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# Molecular profiling and predictive value of circulating tumor cells in patients with metastatic breast cancer: an option for monitoring response to breast cancer related therapies

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**Abstract** *Purpose* We analyzed circulating tumor cells (CTC) in blood of metastatic breast cancer patients ( $n = 42$ ) and determined the ability of this method to predict therapy response. *Methods* CTC from blood were analyzed before and during therapy for EpCAM, MUC1 and HER2 transcripts with the *AdnaTest BreastCancer*. The estrogen (ER) and progesterone (PR) receptor expression was assessed by RT-PCR. *Results* The overall detection rate for CTC was 52% (thereof 86% EpCAM; 86% MUC1; 32% HER2; 35% ER; 12% PR). CTC were ER, PR and HER2 negative in 45% (ER), 78% (PR) and 60% (HER-2) of patients with steroid receptor-positive tumors. 29% of patients with HER2-negative tumors had HER2-positive CTC. The test predicted therapy response in 78% of all cases. Persistence of CTC significantly correlated with shorter overall survival ( $P = 0.005$ ). *Conclusions* Molecular profiling of CTC may offer superior prognostic information with regard to risk assessment for recurrence and predictive judgement of therapeutical regimens.

**Keywords** Circulating tumor cells · Molecular profiling · Therapy monitoring · Metastatic breast cancer

## Introduction

The current therapies in early breast cancer are based on the primary tumor size, the presence or absence of axillary lymph node metastasis, grading of tumor differentiation, the receptor and HER2 status, age as well as the hormonal status of the patient. Most patients with stage I and II breast cancer will be cured of their disease by surgery, adjuvant chemotherapy and hormone therapy. However, a substantial number of them will develop recurrent carcinoma [1]. This recurrence rate is explained by tumor cell dissemination into distant organs, preferentially bone marrow (BM), which often occurs prior to surgery. It has been demonstrated that these cells show a considerable heterogeneity in the expression of carcinoma-associated cell—surface molecules, including HER2, EpCAM, MUC1 and Lewis<sup>Y</sup> [2]. Furthermore, they have a time-limited proliferation potential and, thus, may be dormant [3]. This phenomenon was also observed in blood samples of breast cancer patients even up to 22 years after diagnosis [4]. Since existing therapies are aimed at proliferation pathways only, this might explain why adjuvant chemotherapy fails to prevent relapse in some patients with early breast cancer. In this context, it has been demonstrated that tumor cells frequently survive high-dose chemotherapy [5] and that tumor cells, still present after chemotherapy, represent an independent poor prognostic factor for reduced overall survival [6, 7].

Thus, tumor cell detection in the BM is being regarded increasingly as a clinically relevant prognostic factor for breast cancer and a pooled analysis of BM findings in more than 4,700 patients documented the prognostic value of

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these cells with regard to reduced disease free and overall survival [8]. The above observations indicate that the detection and characterization of disseminated occult tumor cells may be an important marker to determine in patients with breast cancer. On the one hand, it may identify a subgroup of patients with early breast cancer who are at high risk of relapse. On the other hand, in patients with incurable metastatic breast cancer, there is an urgent need to identify a surrogate marker of tumor response to overcome current limitations of radiography and surgical biopsy in the serial monitoring of cancer therapy, especially in trials of costly new and targeted agents.

Since multiple BM aspirations from the same patient during follow-up are not easily tolerated, the peripheral blood would be a more convenient source of sampling for such longitudinal follow-up studies. In primary breast cancer, the predictive value of circulating tumor cells (CTC) still has to be proven. Whereas some groups showed that the presence of CTC in blood before and/or after adjuvant therapy is associated with poor prognosis and the over-expression of the estrogen-receptor [9–12], other groups did not support these data [7, 13, 14]. Furthermore, apoptotic cells significantly contribute to CTC fractions in blood of breast cancer patients [15].

In contrast, the presence of  $>5$  CTC in 7.5 ml of blood from metastatic breast cancer patients has been shown to predict shorter progression-free and overall survival and has superior and independent prognostic value of tumor burden and disease phenotype [16, 17]. However, besides isolating these cells, the need to determine the profiles of expression of genes and/or proteins of tumors is becoming increasingly important since changes in the phenotype of the tumor cells can occur after the original diagnosis (e.g. HER2, and receptors for estrogen and progesterone) and resistance to a treatment can only be inferred after the treatment has failed [18]. PCR methods are generally more sensitive than microscopic methods and have the additional advantage of a high-throughput technology. Furthermore, using a multi-marker panel of cancer associated genes, RT-PCR was found to be the most sensitive technique for the detection of CTC in blood of breast cancer patients [19, 20]. One approach involves the analysis of CTC-enriched samples by gene expression profiling [21, 22].

In this study, we used the technology developed by AdnaGen which combines immunomagnetic tumor cell selection targeting EpCAM and MUC1 (*AdnaTest BreastCancerSelect*), followed by multiplex RT-PCR for the transcripts EpCAM, MUC1 and HER2 (*AdnaTest BreastCancerDetect*). In addition, the transcripts of the estrogen (ER) and progesterone (PR) receptor were analyzed by an additional RT-PCR.

It was the purpose of the study to (a) detect and analyze CTC in the blood of metastatic breast cancer patients by molecular profiling during a follow-up of palliative chemo-, antibody- or

hormonal therapy and (b) determine the ability of this method to predict the response to breast cancer related therapies.

## Patients, materials and methods

### Study design

We conducted a prospective single institution trial to purpose the molecular analysis of circulating tumor cells (CTC) in blood of breast cancer patients during palliative therapy and to determine the ability of this method to predict the response to breast cancer related therapies.

### Patient population

The study was conducted at the Department of Obstetrics and Gynecology in collaboration with the Department of Internal Medicine (Cancer Research) at the University Hospital in Essen. In total, 42 patients have been studied since August 2006. The median time of follow up and sample collection was 9 months (range 5–14).

