, who did not receive (neo)adjuvant systemic treatment (n=551), CD146 expression was a prognostic factor for both metastasis-free survival (MFS) and overall survival (OS) in univariable analysis but not in multivariable analysis. Also in these clinical specimens, no correlation between CD146 expression and EMT nor a difference in outcome to first-line tamoxifen was seen. So, CD146 is present in primary breast cancer tissues and associated with more aggressive tumors, but the absence of a relation with EMT suggests that CD146 could be required, but is on its own not sufficient for EMT in breast cancer. Thus, CD146 could still be an interesting marker for CTC isolation, but the added value is weakened based on these results and also the results from the first clinical study using CD146 as a marker to isolate CTCs beyond EpCAM were marginal (62).

In the systematic review described in **chapter 3**, the use of liquid biopsies in muscle-invasive bladder cancer (MIBC) patients was discussed. Since characterization of CTCs or cell-free DNA (cfDNA) can give prognostic and predictive information about responses to a particular therapy, in this review it was evaluated if liquid biopsies can play a role in selecting MIBC patients for neo-adjuvant chemotherapy (NAC) prior to local definitive treatment, since it is known that the overall survival benefit of NAC is modest. Since liquid biopsies can characterize tumor cells and DNA from blood and/or urine in a minimally invasive manner, liquid biopsies are an attractive option to guide the administration of perioperative chemotherapy in MIBC patients. In this review, it was found that the presence of ≥1 CTC before radical cystectomy (detected with the CellSearch technique)

is a robust marker for poor recurrence-free and overall survival. For cfDNA, results are not so clear yet. cfDNA can be detected in the blood of MIBC patients, but the studies were rather small and results on its prognostic value varied. Since there are few hotspot somatic mutations in MIBC, cfDNA-based approaches should likely use (patient-specific) next-generation sequencing panels, containing a select set of genes. Another option that needs more investigation is the determination of copy number alterations (CNAs) in circulating tumor DNA (ctDNA) using for instance shallow whole genome sequencing (sWGS), since this technique is not hampered by limited presence of hotspot mutations. So, liquid biopsies hold promise in selecting MIBC patients for perioperative chemotherapy, however, more prospective studies investigating the true clinical applicability for treatment decision-making are needed.

Another type of cancer that is often subject of investigation is metastatic castration-resistant prostate cancer (mCRPC). Since the number of treatment options for mCRPC have increased rapidly in the last decade, biomarkers that can guide therapy selection are necessary. In **chapter 4** the enumeration and characterization of CTCs in mCRPC patients who receive cabazitaxel therapy was investigated, to determine if CTCs can play a role in selecting patients who may benefit from cabazitaxel therapy. In 114 mCRPC patients, CellSearch CTC count was independently associated with poor progression-free survival (PFS) and OS in multivariable analysis. Also, alkaline phosphatase and hemoglobin were independent associated with PFS and OS. A gene expression profile comprised of five genes was established in CTCs to predict outcome to cabazitaxel therapy in these patients. This profile was associated with OS in univariable analysis, but not in multivariable analysis. So the data presented in this chapter showed that CTC numbers are prognostic in patients receiving cabazitaxel, but characterization of the CTCs lack sufficient power in this setting.

In the next chapters, characterization of CTCs was investigated in metastatic breast cancer (mBC) patients. **Chapter 5** describes the determination of the androgen receptor (AR) on CellSearch-enriched CTCs of mBC patients. Determination of AR in mBC patients could be relevant since AR is a drug target and its expression has been associated with endocrine resistance in mBC patients. To measure AR expression in a minimally invasive manner, and to provide an up-to-date determination of the AR-status, in this chapter AR was measured on CTCs of mBC patients. In 31% of the CTCs from mBC patients, AR expression was above the threshold. To investigate if the AR-status can change over time, the AR-status was measured on matched primary tumor tissues of mBC patients of whom CTC-AR-status was determined. In 58% of the matched CTC and primary tumor samples, the AR-status was discordant, observing both switches from AR-positive primary tumor to AR-negative in CTCs and vice versa. In patients treated with ER-targeting therapies, there was no statistically significant difference in PFS depending on the patient's CTC-AR-status. Thus, 31% of mBC express AR as a drug target on their CTCs.

Furthermore, considering the large discordance of the AR-status between the primary tumor and CTCs, the AR-status of CTCs may be a valuable and minimally invasive tool to select mBC patients who may benefit from AR-targeting agents.

In the final chapter, **chapter 6**, a clinical study in which heavily pretreated mBC patients who start with a new line of cisplatin (cDDP) chemotherapy is described. Of these patients, before the start of the cDDP treatment, CTCs were enumerated and characterized. The primary aim of the study was to determine whether characterization of CTCs could identify patients responding to cDDP and to describe the outcomes to cDDP monotherapy in a large group of heavily pretreated mBC patients. For the characterization of the CTCs, a CTC-cDDP-sensitivity profile was generated based on gene expression data of cDDP-sensitive and cDDP-resistant breast cancer cell lines. The best observed response of the patients treated with cDDP was a partial response in 7% of the patients and stable disease in 56% of the patients. However, the CTC-cDDP-sensitivity profile that was measured on CellSearch-enriched CTCs in these patients was unable to identify patients responding to cDDP. Of the established prognostic factors in mBC, CTC count was the only factor associated with outcome to cDDP therapy.

Overall, these studies show that liquid biopsies are promising but also have their limitations. With the rapid advance of technology in this area, like diagnostic leukapheresis and isolation of single and/or pure CTCs, it is anticipated that in the next few years liquid biopsies can be further developed to become clinically relevant and applicable tools that can help in providing personalized medicine to patients.

