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Circulating Tumor Cells in Patients with Breast Cancer: Monitoring Chemotherapy Success

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Abstract. *Circulating tumor cells (CTCs) are an independent prognostic factor for patients with metastatic breast cancer (MBC). However, the role of CTCs in early breast cancer management is not yet clearly defined. The aim of this study was to assess the CTC-positivity rate in patients undergoing chemotherapy depending on breast cancer stage in the adjuvant and neoadjuvant setting. We evaluated the ability to confirm therapy response by CTC analysis. Patients and Methods: CTCs isolated from blood by means of immunomagnetic separation were further characterized by means of reverse transcriptase – polymerase chain reaction (RT-PCR) for epithelial cell adhesion molecule (EPCAM), mucin 1 (MUC1) and v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (HER2) transcripts with the AdnaTest™ . This prospective study included 179 patients; altogether 419 blood samples were evaluated. Patients with primary tumors were divided into neoadjuvant (n=38), and adjuvant (n=100) groups. Forty-one patients with MBC were evaluated under palliative treatment. Results: CTC positivity was described in 35% of patients with early breast cancer without detected metastases before neoadjuvant chemotherapy; similarly, a 26% positivity rate was found in the adjuvant group. In patients with MBC, we detected CTCs in 43% of them. After completing the therapy, the CTC positivity rate decreased to 5% in the neoadjuvant group, to 13% in the adjuvant group and to 12% in the MBC group. CTC positivity after the therapy may classify a subgroup of patients at high risk of developing metastatic disease. This was even true when a patient was evaluated as being CTC-negative before chemotherapy. The multivariate analysis evaluating the correlation of CTC positivity with clinicopathological characteristics such as tumor size, nodal involvement, hormone receptor status, HER2 expression and number of metastatic sites revealed no statistically significant relationships. Conclusion: CTC status may have a significant impact on early BC management.*

Solid tumors diagnosed at an early stage can be treated by local resection, with or without additional chemotherapy (CHT) aimed at eliminating the potential of micrometastasis generation. Micrometastases are initiated by the invasion of tumor cells into the systemic circulation. Tumor cell dissemination is an early process in breast cancer (BC) and circulating tumor cells (CTCs) are considered to be a surrogate marker for the detection and characterization of minimal residual disease (MRD). Detection and characterization of CTCs may provide important prognostic and predictive information to guide monitoring and treatment (1-7). Thus, tumor cells that are detected after potentially curative surgery either in the bone marrow [disseminated tumor cells (DTCs)] or in the peripheral blood (CTCs), are thought to contribute to disease relapse, and therefore are considered as potential targets of adjuvant treatment (5, 8, 9).

Several strategies to isolates and characterize CTCs have been described (10). We monitored the abundance of CTCs in blood by immunomagnetic separation followed by reverse transcriptase – polymerase chain reaction (RT-PCR) (Adnatest™) in the present study (11, 12). The presence of

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CTCs in the peripheral blood of women with BC has also been linked to a poor prognosis. Although CTCs might be prognostically relevant for some patients with early-stage BC, the link between CTCs and adverse prognosis has been most convincingly shown in metastatic disease (13). The relationship between CTCs and tumor biomarkers ER, PR and HER2 is not conclusive (14, 15). Interestingly, *HER2* mRNA-positive CTCs were not associated with positive HER2 status (p=0.635) or other important clinical or pathological primary tumor parameters (p>0.05) (15). This finding is in agreement with the previously described heterogeneity of CTCs (16, 17) and from the point of administration of biological-targeted therapy it is very important.

Nevertheless, rising levels of CTCs are highly predictive of disease progression. Currently, it remains unknown whether an early change in chemotherapy (CHT), needed due to persistently elevated CTCs, is of benefit, if objective evidence of disease progression (*e.g.* by imaging) is lacking.

We hypothesized that the abundance of CTCs might indicate the need for various treatment strategies in patients undergoing CHT. To demonstrate the potential of personalized therapy, we compared the CTC abundance in patients treated with regimens of neoadjuvant, adjuvant and palliative CHT.

Peripheral blood from patients with early BC was tested before starting CHT and after completing CHT in order to answer the question as to how many of the CTC-positive cases became negative following treatment and *vice versa*. The aim of the present study was to assess the CTC-positivity rate in patients with BC undergoing CHT treatment in relationship to the disease stage, and to confirm therapy response by CTC analysis.