Blood samples of 32/42 patients were analyzed during follow-up of their individual therapies. Ten patients could not be monitored for the following reasons: four patients died within two months after first blood sampling and six patients were lost for follow-up because palliative treatment was applied in other hospitals nearby their hometowns.

### Eligibility criteria

The eligibility criteria were as follows: age  $\geq 18$  years; patients with measurable or evaluable metastatic breast cancer; predicted life expectancy  $\geq 2$  months; Eastern Cooperative Oncology Group (ECOG) scores for performance status of 0–2; no severe uncontrolled co-morbidities or medical conditions; no second malignancies.

Patients had either a relapse of breast cancer diagnosed years before and were to start chemotherapy or a documented progressive breast cancer before receiving a new endocrine, chemo- or experimental therapy. Prior adjuvant treatment, radiation or any other treatment of metastatic disease were permitted.

### Response criteria

Before starting a new treatment, patients underwent an evaluation of metastatic sites by ultrasound, X-ray or computer tomography. Blood samples were collected for laboratory evaluations, including CEA and Ca 15-3 as well as for the isolation and characterization of CTC. Re evaluations of disease status were done by the same techniques every 8–12 weeks, depending on the treatment schedule, until the loss or death of a patient (Table 1).

**Table 1** Flow chart for the evaluation and re-evaluation of patients

Time	Visit no. 1 before start of a new treatment regimen for metastatic breast cancer	Follow up every 8–12 weeks	Follow up every 2–4 weeks <sup>a</sup>
Imaging	X	X	
Clinical investigation	X	X	
Laboratory (CEA, Ca 13–5 e.c.)	X	X	
CTC detection in peripheral blood	X		X

<sup>a</sup> Dependent on treatment regimen

Response to therapy was evaluated according to the *Response Evaluation Criteria in Solid Tumors* (RECIST). Complete Response (CR): disappearance of all target lesions; Partial Response (PR): at least 30% decrease in the sum of the LD (longest diameter) of target lesions, taking as reference the baseline sum LD; Progressive Disease (PD): at least 20% increase in the sum of the LD target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions; Stable Disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started.

### Immunohistochemical analysis

For each of the 42 patients, the tumor type, TNM-staging and grading were assessed according to the WHO-classification of tumors of the breast [23] and the sixth edition of the TNM Classification System [24]. The ER and PR receptor status were determined by immunohistochemistry. The DAKO-score for the expression of HER2 was determined with the HercepTest<sup>®</sup>, and the results were interpreted as follows: 0 = no membrane staining; 1+ = faint, partial membrane staining; 2+ = weak complete membrane staining in greater than 10% of invasive cancer; 3+ = intense complete membrane staining in greater than 10% of invasive cancer cells. FISH analysis in cases of 2+ staining as determined with the HercepTest was performed as described elsewhere [25].

### Sampling of biological material

2 × 5 ml EDTA blood was collected for isolation of CTC before the application of therapeutic substances with an S-Monovette<sup>®</sup> (Sarstedt AG & Co.) and stored at 4°C until

further examination. The samples were processed immediately or not later than 4 h after blood withdrawal.

An additional serum sample was collected to determine tumor markers.

### Healthy controls

5 ml blood was collected from 88 healthy donors aged 23–73 years for the determination of specificity and sensitivity of the *AdnaTest* and from 48 healthy donors for assaying the hormonal receptors.

### Determination of serum tumor markers

CEA and CA 15-3 were determined using the Elecsys CEA/CA 15-3 immunoassays (Roche, Mannheim, Germany) for the quantitative determination of CEA/CA 15-3 in human serum and plasma. The serial measurement of CEA/CA 15-3 was intended to aid in the management of cancer patients. These assays were performed in the central laboratory of our University Hospital on Cobas<sup>®</sup> immunoassay analyzers according to the manufacturer's instruction. The central laboratory has a valid certification for the performance of these assays following international guidelines.

### Tumor cell enrichment/selection

Since August 2006 blood samples were taken from 42 patients with metastatic breast cancer. The *AdnaTest BreastCancer* (AdnaGen AG, Langenhagen, Germany) enables the immunomagnetic enrichment of tumor cells via epithelial and tumor associated antigens. Two antibodies against the epithelial antigen MUC1 and one antibody against the epithelial glycoprotein GA733-2 (EpCAM) are conjugated to magnetic beads (Dynabeads) for the labelling of tumor cells in peripheral blood [26, 27]. In brief, the blood samples were incubated with a ready-to-use antibody mixture commercialized as *AdnaTest BreastCancerSelect* according to the manufacturer's instructions. The labelled cells were extracted by a magnetic particle concentrator (MPC). Subsequently, mRNA isolation from lysed, enriched cells was performed according to the manufacturer's instructions with the Dynabeads mRNA DIRECT<sup>™</sup> Micro Kit (DynaL Biotech GmbH, Hamburg, Germany) that is included in the *AdnaTest BreastCancerDetect*. Reverse transcription resulted in cDNA, which was the template for tumor cell detection and characterization by multiplex RT-PCR. Sensiscript<sup>®</sup> Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany) was used for the reverse transcription because of its high sensitivity (recommended for

amounts of <50 ng RNA) in combination with oligo(dT) coupled Dynabeads of the mRNA DIRECT™ Micro Kit (DynaL Biotech GmbH) according to the manufacturer's instructions [28]. cDNA was synthesized in a thermocycler under the following conditions: Reverse transcription was performed at 37°C for 60 min followed by 3 min at 93°C for inactivation of the reaction. The resulting cDNA was stored at –20°C until further use.

### Tumor cell detection

The *Adnatest BreastCancerDetect* was used for the detection of breast cancer-associated gene expression in immunomagnetically enriched tumor cells by reverse transcription and PCR. The analysis of tumor-associated mRNA isolated from circulating tumor cells was performed in a multiplex PCR for three tumor-associated transcripts: HER2, MUC1 and GA733-2. The thermal profile used for multiplex RT-PCR was as follows: After a 15 min denaturation at 95°C, 35 PCR cycles were carried out by denaturation at 94°C for 1 min, annealing/extension at 60°C for 1 min of and elongation for 1 min at 72°C. Subsequently, termination of the reaction was carried out at 72°C for 10 min followed by storage of the samples at 4°C.