NEDERLANDSE SAMENVATTING

Algemene introductie

Box met uitleg

Primaire tumor: dit is de tumor die als eerste aanwezig is, de 'hoofdtumor'. Bij borstkanker is dit dus de tumor die in de borst ontdekt wordt. Bij borstkanker wordt deze tumor vaak met een operatie verwijderd, als de ziekte in een vroeg stadium wordt ontdekt (voordat er uitzaaiingen zijn). Helaas kunnen patiënten ook later nog uitzaaiingen krijgen, soms pas na jaren. Tussen de operatie en het krijgen van uitzaaiingen kan wel 10 jaar tijd zitten.

Metastasen: uitzaaiingen van de primaire tumor.

Biopt: bij een biopt wordt er hapje uit tumorweefsel genomen met een holle naald. Dit weefsel kan bekeken worden door de patholoog om te kijken of het kwaadaardig is en welke eigenschappen dat weefsel heeft.

Al jaren wordt er veel onderzoek gedaan naar alle typen kanker, maar ondanks dat blijft kanker wereldwijd de grootste doodsoorzaak (1, 2). Door de komst van de bevolkingsonderzoeken en door verbeterde behandelingen is het sterftecijfer wel gedaald, maar nog steeds gaan veel mensen dood doordat ze uitgezaaide kanker (metastasen) ontwikkelen. Voor uitgezaaide ziekte komen steeds meer en verbeterde behandelingen beschikbaar, welke verlenging van het leven en een betere kwaliteit van leven kunnen geven. Echter, de meeste behandelingen hebben ook bijwerkingen, waardoor de kwaliteit van leven juist achteruit gaat. Het is daarom belangrijk om de juiste behandeling voor de patiënt te vinden, zodat patiënten zo lang mogelijk kunnen leven met een goede kwaliteit van leven. Bij het kiezen van de juiste behandeling staat de wens van de patiënt voorop, maar ook de conditie (performance status) van de patiënt en eventuele andere ziekten of aandoeningen (co-morbiditeit) die een patiënt heeft, zijn van belang. Maar naast al die factoren, kan de behandeling het beste gekozen worden op basis van de eigenschappen die de tumor van de patiënt heeft.

Eigenschappen van de tumor

Elke tumor heeft zijn eigen eigenschappen of karakteristieken, waarop de behandeling van een patiënt kan worden aangepast. Op dit moment worden de eigenschappen van de tumor vaak bepaald door een hapje van de primaire tumor te nemen, of worden ze

bepaald op de primaire tumor nadat die verwijderd is met een operatie. Ook als er na een paar jaar een nieuwe behandeling gegeven moet worden, wordt er gekeken naar de eigenschappen die op de primaire tumor ooit zijn bepaald. Een nadeel hiervan is dat de eigenschappen van de tumor in de tussentijd kunnen veranderen of ze kunnen zijn veranderd door de behandelingen die er gegeven zijn. Bij borstkanker en prostaatkanker kan er soms jaren zitten tussen het verwijderen van de primaire tumor en het krijgen van uitzaaiingen. Daardoor is het belangrijk om de eigenschappen van de tumor te weten te komen op het moment dat het nodig is om een nieuwe behandeling te starten. Door alleen naar de eigenschappen van de primaire tumor te kijken, die dus soms jaren daarvoor al bepaald zijn, wordt de nieuwe behandeling mogelijk afgestemd op achterhaalde informatie. Het zou dus het mooiste zijn om voor start van elke nieuwe behandeling voor uitgezaaide ziekte een hapje (biopt) te nemen uit een uitzaaiing, zodat de 'actuele' eigenschappen bepaald kunnen worden. Een probleem hierbij is dat niet uit alle uitzaaiingen makkelijk en veilig een hapje genomen kan worden. Sommige uitzaaiingen zitten op plekken die niet of heel moeilijk te bereiken zijn, of ze liggen diep tussen de organen bijvoorbeeld. Het kan dus pijnlijk en gevaarlijk zijn om zo'n hapje te nemen omdat er altijd een kans is dat er een bloeding ontstaat of dat de plek ontstoken raakt. Hiervoor is gezocht naar andere mogelijkheden om toch de eigenschappen van de uitgezaaide tumor te bepalen, zonder dat het nodig is om een hapje te nemen. Een van die andere mogelijkheden is door het afnemen van 'liquid biopsies' (vloeibare biopten). Deze vloeibare biopten bestaan uit tumorcellen of delen van tumorcellen, die door de bloedvaten circuleren. Door gebruik te maken van specifieke apparatuur in het laboratorium, kunnen die (delen van) tumorcellen uit een buisje bloed worden gehaald. Hieronder volgt een korte beschrijving van twee typen vloeibare biopten: de circulerende tumorcellen en celvrij DNA.

Box met uitleg

Liquid biopsies: de vloeibare biopten bestaan uit tumorcellen of delen van tumorcellen (bijvoorbeeld het DNA van de cellen) die zijn losgeraakt van de tumor en door het bloed circuleren. Deze (delen van) tumorcellen kunnen door specifieke apparatuur uit een buisje bloed worden gehaald van patiënten. Onder de vloeibare biopten vallen onder andere de circulerende tumor cellen (CTC's) en celvrij DNA (cfDNA, "circulating cell-free DNA').

Epitheliale tumor: een borst- en een prostaattumor ontstaan uit epitheel weefsel. Dit weefsel staat er om bekend dat het lichaamsoppervlakken en verschillende lichaamsholten bedekt. Andere voorbeelden van typen weefsel zijn bindweefsel of spierweefsel.