Patients and Methods

Eligibility criteria. The eligibility criteria were the following: age ≤18 years; patients with early BC, eligible for adjuvant or neoadjuvant CHT; patients with measurable or evaluable MBC; predicted life expectancy ~2 months; no severe uncontrolled co-morbidities or medical conditions; no second malignancies. Patients with MBC had either a relapse of BC diagnosed years before and were to start CHT, or had documented progressive BC before receiving a new endocrine, chemo- or experimental therapy. Prior adjuvant treatment, radiation or any other treatment of metastatic disease was permitted.

Blood sampling schedule. In patients with primary BC (n=138), the sampling of peripheral blood (5 ml) was preferably carried-out before and during therapy, prior to the third CHT cycle. In 30 patients with primary BC a bone marrow sample has been tested in parallel for DTC abudance. In patients with local recurrence, the blood was collected prior to treatment of recurrent disease. The results of patients with tumor duplicities were correlated with the course of the recent treatment regardless of therapy of the previous tumor. Patients with generalized disease (n=41) were tested within the progression of disease or suspected progression, before starting the next line of therapy.

Tumor cell enrichment and detection. From December 2008, blood samples were taken from 138 patients with early BC (neoadjuvant CHT, n=38; adjuvant CHT, n=100) and 41 patients with MBC. AdnaTest BreastCancerSelect™ (AdnaGen, Langenhagen, Germany) enables the immunomagnetic enrichment of tumor cells *via* epithelial- and tumor-associated antigens. Two antibodies against the epithelial antigen MUC1 and one against the epithelial glycoprotein GA733-2 (EpCAM) are conjugated to magnetic beads (Dynabeads) for the labeling of tumor cells in peripheral blood. In brief, the blood samples and or bone marrow samples were incubated with a ready-to-use antibody mixture commercialized as AdnaTest BreastCancerSelect™ according to the manufacturer's instructions. A magnetic particle concentrator extracted the labeled cells.

The Adnatest BreastCancerDetect™ was used for the detection of BC-associated gene expression in immunomagnetically-enriched CTCs by reverse transcription and polymerase chain reaction (PCR). mRNA isolation from lysed, enriched cells was performed according to the manufacturer's instructions with the Dynabeads mRNA DIRECT™ Micro Kit (Dynal Biotech GmbH, Hamburg, Germany) that is included with AdnaTest BreastCancerDetect™. Reverse transcription resulted in cDNA, which was the template for detection and characterization of CTCs by multiplex RT-PCR. Sensiscript Reverse Transcriptase (QIAGEN GmbH, Hilden, Germany) was used for the reverse transcription (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. 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. The analyses of three tumor-associated transcripts: *HER2, MUC1* and *EpCAM* was performed in a multiplex PCR using prepared cDNA from enriched CTCs. The thermal profile used for multiplex -PCR was as follows: After a 15 min denaturation at 95˚C, 35 PCR cycles followed, starting 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, the reaction was terminated at 72˚C for 10 min. The samples were stored at 4˚C. The primers generate fragments of the following sizes: *EpCAM*: 395 base pairs (bp), *MUC1*: 293 bp, *HER2*: 270 bp, and actin: 114 bp. An Actin gene was used as internal positive control for PCR as a part of the Adnatest™. The PCR fragments were visualized and measured by capillary electrophoresis using 2100 Bioanalyzer with the DNA 1000 LabChips and the Expert Software Package (version B.02.03.SI307) (Agilent Technologies Inc, Santa Clara, USA). If any of the 3 tumor-associated genes PCR- transcripts has been detected in an amount >0.15 ng/l, the samples was considered positive.

Tumor cell visualization. An innovation has been introduced into the CTCs-detection process to enable CTCs-visualization. We have additionally withdrawn 1 ml of the peripheral blood. The blood has been processed following: 10 ul of immumomagnetic beads (Adnatest™) were added and were incubated for 15-30 min. The enriched cells have been evaluated under the inversion microscope (Figure 1). We dissolved the cells in the PBS and did standard trypan blue staining for viability assessment immediately after isolation (Figure 1D, 1E, 1F).

Histopathology. To evaluate the histopathological characteristics of primary tumors, routinely processed paraffin samples stained with hematoxylin-eosin were used. Apart from histology, the degree of

differentiation of tumor cells, according to the Nottingam scoring system (scoring 1- 3) was evaluated. Expression of ER, PR and HER-2 was examined by immunohistochemistry. The sample was evaluated as ER/PR-positive for nuclear expression if at least 1% of the tumor cells were positively stained. HER2 expression was described as 1+ in case of HER2 membranous positivity in fewer than 10% of cells, 2+ if more than 10% of cell positivity, and 3 + on 30% or more positivity. The 2+ or 3+ scored samples were further examined by fluorescence *in situ* hybridization. To evaluate the macro- or microinvolvement of lymph nodes, samples from patients with primary tumors were evaluated. Lymph node affected by metastases of 0.2-2 mm size were considered as micrometastasis, nodes affected by tumor size greater than 2 mm were evaluated as macrometastases.