The primer sets for the ER and PR receptor were a gift from AdnaGen® AG (Langenhagen, Germany). ER and PR were detected on CTC performed with these reagents after the preparation of the cDNA and according to the instructions of the *AdnaTest BreastCancerDetect*. PCR was performed with the HotStarTaq Master Mix (QIAGEN GmbH). Actin was used as internal PCR positive control. The thermal profile used for the nested RT-PCR was as follows: After a 15-min denaturation at 95°C, 37 cycles of PCR were carried out by denaturation at 94°C for 30 s, annealing/extension at 60°C for 30 s and elongation for 30 s at 72°C. Subsequently, termination of the reaction was carried out at 72°C for 5 min followed by storage of the samples at 4°C.

The primers generate fragments of the following sizes: GA733-2: 395 base pairs (bp), MUC1: 293 bp, HER2: 270 bp, PR: 270 bp, ER: 305 bp and actin: 114 bp. Visualization of the PCR fragments was carried out with a 2100 Bioanalyzer using the DNA 1000 LabChips (Agilent Technologies) and the Expert Software Package (version B.02.03.SI307).

### Evaluation of data

The test is considered positive if a PCR fragment of at least one tumor associated transcript is clearly detected. Using the software package for evaluation of the data on the Agilent 2100 Bioanalyzer, peaks with a concentration of >0.15 ng/μl are positive for the transcripts GA733-2,

MUC1 and HER2. Peaks that are not detected at the above setting are negative (concentration of <0.15 ng/μl). Peaks with a concentration of >0.60 ng/μl are positive for the ER transcript. The PR expression is considered positive when the transcript is detected without applying any cut-off.

### Statistical analysis

Survival analysis was performed by analyzing Kaplan–Meier-life tables, whereby death is used as endpoint. Overall survival has been described as the overall survival during the time of monitoring. Kaplan–Meier survival curves separated for CTC(+) and CTC(–) are presented and compared via log rank test. Null-hypothesis of non different survival times is rejected, when *P*-values < 0.05. The results were verified by Dr. Keller (ACOMED Statistics, Leipzig, Germany) using Software NCSS 2007 (Hintze J. (2007) NCSS, PASS and GESS. NCSS. Kaysville, Utah, US. [www.ncss.com](http://www.ncss.com)).

### Results

#### Patient characteristics

A total of 42 patients was enrolled since August 2006. As shown in Table 2, the patients ranged in age from 29 to 74 years. All four initial tumor stages were included, with a predominance of stage II. Only eight patients had a primary metastatic breast cancer. Most patients had ductal breast cancer. Moderately and poorly differentiated tumors were predominant. The median number of sites of metastasis was two with a range from 1 to 4 sites. Most patients had visceral metastasis. 27 and 23 primary tumors were ER- and PR-positive, respectively; and 12 primary tumors had an over-expression of HER2 (DAKO–Score 3+). All patients with BET (breast conserving surgery) received an adjuvant radiation. Patients with hormone receptor—positive tumors (stage I–III) received an adjuvant hormonal treatment with tamoxifen or an aromatase inhibitor. The chemotherapeutic adjuvant treatment mostly contained anthracyclines and taxanes. Patients with metastatic tumors of the breast received different chemotherapeutic treatments including anthracyclines, taxane, vinorelbine and 5-FU. Most patients were extensively pretreated before starting the collection of the blood samples. 27 patients received an anthracycline- or taxane-based chemotherapy in an adjuvant or metastatic setting before study start. Nearly all patients with HER2 3 + tumors received trastuzumab. One patient with HER2 overexpression of breast cancer did not receive trastuzumab because of a coronary artery disease and reduced general condition.

After starting measurement of CTC, patients underwent individual chemotherapeutic or hormonal treatment



**Table 2** Patient characteristics at baseline

	<i>n</i> (Patients)
Total numbers of pts	42
Median age (years)	50 (range 29–74)
<i>Initial staging</i>	
I	4
IIA	14
IIB	7
IIIA	4
IIIB	4
IV	8
Not known	1
<i>Primary tumor</i>	
<i>Histology</i>	
Ductal	28
Lobulär	6
Medullär	1
Not known	7
<i>Grading</i>	
1	2
2	20
3	18
Not known	2
<i>Estrogen receptor</i>	
Pos	27
<i>Progesteron receptor</i>	
Pos	23
<i>HER2 overexpression</i>	
Pos (3+)	12
<i>Sites of metastasis</i>	
Median number	2 (range 1–4)
Visceral	34
Nonvisceral	9
Cerebral	2
<i>Elevated markers</i>	
CEA	13
CA 15-3	20
Not known	10
<i>Adjuvant treatment</i>	
Chemotherapy	23
Radiation	14
Hormone	17
Trastuzumab	2
Not known	6
<i>Metastatic treatment</i>	
<i>Chemotherapy line</i>	
1st	9
2nd	7
3rd	4
4th and more	16

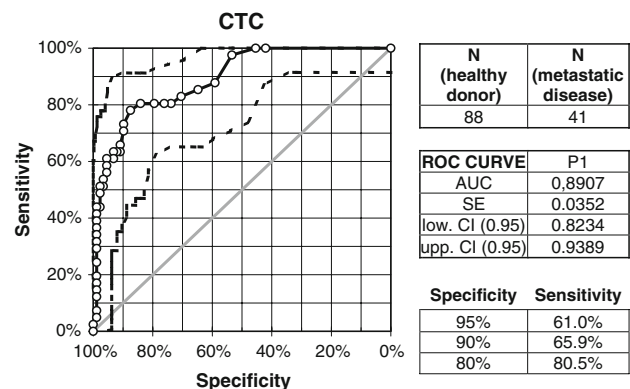
**Table 2** continued

	<i>n</i> (Patients)
Trastuzumab	10
Hormone therapy	15
Not known	6

depending on the pre-treatment. A sub-group of 32/42 patients was monitored for CTC during chemotherapy over a median time period of 9 months (5–14 months). 10 patients could not be monitored for the following reasons: four patients died within two months after first blood sampling and six patients were lost for follow-up because palliative treatment was applied in other hospitals nearby their hometowns.