Circulerende tumorcellen

Circulerende tumorcellen (CTC's) zijn tumorcellen die loslaten van de primaire tumor of de uitzaaiingen en daarna circuleren door het bloed. Bij bepaalde typen tumoren, de zogenaamde epitheliale tumor, kunnen CTC's uit het bloed worden gehaald. Voor het isoleren van deze CTC's wordt op dit moment meestal het CellSearch systeem gebruikt, wat gebruik maakt van de epitheliale marker EpCAM. EpCAM is een eiwit wat specifiek is voor epitheliale cellen. Door antilichamen tegen EpCAM waar ijzeren bolletjes aan vastgehecht zijn, toe te voegen aan het bloed, worden de CTC's bedekt met de antilichamen, een daarmee met de ijzeren bolletjes. Hierdoor kunnen de CTC's uit het bloed worden gehaald met behulp van een magneet. Omdat er ook altijd wel wat witte bloedcellen (leukocyten) mee worden getrokken door de magneet, worden er na het isoleren van de CTC's nog verschillende kleuringen op de cellen gedaan om onderscheid te kunnen maken tussen een CTC en een witte bloedcel.

Box met uitleg

Progressie: progressie van een tumor betekent dat de tumor is gegroeid, of dat er nieuwe uitzaaiingen zijn ontstaan. De behandeling die op dat moment gegeven wordt, remt de groei van de tumor dan dus niet meer af.

Nadat de CTC's uit het bloed zijn geïsoleerd, kunnen de cellen worden geteld. Bij vrouwen met uitgezaaide borstkanker die starten met een nieuwe behandeling kan op die

manier onderscheid worden gemaakt tussen patiënten met ≥5 CTC's en patiënten met <5 CTC's. Uit onderzoek is inmiddels bekend dat de patiënten met ≥5 CTC's bij start van een nieuwe behandeling, sneller progressie hebben op die therapie (PFS, progressie vrije overleving, "progression free survival") en een kortere algehele overleving (OS, "overall survival") dan patiënten met <5 CTC's (17). Dit geldt niet alleen voor uitgezaaide borstkanker, maar ook voor vele andere tumortypen (19-31). Op basis van veel onderzoeken, is het CellSearch systeem door de FDA (Food and Drug administration; United States) goedgekeurd voor gebruik in de kliniek voor uitgezaaide borstkanker, prostaatkanker en dikke darmkanker.

Naast het tellen van CTC's, kan er ook gekeken worden naar de eigenschappen van deze tumorcellen. Hierbij kan er bijvoorbeeld gekeken worden naar de aanwezigheid van bepaalde receptoren, het aantal kopieën van een bepaald gen en naar bepaalde eiwit- en genexpressie patronen in CTC's (32, 34-40). Uit onderzoek blijkt dat de eigenschappen van de CTC's meer lijken op die van de uitzaaiingen dan die van de primaire tumor (41). Hierdoor is het bekijken van de eigenschappen in CTC's een veelbelovende manier om te kijken naar de eigenschappen van de tumor op het moment dat er een nieuwe behandeling gestart wordt.

Celvrij DNA

Box met uitleg

NGS: "Next-generation sequencing". Om te kijken of er een afwijking in het DNA van een patiënt zit, kan de volgorde van de bouwstenen van het DNA van die patiënt worden bekeken. Ieder mens heeft een eigen volgorde van bouwstenen waaruit DNA is opgebouwd. Met "sequencen" wordt het in kaart brengen van die volgorde bedoeld. Omdat de nieuwere technieken waarmee dit sequencen kan worden uitgevoerd steeds sneller en goedkoper worden, waarmee van grotere stukken DNA de volgorde in een keer kan worden bepaald, noemen we deze nieuwere techniek: "next-generation sequencing" (sequencen van de volgende generatie).

dPCR: "digital PCR". Dit is een andere techniek waarbij specifiek gezocht kan worden naar bepaalde afwijkingen in de bouwstenen van een gen. Omdat er alleen gezocht wordt naar die afwijking, kan dit heel gevoelig kunnen worden bekeken. Het cfDNA van een patiënt wordt hierbij op een plaatje toegevoegd met heel veel verschillende hokjes. In alle hokjes wordt een beetje cfDNA toegevoegd en als de afwijking waar naar gezocht wordt aanwezig is in de bouwstenen, krijgt het hokje een bepaalde kleur die met specifieke technieken zichtbaar kan worden gemaakt. Als die kleur niet gevonden wordt in een van de hokjes, is de afwijking niet aanwezig.

Een ander vloeibaar biopt waarnaar gekeken is in dit proefschrift, is celvrij DNA (cfDNA, "circulating cell-free DNA"). Dit is DNA wat vrijkomt nadat cellen overtollig zijn geworden en daardoor doodgegaan (wat een natuurlijk proces is) (42). Nadat dit DNA is vrijgekomen, kan het worden geïsoleerd uit het bloed (uit het plasma of serum). Bij patiënten met kanker is het DNA wat vrijkomt niet alleen afkomstig uit 'normale' cellen, maar ook van tumorcellen. Dit deel van het cfDNA wordt ook wel circulerend tumor DNA genoemd (ctDNA, "circulating tumor DNA"). Als het circulerende DNA bij patiënten met kanker wordt bekeken, is het DNA vooral afkomstig van erfelijk DNA uit 'normale' bloedcellen en slechts een klein deel is afkomstig van de tumorcellen (het deel ctDNA). Hierdoor zijn er hele gevoelige technieken nodig om dit ctDNA te kunnen bekijken vanuit het bloed, zoals next-generation sequencing (NGS) en digital PCR (dPCR).

Mogelijke toepassingen van de bepaling van tumor eigenschappen

Het doel van dit proefschrift is om het belang te laten zien van het bepalen van de eigenschappen van tumorcellen of tumor DNA en de rol die de vloeibare biopten hierbij kunnen spelen. De verschillende hoofdstukken zijn voorbeelden hoe de eigenschappen

van tumoren gebruikt kunnen worden bij het zoeken van de juiste behandeling voor de juiste patiënt.