### Determination of the specificity for tumor cell selection and detection

A ROC analysis was performed using the highest fragment concentration obtained among the three markers (GA733-2, MUC1, and HER2) from 41 patients with metastatic breast cancer and 88 healthy donors (Fig. 1). The fragment concentrations (ng/μl) obtained from the 2100 Expert Software Package (version B.02.03.SI307) were used instead of the peak heights for the calculations in order to minimize device to device variations. The resulting ROC curve is shown in Fig. 1. The *AdnaTest BreastCancer* ROC curve yields an AUC (area under the curve) of 0.89, which suggests that the *AdnaTest BreastCancer* has reasonably small false positive and false negative rates across a reasonable range of cut off values. At a specificity level of 90% (>0.15 ng/μl) a clinical sensitivity of roughly 65.9% can be shown and even at 99% specificity level (>0.54 ng/μl) a sensitivity of 43.9% would be reached. The statistical maximum for the diagnostic value (highest distance to



**Fig. 1** ROC curve for AdnaTest Breast Cancer. The ROC curve is shown as a grey line. The dashed lines describe the 95% confidence intervals. Data obtained from 41 women with metastatic breast cancer and 88 healthy donors were used for the calculation

random guess line) is reached at a sensitivity of 80.5% and a corresponding specificity of 80%, respectively.

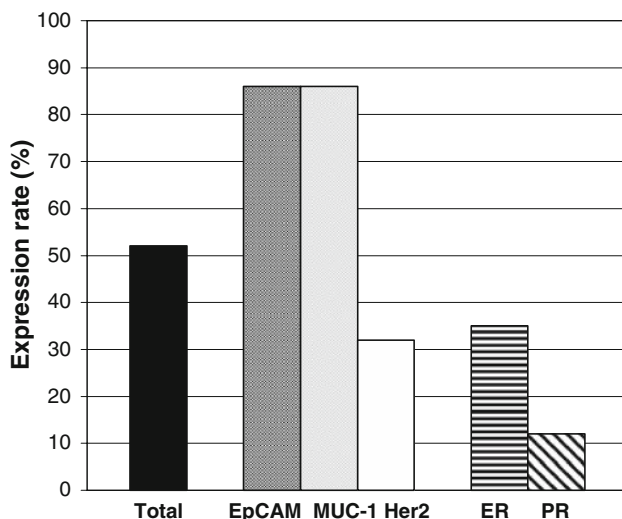
For the determination of the specificity of the ER and PR assay 48 healthy donor samples were analyzed yielding to negative results for ER in 44/48 cases (92% specificity) if a cut-off value of 0.6 ng/μl was applied. For PR all healthy donor samples were found to be negative (data not shown).

### Expression profiling of CTC of metastatic breast cancer patients

The results for CTC detection in 42 patients with metastatic breast cancer at the time of tumor progression or therapy switch are shown in Fig. 2. The overall detection rate for CTC was 52% (22/42 patients) with the expression rates of 86% for EpCAM (19/22 patients), 86% for MUC1 (19/22 patients) and 32% for HER2 (7/22 patients), respectively. Results for ER and PR could be obtained in a subset of 37 patients of which 46% were positive for CTC (17/37 patients) with a detection rate of 35% (6/17 patients) for ER and 12% (2/17 patients) for PR, respectively.

### Comparison of antigen expression on the primary tumor with the expression on CTC

Table 3 shows the results for the expression of ER, PR and HER2 on the primary tumor as compared to the expression



**Fig. 2** Expression of breast cancer related transcripts on CTC. Figure 1 shows the expression rate for the different transcripts analyzed on CTC. Multiplex-PCR for EpCAM, MUC1 and HER2 could be analyzed in all 42 patients. The black column shows the total positive rate for CTC found prior to application of chemotherapy. The dark grey column demonstrates the expression rate for EpCAM on CTC, the light grey column for MUC1, the white column for HER2, the cross-striped column for ER and the diagonally-striped column for PR, respectively

**Table 3** Comparison of antigen expression on the primary tumor with the expression on CTC

PCR-results	Primary tumor IHC-positive	Primary tumor IHC-negative
ER-positive (PCR)	6	0
ER-negative (PCR)	5	6
PR-positive (PCR)	2	0
PR-negative (PCR)	7	7
Her2-positive (PCR)	2	5
Her2-negative (PCR)	3	12

CTC, Circulating tumor cells; ER, estrogen receptor; IHC, immunohistochemistry; PCR, polymerase chain reaction; PR, progesterone receptor