Box met uitleg

Neo-adjuvante therapie: een behandeling die wordt gegeven voordat een operatie plaatsvindt om de tumor te verwijderen. Het doel van deze behandeling is om de tumor zo klein mogelijk te maken, zodat die makkelijker kan worden verwijderd. En om alle losse tumorcellen dood te maken, zodat de kans zo klein mogelijk is dat de kanker terug komt.

Adjuvante therapie: behandeling die wordt gegeven na een operatie om de tumor te verwijderen. Deze behandeling is er op gericht om eventuele achtergebleven tumorcellen ook nog dood te maken. Het doel hierbij is om de kans zo klein mogelijk te maken dat de kanker terug komt en de kans op genezing dus zo groot mogelijk te maken.

Systemische therapie: behandelingen die in het hele lichaam terecht komen, dus niet lokaal toegepast, maar in het hele systeem (lichaam).

Resistentie: er wordt gesproken over resistentie van een behandeling als de tumor weer gaat groeien, en dus ongevoelig wordt, terwijl de behandeling doorgegeven wordt. Resistentie kan vanaf het begin af aan optreden, er is dan helemaal geen verminderde groei of verkleining van de tumor op die behandeling. Maar resistentie kan ook na verloop van tijd optreden, waarbij de tumor eerst kleiner wordt, maar na een tijdje toch weer gaat groeien onder dezelfde behandeling.

Om de eigenschappen van CTC's te kunnen bekijken, moeten deze CTC's wel eerst gevonden kunnen worden. Zelfs met de meest gevoelige technieken die we tot nu toe beschikbaar hebben, kunnen er niet in alle patiënten met (uitgezaaide) kanker CTC's geïsoleerd worden. De vraag is of er in deze patiënten geen CTC's aanwezig zijn, of dat we ze niet kunnen aantonen. In de patiënten waarin wel CTC's worden gevonden, is het aantal gevonden CTC's vaak laag (in alle verschillende tumor typen), waardoor de vraag ontstaat of daadwerkelijk alle CTC's gevonden worden in deze patiënten. Mogelijk zou een andere marker dan EpCAM gebruikt kunnen worden om meer CTC's kunnen isoleren, of zou er een extra marker samen met EpCAM gebruikt kunnen worden. Uit eerder onderzoek naar genexpressie op cellijnen was gebleken dat CD146 een mogelijke kandidaat is om als nieuwe of extra marker te gebruiken voor CTC-isolatie.

Uit de literatuur is gebleken dat CD146 een relatie heeft met een slechte uitkomst en hoe agressief een tumor is. Echter, al het onderzoek wat gedaan was, was in patiënten die al eerdere een systemische behandeling hadden gekregen met adjuvante therapie. Omdat uit dit eerdere onderzoek is gebleken dat CD146 een rol lijkt te spelen bij hormonale resistentie is ook onderzocht of CD146, naast een marker voor CTC-isolatie, ook een nuttige marker zou kunnen zijn voor het voorspellen van de uitkomst die een patiënt heeft op de behandeling met hormonale therapie (dus of er verwacht wordt dat een patiënt respons heeft op die behandeling, of dat de tumor snel weer gaat groeien na start van die behandeling). Omdat het meeste eerdere onderzoek was uitgevoerd op cellijnen, is in **hoofdstuk 2** daarom op een grote serie primaire borsttumoren (n=1342) door middel van immunohistochemische kleuring de expressie van CD146 bepaald om de rol van CD146 in de biologie van borstkanker beter in beeld te krijgen. Van de onderzochte tumoren liet 11% CD146 expressie zien. Bij de meer agressieve borsttumoren, de triple negatieve tumoren, werden relatief de meeste tumoren met CD146 expressie gezien (met 64% de hoogste prevalentie).

Box met uitleg

EMT: epitheliale naar mesenchymale transitie. Dit is een biologisch proces waarbij cellen die epitheliale eigenschappen hebben, veranderen naar cellen die mesenchymale eigenschappen hebben. Dit is een van de bekendste processen die een rol spelen bij het ontstaan van uitzaaiingen. Als een epitheliale tumor gaat uitzaaien, moet de tumorcellen eerst mesenchymale eigenschappen ontwikkelen om beter te kunnen verplaatsen door de bloedbaan en zo op andere plekken in het lichaam te kunnen komen.

In alle patiënten die geen lymfeklieruitzaaiingen hadden bij diagnose en geen (neo) adjuvante systemische therapie hadden gehad (n=551) was CD146 expressie in de univariabele analyse een significante prognostische factor voor zowel metastase-vrije overleving (MFS, "metastasis-free survival") als algehele overleving (OS), maar deze associatie hield niet stand in een multivariabele analyse met alle andere bestaande klinische prognostische factoren. Er werd in deze studie verder geen relatie gevonden tussen CD146 expressie en epitheliale-mesenchymale transitie (EMT).

Verder werd er ook geen verschil gezien in uitkomst op eerstelijns tamoxifen (hormonale) therapie in deze patiënten. Dit hoofdstuk laat dus zien dat CD146 aanwezig is op een deel van de primaire weefsel van borsttumoren. Maar gebaseerd op deze data lijkt het echter verlies van CD146 alleen onvoldoende voor het optreden van EMT in borsttumoren. CD146 lijkt daarom mogelijk niet de marker die we zoeken voor het vergroten

van het aantal CTC's bij de patiënten, hetgeen inmiddels ook in ons laboratorium is aangetoond in een klinische studie (62). Ons onderzoek toonde we wel aan dat CD146 expressie een associatie had met een agressievere vorm van borstkanker maar een klinische meerwaarde, boven de bekende karakteristieken, had deze marker helaas niet.

Box met uitleg

Bij patiënten met borstkanker, kunnen de volgende receptoren aanwezig zijn op hun tumor:

ER: oestrogeen receptor ("estrogen receptor"). Deze receptor bepaald of een tumor hormoongevoelig is. Bij een hormoongevoelige tumor kan de tumor groeien onder invloed van hormonen. Daarom wordt bij een ER-positieve, hormoongevoelige, tumor een behandeling gegeven met hormonale therapie (of beter: anti-hormoon therapie).

PR: progresteron receptor.

HER2: HER2 staat voor "human epidermal growth factor Receptor 2". Bij patiënten met een HER2-positieve tumor is er overmatig veel van het HER2 eiwit aanwezig op de tumor. Er bestaat een doelgerichte behandeling die werkt tegen dit HER2 eiwit.

Triple negatief: bij patiënten met een triple negatieve borsttumor, zijn geen van de bovenstaande receptoren aanwezig op de borsttumor. Dat betekent dat hormoontherapie of een doelgerichte behandeling tegen HER2 dus ook niet werken bij deze patiënten.

In **hoofdstuk 3** wordt in een systematische review beschreven hoe vloeibare biopten gebruikt kunnen worden in patiënten met spierinvasieve blaaskanker (MIBC, "muscle-invasive bladder cancer"). Aangezien de eigenschappen van CTC's of cfDNA prognostische of predictieve informatie kunnen geven, werd in dit review onderzocht of vloeibare biopten een rol konden spelen in welke van de MIBC-patiënten neo-adjuvante chemotherapie (NAC) zouden moeten krijgen voor de operatie. Uit de literatuur is het namelijk bekend dat de overlevingswinst van NAC beperkt is. Hierdoor zou in de meest optimale situatie NAC alleen moeten worden toegediend aan de patiënten die baat hebben bij die behandeling, om zo ook bijwerkingen te voorkomen in de patiënten die niet reageren op de behandeling. Omdat met vloeibare biopten tumorcellen en tumor DNA in het bloed en/of in de urine gemakkelijk verkregen kunnen worden, zijn de

vloeibare biopten een aantrekkelijke optie om te gebruiken bij de beslissing welke MIBC patiënten een behandeling zouden moeten krijgen voordat de operatie plaatsvindt om de tumor te verwijderen.

Box met uitleg

Prognostisch: dit zijn factoren die voorspellen hoe de ziekte bij iemand gaat verlopen, zonder dat daarbij rekening wordt gehouden met de behandeling. Hiermee kan dus worden bekeken wat de prognose van iemand is.

Predictief: dit zijn factoren die voorspellen hoe een tumor op een behandeling gaat reageren. Hiermee kan worden bekeken of de verwachting is dat iemand op een behandeling gaat reageren, of dat er beter een andere behandeling gegeven kan worden.

In het review werd gevonden dat patiënten met ≥1 CTC in het bloed (aangetoond met het CellSearch-systeem) voordat de operatie plaatsvond om de tumor te verwijderen, een slechtere recidiefvrije overleving (RFS, "recurrence-free survival") en algehele overleving (OS) hadden. CTC's waren dus een robuuste marker om RFS en OS te voorspellen. Voor cfDNA waren de resultaten minder duidelijk. Het werd wel aangetoond dat cfDNA gevonden kan worden in het bloed van MIBC-patiënten, maar aan alle gerapporteerde studies namen te weinig patiënten deel om harde uitspraken te kunnen doen. Ook waren de uitkomsten over de prognostische waarde van cfDNA in de verschillende studies niet eenduidig. Bij sommige tumoren heb je bepaalde afwijkingen (mutaties) in het genoom die vaak in dezelfde regio van het genoom voorkomen. Deze vaker voorkomende mutaties worden hotspot mutaties genoemd. Bij MIBC zijn er maar weinig somatische hotspot mutaties aanwezig. Daardoor is het waarschijnlijk nodig dat NGS-technieken worden gebruikt voor de detectie van cfDNA in MIBC. Een andere optie zou kunnen zijn om te kijken naar kopie nummer veranderingen (CNA, "copy number alterations"). Bij CNA's ontbreekt er een stukje DNA op een chromosoom, of er is te veel DNA op een chromosoom aanwezig. Deze CNA's kunnen in ctDNA worden onderzocht met een bepaalde manier van NGS, waarbij er niet heel diep in het genoom hoeft te worden gekeken ("shallow whole genome sequencing", (sWGS). Omdat deze techniek niet afhankelijk is van de weinige aanwezige hotspot mutaties, is dit ook zeker een optie waar meer onderzoek naar zou moeten worden gedaan. Concluderend zijn de vloeibare biopten veelbelovend voor het selecteren van MIBC-patiënten voor chemotherapie rondom de operatie om de tumor te verwijderen. Maar er zijn meer (prospectieve) studies nodig waarbij een grotere hoeveelheid patiënten worden bekeken om echt uit

te kunnen zoeken of vloeibare biopten kunnen worden toegepast in de kliniek bij het helpen besluiten of die patiënten een behandeling rondom de operatie zouden moeten krijgen of niet.