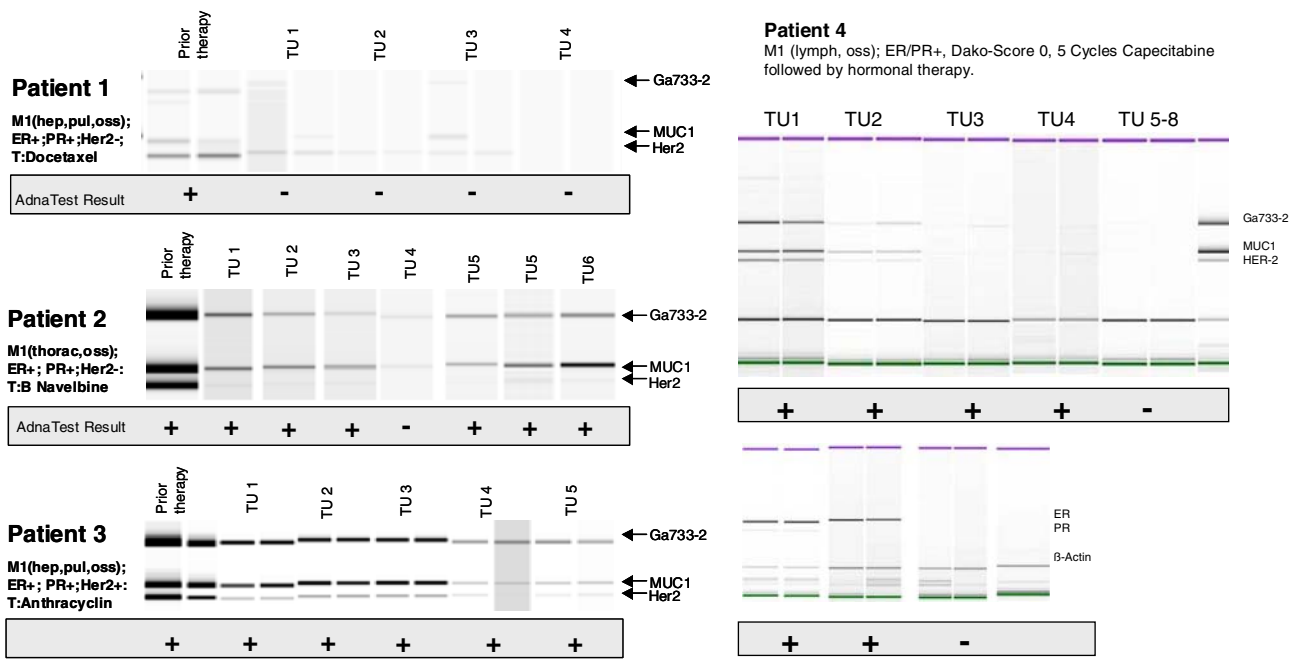
on CTC. Comparisons with the primary tumor were only performed in CTC+ patients ( $n = 22$ ). In 5/11 (45%) patients with ER+ tumors, CTC were ER- and 7/9 (78%) patients with PR+ tumors did not express PR on CTC. Similar results were found for HER2 where 3/5 (60%) patients with HER2-positive tumors were HER2-negative on CTC. Interestingly, 5/17 (29%) patients with HER2-negative primary tumors had HER2-positive CTC.

### Palliative therapy monitoring of metastatic breast cancer patients

Figure 3 illustrates that the efficacy of therapy can be assessed through the detection of tumor cells. Patient 1 suffered from multiple liver (HEP), lung (PUL) and bone (OSS) metastases and was positive for ER and PR. Prior to therapy with paclitaxel, patient 1 showed an expression of GA733-2 and MUC1. After the onset of therapy, CTC immediately disappeared and in the course of four taxane therapy sessions, patient 1 was only positive again for CTC on one occasion.

Example 2 describes the therapy follow up of a patient that initially responded to a vinorelbine based therapy which was paralleled by a decreasing CA 15-3 level. However, after two months and seven therapy sessions no effect on the metastases was observed. The CTC findings confirm an initial response of that patient to the therapy, which eventually led after the third vinorelbine session to the disappearance of CTC. However, a reappearance of CTC after the fourth therapy session indicated an onset of therapy resistance.

Patient 3 had liver (HEP), lung (PUL) and bone (OSS) metastases and was positive for ER, PR and HER2. Prior to therapy with an anthracycline as well as during treatment, patient 3 showed an expression of three tumor-associated transcripts. Whereas CTC disappearance was related to clinical response in patient 1, progressive disease under therapy was related with positive findings of CTC in patient 3.



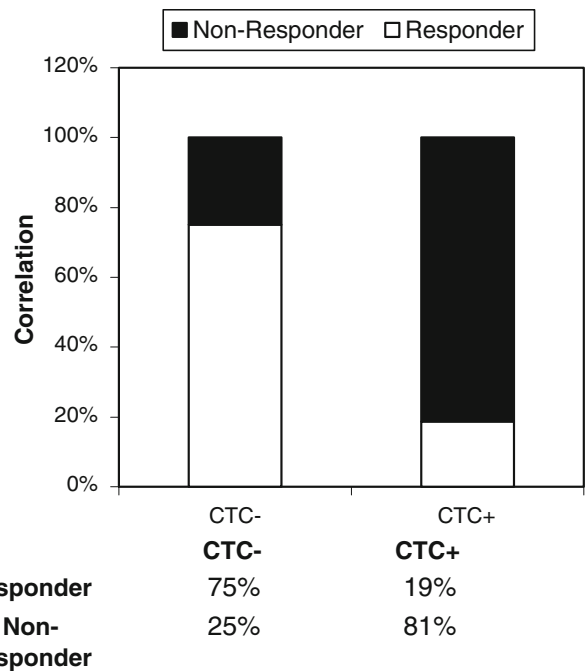
**Fig. 3** Monitoring of palliative therapy of breast cancer patients. Before each therapy unit (TU) blood samples were analyzed in duplicates. The time course of treatment including the analysis of ER and PR (lower part) is shown for Patient 4

Patient 4 had bone (OSS) and lymph node metastases, was positive for ER and PR but negative for HER2 on the primary tumor. Before therapy with capecitabine, this patient showed the expression for all markers analyzed. The upper part of the gel shows the results for the expression of GA733-2, MUC1 and HER2 and the lower part for the expression of the hormone receptors during the course of treatment. CTC completely disappeared after five chemotherapy sessions, which correlated with a decrease in CA 15-3 and CEA levels and a good clinical response. Therapy was then switched to hormonal therapy with an aromatase inhibitor for maintenance of the disease.

**Correlation between CTC detection and therapy response**

As shown in Fig. 4, CTC were not detected or disappeared shortly after onset of the therapy (CTC-) in 12/16 (75%) patients in the responder group. In 4/16 (25%) non-responding patients no CTC were detected at any time point studied. In contrast, CTC were found in 13/16 (81%) patients with clinically confirmed progression during or after therapy (CTC+) whereas in 3/16 (19%) patients of this responder group CTC were still present after clinically confirmed partial remission.