In de vorige hoofdstukken is er gekeken naar de eigenschappen van tumorcellen en tumor DNA bij borstkanker en bij blaaskanker. Een andere type kanker waar veel onderzoek naar wordt gedaan is uitgezaaide castratie resistente prostaatkanker (mCRPC, metastatic castration-resistant prostate cancer). Aangezien het aantal behandelingen voor mCRPC de laatste jaren sterk is toegenomen, zijn markers die kunnen helpen bij de selectie van de juiste behandeling hard nodig. In **hoofdstuk 4** is onderzoek gedaan naar het aantal CTC's en de eigenschappen van die CTC's in mCRPC patiënten die behandeld worden met cabazitaxel (een chemotherapie). In de 114 mCRPC patiënten die werden onderzocht in deze studie werd gevonden dat het aantal CTC's onafhankelijk van bekende klinische factoren geassocieerd was met een slechte progressie vrije overleving (PFS) en algehele overleving (OS). Naast CTC-aantallen waren ook alkalisch fosfatase en hemoglobine onafhankelijk geassocieerd met PFS en OS. Om de uitkomst op een behandeling met cabazitaxel te voorspellen, werd er ook een genexpressie profiel van vijf genen gemeten in de CTC's van deze patiënten. In de univariabele analyse was dit profiel geassocieerd met OS, maar in de multivariabele analyse was er geen onafhankelijke associatie met PFS of OS. De resultaten uit dit hoofdstuk laten zien dat het aantal CTC's geassocieerd is met uitkomst op cabazitaxel. Maar het bekijken van de eigenschappen van de CTC's (karakteriseren) door middel van het gemaakte genexpressie profiel kon de patiënten met een goede uitkomst niet van de patiënten met een slechte uitkomst op cabazitaxel behandeling onderscheiden, en had dus geen meerwaarde.

In de laatste twee hoofdstukken werd er gekeken naar de eigenschappen van CTC's in uitgezaaide borstkanker patiënten. In **hoofdstuk 5** werd gekeken naar de androgeen receptor (AR), wat een bekende receptor is waar behandelingen op aangrijpen (drug target) in prostaatkanker. Eerder onderzoek heeft laten zien dat deze receptor ook aanwezig kan zijn in borst tumoren, en dat het geassocieerd lijkt te zijn met resistentie tegen hormonale behandelingen bij uitgezaaide borstkanker patiënten. Daarnaast is het mogelijk dat AR ook een target kan zijn voor behandelingen bij uitgezaaide borstkanker patiënten. Om AR-expressie te meten op een weinig invasieve manier, en om een zo up-to-date bepaling te hebben van de AR-status van een tumor, werd in deze studie AR gemeten op CTC's van uitgezaaide borstkanker patiënten. In 31% van de uitgezaaide borstkanker patiënten werd expressie van AR gevonden op hun CTC's. Om te onderzoeken of de AR-status in de tijd verandert, is de AR-status ook gemeten in primaire tumoren van dezelfde patiënten. In 58% van de patiënten van wie de AR-status op CTC's en de primaire tumor bekend was, was deze status niet hetzelfde. Hierbij werden zowel veranderingen van AR-positief naar AR-negatief gezien, als andersom. Bij de patiënten die een hormonale behandeling kregen (gericht tegen de ER) was geen

significant verschil te zien in PFS afhankelijk van de CTC-AR-status. De resultaten uit dit hoofdstuk laten zien dat AR gemeten kan worden in CTC's en dat in deze set 31% van de uitgezaaide borstkanker patiënten AR-positieve CTC's heeft. Omdat er veel verschil zit tussen de AR-status van de primaire tumor en die van CTC samples, en we uit onderzoek weten dat de eigenschappen van de uitzaaiingen meer voorspellend zijn dan die van de primaire tumor of een behandeling bij patiënten die uitgezaaid zijn aanslaat of niet, is de verwachting dat de AR-status van de CTC's een waardevolle, weinig invasieve manier is om uitgezaaide borstkanker patiënten te selecteren die baat zouden kunnen hebben van behandeling gericht op de AR-receptor.

In het laatste hoofdstuk, **hoofdstuk 6**, wordt een klinische studie beschreven waarin uitgezaaide borstkanker patiënten, die al veel behandeling hebben gehad voor hun borstkanker, starten met een nieuwe lijn cisplatin (cDDP) chemotherapie. Voordat deze patiënten begonnen aan hun cDDP behandeling, werden CTC's afgenomen om te tellen en om naar de eigenschappen te kijken. Het doel van de studie was om te bepalen of een respons op cDDP behandeling voorspeld kon worden door middel van de eigenschappen van de CTC's en verder om de uitkomsten op cDDP behandeling te beschrijven in een grotere groep van uitgezaaide borstkanker patiënten. Al de patiënten uit deze studie hadden eerder al meerdere behandelingen gekregen vanwege borstkanker. Op basis van genexpressie data van cDDP-gevoelige (sensitieve) en –ongevoelige (resistente) cellijnen, werd een CTC-cDDP-sensitiviteitsprofiel ontworpen. In de gehele set patiënten die werden behandeld met cDDP, was de beste geobserveerde respons op de behandeling een partiele (gedeeltelijke) respons in 7% van de patiënten en stabiele ziekte in 56% van de patiënten. (Waarbij bij een respons een (gedeeltelijke) afname van de ziekte werd gezien, en bij een stabiele ziekte dus geen afname, maar ook geen groei). Het CTC-cDDP-sensitiviteitsprofiel, welke werd gemeten op de CTC's geïsoleerd met het CellSearch-systeem in deze patiënten, kon geen onderscheid maken tussen patiënten waarbij de tumor een reactie liet zien op cDDP behandeling (respons) en de patiënten waarbij de tumor geen reactie liet zien. Van alle bekende prognostische factoren in uitgezaaide borstkanker, was alleen het aantal CTC's geassocieerd met uitkomst op cDDP behandeling. Patiënten met <5 CTC's voor start van de cDDP behandeling hadden een significant langere PFS en OS dan patiënten met ≥5 CTC's voor start van de cDDP behandeling.