In summary, breast cancer patients, CTC positive either before or during therapy, showed a correlation between presence, persistence or early disappearance of CTC with



**Fig. 4** Correlation between CTC detection and therapy response

therapy response in 12 cases in the CTC negative group and in 13 cases in the CTC positive group, corresponding to a prediction rate of 78%. The concordance between clinical response and serum tumor marker levels of CA 15-3 and CEA measured during follow up was 67% in these patients (data not shown).



## Prognostic value of CTC detection

Overall survival was determined for the time frame of monitoring starting from the time point when patients had either a relapse of breast cancer or a documented progressive breast cancer before receiving a new therapy. The Kaplan–Meier analysis shown in Fig. 5 illustrates a shorter overall survival for CTC+ patients indicating an additional and highly significant prognostic value of CTC detection ( $P = 0.005$ ). 16 of the 32 patients remained positive for CTC and 16 turned negative during therapy. During 14 months after therapy onset eight CTC+ patients have died in contrast to three patients of the CTC– group. Both patient groups did not differ in age and in the number of different therapies before entering the study.

## Discussion

Our study shows that the presence or disappearance of CTC during the time course of individual treatment is a predictor of therapy response in metastatic breast cancer. Furthermore, molecular profiling of CTC with the *Adna-Test* may provide even more differentiated prognostic information and may help to monitor breast cancer related therapies, including chemotherapy, antibody-based chemotherapy and hormone therapy.

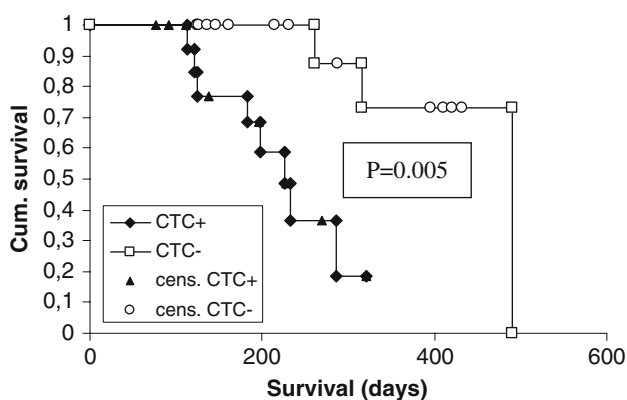
With this test principle, we could demonstrate that CTC were detectable in 52% of the patients. Besides characterizing these cells for three different tumor cell associated transcripts by multiplex PCR, this test enables the additional analysis of transcripts, e.g. hormone receptors, which

may be essential in controlling different kinds of breast cancer related therapies. In this regard, we here demonstrate for the first time that ER was expressed on 35% and PR on 12% of the CTC detected.

The *AdnaTest BreastCancer* was designed to detect rare epithelial cells in whole blood. To ensure the specificity required for the clinical application of mRNA expression assays for occult tumor cells, blood samples from 88 healthy controls were analyzed confirming a high specificity and sensitivity of this test. Thus, we can assume that the identified epithelial and cancer related transcripts in our patients derived from CTC.

Some recent studies showed the prognostic value of CTC in metastatic breast cancer. In this regard, the presence of >5 circulating tumor cells in 7.5 ml of blood from metastatic breast cancer patients has been shown to predict shorter progression-free and overall survival and has superior and independent prognostic value of tumor burden and disease phenotype, thus representing an important marker of tumor biology [16, 17]. The prognostic value of CTC could be confirmed in this study, demonstrating the validity of the RT-PCR method used in this study. However, besides showing the presence of CTC, there is an urgent need to characterize these cells to answer the efficacy of the applied therapy. Our results show that reliance on the immunophenotype of the primary tumor can be misleading since HER2 or the hormone receptors were often differently expressed on CTC as compared to the primary tumor. Similar data for the expression of HER2 on micrometastatic tumor cells from BM of patients with primary breast cancer have been recently published by different groups who independently indicated that HER2 expression on disseminated tumor cells differed from HER2 expression in the primary tumor and that the expression of HER2 in micrometastasis was correlated with poor prognosis [29–32]. These observations might have clinical relevance for trastuzumab treatment. In this context, Meng et al. [4] have recently shown that HER2-positive cells could be eliminated by trastuzumab. The expression of HER2 on CTC as compared to the primary tumor has rarely been investigated. Hayes et al. [18] demonstrated in a monitoring study in patients with advanced breast cancer that it is possible to quantify HER2-positive cells for rationally designed therapy using CTC. Interestingly, they found a phenotype conversion from HER2-negative to HER2-positive CTC in the time course of therapy in a subset of patients. These phenotypical differences can also be seen in three of our patients and have also been documented for EGFR in colorectal cancer [33].

Studies comparing the expression of hormone receptors on the primary tumor with those on CTC rarely have been published up to now. Using the CellSearch SM system followed by PCR and FISH analysis, Reuben et al. [34] demonstrated that they were able to find ER in 15%, PR in



**Fig. 5** Kaplan–Meier analysis. Overall survival is describing the overall survival during the time of monitoring. 8/16 CTC+ patients died within 12 months in contrast to 3/16 patients in the CTC–negative group. A Kaplan–Meier survival plot was calculated to estimate the significance of this observation. The presence of CTC was a significant prognostic factor with respect to poor overall survival ( $P = 0.005$ ) (censored patients: still alive and under observation)

0% and HER2 in 10% of CTC in 15 patients with metastatic sites. Whereas a significant association between primary and metastatic tumors could be shown with respect to the presence of ER, PR and HER2, it seemed that CTC differed significantly from those tumor cells with regards to the expression of hormone-receptor and HER2 status. Analyzing a comparable number of patients, our study shows a higher percentage of hormone receptor positive CTC, which might be explained by the different methods used. Nevertheless, both studies clearly show that HER2, ER and PR were differently expressed on the tumor and on CTC. In contrast to the documented switch in antigen expression, it has been reported that chromosomal abnormalities in CTC obtained from patients with metastatic epithelial cancers matched those in primary lesions [35]. Thus, these data suggest that CTC may represent a unique and heterogeneous cell population which should be investigated further.