Deze studies laten zien dat vloeibare biopten veelbelovend zijn, maar ook dat er nog beperkingen zijn. Nieuwe technische ontwikkelingen die recent beschikbaar zijn gekomen, zullen de komende jaren uitwijzen of de "liquid biopsies" inderdaad klinisch relevant zijn, in de kliniek kunnen worden toegepast en dus ingezet kunnen worden om patiënten een gepersonaliseerde behandeling te geven.

Appendices

Verklarende woordenlijst
Dankwoord
Curriculum Vitae
PhD portfolio
List of publications

VERKLARENDE WOORDENLIJST

AC	adjuvant chemotherapy
AE	adverse event
AI	aromatase inhibitor
AIC	Akaike information criterion
ALK	anaplastic lymphoma kinase
ALP	alkaline phosphatase
AR	androgen receptor
AR-V7	androgen receptor splice variant 7
AUC	area under the curve
BC	breast cancer
BCG	bacillus Calmette-Guerin
BPH	benign prostate hyperplasia
BR	Bloom-Richardson
CB	clinical benefit
CBR	clinical benefit rate
CD146	cluster of differentiation 146
CD45	cluster of differentiation molecule 45
cDDP	cisplatin
cfDNA	circulating cell-free DNA
CI	confidence interval
CIF	cumulatice incidence function
CK	cytokeratin
CMA	cell line microarray
cMIBC	clinical MIBC
CNA	copy number alteration
CR	complete response
CSS	cancer-specific survival
CT	computed tomography
CTC	circulating tumor cell
CTCAE	national cancer institute common toxicity criteria
ctDNA	circulating tumor DNA
DAPI	4′,6-diamidino-2-phenylindole
DLA	diagnostic leukapheresis
DLDA	diagonal linear discrimination analysis
DMFS	distant metastasis-free survival
DNA	deoxyribonucleic acid
dPCR	digital polymerase chain reaction

EAU	European association of urology
EGFR	epidermal growth factor receptor
EMT	epithelial–mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ER	estrogen receptor
FDA	food and drug administration
FDR	false discovery rate
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescent in situ hybridization
GEO	gene expression omnibus
HBD	healthy blood donor
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
IHC	immunohistochemistry
IQR	interquartile range
LDH	lactate dehydrogenase
LHRH	luteinizing hormone-releasing hormone
mBC	metastatic breast cancer
MCAM	melanoma cell adhesion molecule
mCRC	metastatic colorectal cancer
mCRPC	metastatic castration-resistant prostate cancer
METC	medical research ethics committee
MFS	metastasis-free survival
MIBC	muscle invasive bladder cancer
MLPA	multiplex ligation-dependent probe amplification
mRNA	messenger RNA
NAC	neoadjuvant chemotherapy
NGS	next generation sequencing
nmBC	nonmetastatic MIBC
NMIBC	non m
NSCLC	non-small cell lung cancer
OS	overall survival
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD	progressive disease
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PFS	progression-free survival
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

PLP	padlock probes
pMIBC	pathological MIBC
PR	progesterone receptor
PR	partial response
PRISMA	preferred reporting items for systematic reviews and meta-analyses
PSA	prostate specific antigen
PTPRC	protein tyrosine phosphatase receptor type C
RC	radical cystectomy
RECIST	response evaluation criteria in solid tumors
RFS	recurrence-free survival
RNA	ribonucleic acid
ROC	receiving operating characteristic
RR	response rate
RT-qPCR	quantitative reverse transcription polymerase chain reaction
SAE	serious adverse event
SD	standard deviation
SD	stable disease
SNV	single nucleotide variant
SRB	sulforhodamine B
SRP	survival risk prediction
STR	short tandem repeat
sWGS	shallow whole-genome sequencing
TMA	tissue microarray
TNBC	triple negative breast cancer
TPC	treating physician
TURB	transurethral resection of the bladder
WES	whole exome sequencing
WGS	whole-genome sequencing

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CURRICULUM VITAE

Inge de Kruijff werd geboren op 7 juli 1989 te Heemstede. Ze behaalde in 2007 haar gymnasiumdiploma op het Herbert Vissers College te Nieuw-Vennep met het profiel natuur en gezondheid en keuzevakken Latijn en economie. Hierna begon zij haar studie geneeskunde aan de Vrije Universiteit te Amsterdam. Vanaf 2010 begon zij aan haar master geneeskunde waarbij ze haar wetenschappelijke stage bij de afdeling endocrinologie van de Vrije Universiteit heeft volbracht met als onderwerp 'Het verschil tussen etniciteiten in de behandeling van diabetes gravidarum' (begeleiding: Dr. E.M.W. Eekhoff, internist-endocrinoloog). Hierna begon zij aan haar coschappen waarbij ze een keuzestage heeft gedaan op de spoedeisende hulp van het VUmc (begeleiding: Drs. T.H. Biesheuvel, chirurg, MMT-arts) en haar semi-arts stage op de afdeling oncologie van het Spaarne ziekenhuis te Hoofddorp (begeleiding: Dr. B. de Valk, oncoloog). Nadat ze in 2013 haar diploma behaalde is ze in 2014 gaan werken als arts-assistent interne geneeskunde in het Amphia ziekenhuis te Breda (begeleiding: Dr. J.W.J. van Esser, internist-hematoloog). In 2015 is ze begonnen aan haar promotietraject in het Erasmus MC te Rotterdam bij de afdeling interne oncologie onder begeleiding van Prof. Dr. S. Sleijfer en Prof. Dr. J.W.M. Martens waarbij zij onderzoek heeft gedaan naar circulerende tumorcellen zoals in dit proefschrift is uitgewerkt. Tijdens haar promotieonderzoek heeft zij de Pieter de Mulder award gewonnen in 2016 voor onderzoek naar de 'Padlock Probe Assay' waarna zij voor twee maanden een stage heeft gelopen aan het Science for Life Laboratory te Stockholm, Zweden (begeleiding: Prof. Dr. M. Nilsson) om deze techniek verder uit te werken op circulerende tumorcellen. Verder heeft ze meegeschreven aan een gehonoreerde subsidie aanvraag voor *A Sister's Hope*. Daarnaast heeft ze op meerdere (inter)nationale congressen mogen presenteren, waaronder een posterpresentatie op de European Society for Medical Oncology meeting in Madrid, Spanje en een posterpresentatie op de American Society for Clinical Oncology meeting in Chicago, USA. Tijdens haar promotieonderzoek heeft ze zich verder beziggehouden met onderwijs en begeleiding van de Junior Med School studenten en als tutor van de eerstejaars geneeskunde studenten van het Erasmus MC. Hiermee heeft zij ook haar deel basiskwalificatie onderwijs (BKO) behaald. Vanaf 1 februari 2020 is zij werkzaam zijn als wetenschappelijk onderzoeker aan het Rijksinstituut voor Volksgezondheid en Milieu (RIVM).