Little is known about the prognostic significance of CTC in primary breast cancer. A very recently published study by Pachmann et al. [36] analyzed CTC in 91 non-metastatic primary breast cancer patients by laser scanning cytometry of anti-epithelial cell adhesion molecule-stained epithelial cells from whole unseparated blood before and during adjuvant chemotherapy. They concluded that an increase of CTC of 10-fold or more at the end of therapy was a strong predictor of relapse and a surrogate marker for the aggressiveness of the tumor cells. Although a different method has been applied, this study clearly underlines the importance of CTC as a new predictive factor even in primary disease. Furthermore, this study confirms the conclusions drawn in CTC studies for metastatic breast cancer patients.

Generally, serum tumor markers and radiological determination of sites and number of metastatic lesions are used for the assessment of tumor burden by clinical and laboratory methods. Whereas cost effective radiological assessments can only be performed to a certain degree, the clinical utility of serum tumor markers in predicting prognosis and monitoring treatment still remains controversial [37]. The concordance between clinical response and serum tumor marker levels of CA 15-3 and CEA measured during follow up was 67% in our patients as compared to the CTC detection which predicted 78% of the therapy outcome. Although serum markers are helpful and easily measurable from the clinical point of view, the detection and especially the characterization of CTC may be more helpful in individual therapy decisions. Furthermore, the detection and molecular characterization of CTC allows an access to expression profiles of many potential therapy targets (like ER, PR and HER2). CTC might be useful as a surrogate marker for patient stratification and for stratifying patients into well defined clinical studies. In this context, it recently has been demonstrated that assessment of CTC is an earlier, more reproducible indication of disease status than current

imaging methods [38]. Furthermore, Hayes et al. [39] clearly demonstrated that the detection of elevated CTC at any time during therapy is an accurate indication of subsequent rapid disease progression and mortality for metastatic breast cancer patients. These results are similar to our findings showing a significant shorter overall survival for CTC positive patients over the time course of therapy as compared with patients showing a disappearance of or no CTC. In addition, our test system also offers a “semi-quantification” of circulating tumor load during the time course of therapy by the calculation of marker concentration so that even in a patient with the persistence of CTC a partial response could be documented.

Of course, it should be emphasized that our study mainly consisted of patients having experienced different kinds of first up to fourth line therapies and are now undergoing palliative treatment. Thus, there is a limitation of the study when patients have progressive disease and do not respond to any kind of treatment anymore. At that time point, the assessment of CTC will not be useful anymore. Nevertheless, including CTC detection at first diagnosis of metastasis might help to better treat a patient individually in addition to standard regimens.

In general, it is difficult to draw any conclusions concerning the prognostic value of CTC at the moment since there is no “gold standard” for a comparison making interpretations of the published data difficult. Nevertheless, following the recommendations of the *REMARK criteria for tumor marker studies* [40] we have shown that the detection and analysis of CTC in the time course of breast cancer related therapies is not only a useful prognostic tool but also predictive for response to therapy in metastatic breast cancer. Furthermore, the molecular profiles of CTC can be helpful for the identification of drug targets present during the adjuvant and palliative situation. In addition, phenotype changes as described for HER2 and EGFR in the course of primary disease versus metastatic disease may have a crucial influence on the efficacy of drugs. Since this study is an ongoing trial, the evaluation of more patients will show whether the significant differences already documented for CTC+ and CTC– patients will be further confirmed and strengthened by more cases.

## References

1. Goldhirsch A, Glick JH, Gelber RD et al (2005) Meeting highlights: international expert consensus conference on the primary therapy of early breast cancer. *Ann Oncol* 16:1569–1583
2. Braun S, Hepp F, Sommer HL et al (1999) Tumor-antigen heterogeneity of disseminated breast cancer cells: implications for immunotherapy of minimal residual disease. *Int J Cancer* 84:1–5
3. Pantel K, Schlimok G, Braun S et al (1993) Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. *J Natl Cancer Inst* 85:1419–24