PHD PORTFOLIO

PhD Portfolio	Year	Workload (ECTS)
General Courses		
- English writing Course	2017	3.0
- Scientific Integrity	2015	0.3
Specific Courses		
- Biomedical Research Techniques	2015	1.5
- Patient Oriented Research (CPO course)	2015	0.3
- OpenClinica course	2015	0.3
- BROK course	2015	1.5
- Writing Successful Grant Proposals	2015	0.5
- Cursus omgaan met groepen	2015	0.2
- Biostatistics course	2016	5.7
- Photoshop and Illustrator CS6 workshop	2017	0.3
- Course on R	2017	1.8
- BROK recertification	2018	1.0
Seminars and workshops		
- MolMed Day	2015	0.3
- Nederlandse Internisten Dagen	2015	0.3
- Wetenschapsmiddag Interne Oncologie	2015	0.2
- CPCT Symposium	2015	0.2
- Transatlantic conference on personalized medicine	2015	0.3
- Nederlandse Oncologiedagen	2016	0.7
- DUOS symposium	2015	0.3
- CGC annual meeting	2016	0.3
- MolMed Day	2016	0.3
- Daniel den Hoed Day	2016	0.3
- Course on CellSearch system	2016	1.0
- Wetenschapsmiddag Interne Oncologie	2016	0.2
- NGS symposium	2016	0.3
- EORTC meeting	2016	0.7
- CPCT symposium	2016	0.2
- DUOS symposium	2016	0.3
- CfDNA meeting	2017	0.3
- Wetenschapsmiddag Interne Oncologie	2017	0.2
- LKI Symposium Leuven	2017	1.0
- ESMO Congress Madrid	2017	1.0

- Daniel den Hoed Day	2017	0.3
- DUOS symposium	2017	0.3
- MolMed Day	2018	0.3
- ASCO Congress Chicago	2018	1.0
- NVU Annual Meeting	2018	0.3
- Tour D'Europe symposium	2018	0.3
- Teach the Teacher course	2018	0.7

Presentations		
Poster presentations		
- ESMO, Madrid, Spain	2017	1.0
- MolMed day	2018	0.6
- ASCO, Chicago, USA	2018	1.0

Oral presentations		
- Borstkanker Behandeling Beter Symposium, Rotterdam	2015	0.2
- Interne geneeskunde meeting Erasmus MC, Rotterdam	2016	0.2
- Nederlandse Oncologiedagen, Arnhem	2016	0.2
- EORTC meeting, Rotterdam	2016	0.2
- Dutch Uro-Oncology Studygroup (DUOS) symposium, Utrecht	2017	0.2
- NVU voorjaarsvergadering, Nijmegen	2018	0.2
- Tour d'Europe symposium, Rotterdam	2018	0.2
- Medical Oncology Research Meeting, Rotterdam	2017-2018	0.4
- Josephine Nefkens Institute Oncology Meeting, Rotterdam	2019	0.2
Meetings		
- Medical Oncology Research Meeting, Rotterdam	2017-2019	2.0
- Josephine Nefkens Institute Scientific Lab Meetings, Rotterdam	2015-2019	3.0
- Journal Club	2015-2019	3.0
- Onderwijs Multidisciplinaire Behandeling in de Oncologie (OMBO); standaard en nieuwe ontwikkelingen	2015-2016	0.2
Teaching		
- Supervision/coordinator Junior Med School Medical Oncology Research Program (four weeks)	2016-2017	1.5
- Tutor first-year medical students	2015-2017	3.0
- Deel Basiskwalificatie Onderwijs (BKO)	2019	1.0

LIST OF PUBLICATIONS

De Kruijff IE, Sieuwerts AM, Beije N, Prager-van der Smisse WJC, Angus L, Beaufort CM, Van MN, Oomen-de Hoop E, Jager A, Hamberg P, de Jongh FE, Kraan J, Martens JWM, Sleijfer S. Prospective evaluation of a circulating tumor cell sensitivity profile to predict response to cisplatin chemotherapy in metastatic breast cancer patients. *Submitted*.

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El-Heliebi A, Hille C, Laxman N, Svedlund J, Haudum C, Ercan E, Kroneis T, Chen S, Smolle M, Rossmann C, Krzywkowski T, Ahlford A, Darai E, von Amsberg G, Alsdorf W, König F, Löhr M, **de Kruijff I**, Riethdorf S, Gorges TM, Pantel K, Bauernhofer T, Nilsson M, and Sedlmayr P. In Situ Detection and Quantification of AR-V7, AR-FL, PSA, and *KRAS* Point Mutations in Circulating Tumor Cells. *Clin Chem. 2018 Mar;64(3):536-546*.

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