4. Meng S, Tripathy D, Frenkel EP et al (2004) Circulating tumor cells in patients with breast cancer dormancy. *Clin Cancer Res* 10:8152–8162
5. Kasimir-Bauer S, Mayer S, Bojko P et al (2001) Survival of tumor cells in stem cell preparations and bone marrow of patients with high-risk or metastatic breast cancer after receiving dose-intensive or high-dose chemotherapy. *Clin Cancer Res* 7:1582–1588
6. Braun S, Kantenich C, Janni W et al (2000) Lack of effect of adjuvant chemotherapy on the elimination of single dormant tumor cells in bone marrow of high-risk breast cancer patients. *J Clin Oncol* 18:80–86
7. Wiedswang G, Borgen E, Schirmer C et al (2006) Comparison of the clinical significance of occult tumor cells in blood and bone marrow in breast cancer. *Int J Cancer* 118:2013–2019
8. Braun S, Vogl FD, Naume B et al (2005) A pooled analysis of bone marrow micrometastasis in breast cancer. *N Engl J Med* 53: 793–802
9. Stathopoulou A, Vlachonikolis I, Mavroudis D et al (2002) Molecular detection of cytokeratin-19-positive cells in the peripheral blood of patients with operable breast cancer: evaluation of their prognostic significance. *J Clin Oncol* 20:3404–3412
10. Giatromanolaki A, Koukourakis MI, Kakolyris S et al (2004) Assessment of highly angiogenic and disseminated in the peripheral blood disease in breast cancer patients predicts for resistance to adjuvant chemotherapy and early relapse. *Int J Cancer* 108:620–627
11. Gaforio J-J, Serrano M-J, Sanchez-Rovira P et al (2003) Detection of breast cancer cells in the peripheral blood is positively correlated with estrogen-receptor status and predicts for poor prognosis. *Int J Cancer* 107:984–990
12. Jotsuka T, Okumura Y, Nakano S et al (2004) Persistent evidence of circulating tumor cells detected by means of RT-PCR for CEA mRNA predicts early relapse: a prospective study in node-negative breast cancer. *Surgery* 135:419–426
13. Pierga J-Y, Bonneton Ch, Vincent-Salomon A et al (2004) Clinical significance of immunocytochemical detection of tumor cells using digital microscopy in peripheral blood and bone marrow of breast cancer patients. *Clin Cancer Res* 10:1392–1400
14. Benoy IH, Elst H, Philips M et al (2006) Real-time RT-PCR detection of disseminated tumour cells in bone marrow has superior prognostic significance in comparison with circulating tumour cells in patients with breast cancer. *Br J Cancer* 94:672–680
15. Mehes G, Witt A, Kubista E et al (2001) Circulating breast cancer cells are frequently apoptotic. *Am J Pathol* 159:17–20
16. Cristofanilli M, Budd GT, Ellis MJ et al (2004) Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med* 351:781–791
17. Cristofanilli M, Broglio KR, Guarneri V et al (2007) Circulating tumor cells in metastatic breast cancer: biologic staging beyond tumor burden. *Clin Breast Cancer* 7(6):471–479
18. Hayes DF, Walker TM, Singh B et al (2002) Monitoring expression of HER-2 on circulating epithelial cells in patients with advanced breast cancer. *Int J Oncol* 21:1111–1117
19. Bosma AJ, Weigelt B, Lambrechts AC et al (2002) Detection of circulating breast tumor cells by differential expression of marker genes. *Clin Cancer Res* 8:1871–1877
20. Ring AE, Zabaglo L, Ormerod MG et al (2005) Detection of circulating epithelial cells in the blood of patients with breast cancer: comparison of three techniques. *Br J Cancer* 92:906–912
21. Baker MK, Mikhitarian K, Osta W et al (2003) Molecular detection of breast cancer cells in the peripheral blood of advanced-stage breast cancer patients using multimarker real-time reverse transcription-polymerase chain reaction and a novel porous barrier density gradient centrifugation technology. *Clin Cancer Res* 9:4865–4871
22. Hauch S, Zimmermann S, Lankiewicz S et al (2007) The clinical significance of circulating tumour cells in breast cancer and colorectal cancer patients. *Anticancer Res (Greece)* 27:1337–1341
23. Ellis IO, Schnitt SJ, Sastre-Garau X, Bussolati G, Tavassoli FA, Eusebi V (2003) Invasive breast carcinoma. In: Tavassoli FA, Devilee P (eds) World health organization classification of tumours. tumours of the breast and female genital organs. IARC Press, Lyon
24. Sobin LH, Wittekind C (2002) International union against cancer. TNM classification of malignant tumours, 6th edn. Wiley, New York
25. Lal P, Salazar PA, Hudis CA et al (2004) HER-2 testing in breast cancer using immunohistochemical analysis and fluorescence in situ hybridization: a single-institution experience of 2, 279 cases and comparison of dual-color and single-color scoring. *Am J Clin Pathol* 121:631–636
26. Demel U, Tilz GP, Foeldes-Papp Z et al (2004) Detection of tumour cells in the peripheral blood of patients with breast cancer. Development of a new sensitive and specific immunomolecular assay. *J Exp Clin Cancer Res* 23:465–468
27. Zieglschmid V, Hollmann C, Gutierrez B et al (2005) Combination of immunomagnetic enrichment with multiplex RT-PCR analysis for the detection of disseminated tumor cells. *Anticancer Res* 25:1803–1810
28. Lankiewicz S, Rivero BG, Bocher O (2006) Quantitative real-time RT-PCR of disseminated tumor cells in combination with immunomagnetic cell enrichment. *Mol Biotechnol (USA)* 34(1): 15–27
29. Braun S, Schlimok G, Heumos I et al (2001) ErbB2 overexpression on occult metastatic cells in bone marrow predicts poor clinical outcome of stage I–III breast cancer patients. *Cancer Res* 61:1890–1895
30. Vincent-Salomon AJ, Couturier C, Nos X et al (2004) HER2 gene status assessment in micrometastatic cells in bone marrow (BM) of breast cancer patients by fluorescence in situ hybridization. *J Clin Oncol* 22:9520
31. Becker S, Becker-Pergola G, Fehm T et al (2005) HER2 expression on disseminated tumor cells from bone marrow of breast cancer patients. *Anticancer Res* 25:2171–2176
32. Solomayer EF, Becker S, Pergola-Becker G et al (2006) Comparison of HER2 status between primary tumor and disseminated tumor cells in primary breast cancer patients. *Breast Cancer Res Treat* 98(2):179–184
33. Italiano A, Saint-Paul MC, Caroli-Bosc FX et al (2005) Epidermal growth factor receptor (EGFR) status in primary colorectal tumors correlates with EGFR expression in related metastatic sites: biological and clinical implications. *Ann Oncol* 16(9):1503–1507
34. Reuben JM, Lee BN, Li C et al. (2007) Genomic of circulating tumor cells in metastatic breast cancer. *J Clin Oncol ASCO annual meeting proceedings part I 25(18S):1002 (June 20 Supplement)*
35. Fehm T, Morrison L, Saboorian H (2002) Patterns of aneusomy for three chromosomes in individual cells from breast cancer tumors. *Breast Cancer Res Treat* 75(3):227–239
36. Pachmann K, Oumar C, Kavallaris A et al (2008) Monitoring the response of circulating epithelial tumor cells to adjuvant chemotherapy in breast cancer allows detection of patients at risk of early relapse. *J Clin Oncol* 26(8):1208–1215
37. Bast RC, Ravdin P, Hayes DF et al (2001) Update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *J Clin Oncol* 19(6):1865–1878
38. Budd GT, Cristofanilli M, Ellis MJ et al. Circulating tumor cells versus imaging-predicting overall survival in metastatic breast cancer. *Clin Cancer Res* 12(21):6403–6409
39. Hayes DF, Cristofanilli M, Budd GT et al (2006) Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. *Clin Cancer Res* 12(14 Pt 1):4218–4224
40. McShane LM, Altman DG, Sauerbrei W et al (2006) REporting recommendations for tumor MARKer prognostic studies (REMARK). *Breast Cancer Res Treat* 100(2):229–235