Statistical analysis. Chi-squared test and Fisher's exact test were used to evaluate the relationship between CTC positivity and clinicopathological factors. The McNemar test was used to compare the relationship of CTC positivity before and after surgery. Statistical analysis was performed by SPSS, version 11.5 (SPSS Inc., Chicago, IL, USA). *p*-Values below 0.05 were considered statistically significant and the null hypothesis of no difference was rejected at that level.

Results

Characteristics of the tested patients. Within the period 2008-2010, a total of 179 patients with BC were enrolled into the study. Patients with early BC represented 77% of the samples, patients with generalization represented 23%. The study included 13 patients with local recurrence and 5 with tumor duplicities without generalization of disease. The average age of our group was 49.1 years; 19% patients were aged less than 35 years. The ratio of pre-menopausal and post-menopausal patients was 79 (40%) / 103 (52%), and the study included 4 men. The most common histological type was ductal carcinoma (71%) with a low degree of differentiation, grade 3 (42%). The average tumor size corresponded to stage T1 to T2 according to the TNM classification.

Lymph node involvement was demonstrated in 50% of patients, including those with disease generalization. In MBC with lymph node metastasis presence, macro- or micrometastasis have not been distinguished. The positive correlation of CTC abundance and nodal status was found only in patients with primary tumors. One positive sentinel lymph node was found in 20 patients (29%), two to three nodes in 18 patients (26%) and three or more nodes in 22 patients (32%). Detailed information of clinicopathological characteristics of patients enrolled in the study given in Table I.

Peripheral blood samples for testing the presence of CTCs were examined in patients one or more times according to the study protocol. A blood sample was considered positive if the expression level of at least one of the measured genes was above the cut-off level in the sample.

CTC detection. The CTC results were obtained from 179 patients, using 419 samples, 16 of which were bone marrow samples. Eighty-eight (21%) samples were positive for the presence of CTCs, 259 samples (62%) were negative, the rest of the samples was inconclusive and should be repeated. Patients with primary tumors were divided according to the type of therapeutic approach into the neoadjuvant (n=38) and adjuvant (n=100) group. The CTC positivity rate decreased in patients with early BC (M0) undergoing adjuvant chemotherapy from 26% to 13% after completing CHT. In the neoadjuvant group, 35% of samples were positive before therapy; after two CHT cycles, only 5% remained positive. In the patients with MBC, CTCs were described in 43%, in at least one sampling; after treatment, the positivity rate decreased to 12% (Table II).

Correlation of CTC abundance with tumor size, hormonal receptor status, and lymph node involvement was not statistically significant in the adjuvant setting.

DTC detection. The DTCs presence has been tested in parallel with CTCs abundance in early BC patients (n=16). Based on the results of the Adnatest™ six patients were evaluated as DTC–positive (37,5%), out of the DTC-positive patients four CTC-positive patients were described (66% of the DTC-positive patients). Similarly, in the DTC negative group four CTC-positive patients have been found (40% of the DTC-negative patients is CTC-positive) (Table III).

Concordance of HER2 status. HER2-positive CTCs were detected in 35% (13/37) of patients with HER2-negative primary tumors. In those with HER2-positive primary tumors, the concordance of HER2 expression was 68.2% (8/12) (Table IV). Considering the fact that also in the triple-negative cases, 33% of the detected CTCs expressed HER2, we may expect that one-third of metastases arising in patients with HER2-negative primary tumors may be HER2-positive. If DTCs were detected in bone marrow of HER2-negative patients, they have been HER2-positive in 100% cases.

Dynamics of CTC abundance during therapy. Focusing on the CTC abundance examined during CHT, we have evaluated conversion rates (CTC-positivity became CTC-negativity and conversely) in patients with at least 2 or 3 different blood withdrawals during the CHT. Similarly we have evaluated conversion rates in the group of early BC and the MBC group.

From the results presented in detail in Table V we may summarize the following. We have evaluated conversion rates (CTC-positivity became CTC-negativity and conversely) in patients with atleast 2 or 3 different blood withdrawals during the CHT. Similarly we have evaluated conversion rates in the early BC and MBC group.

Our results show that in 30-50% of cases, the CTC status changed depending on the type of disease during therapy. CTC positivity became negative in 50%, and conversely

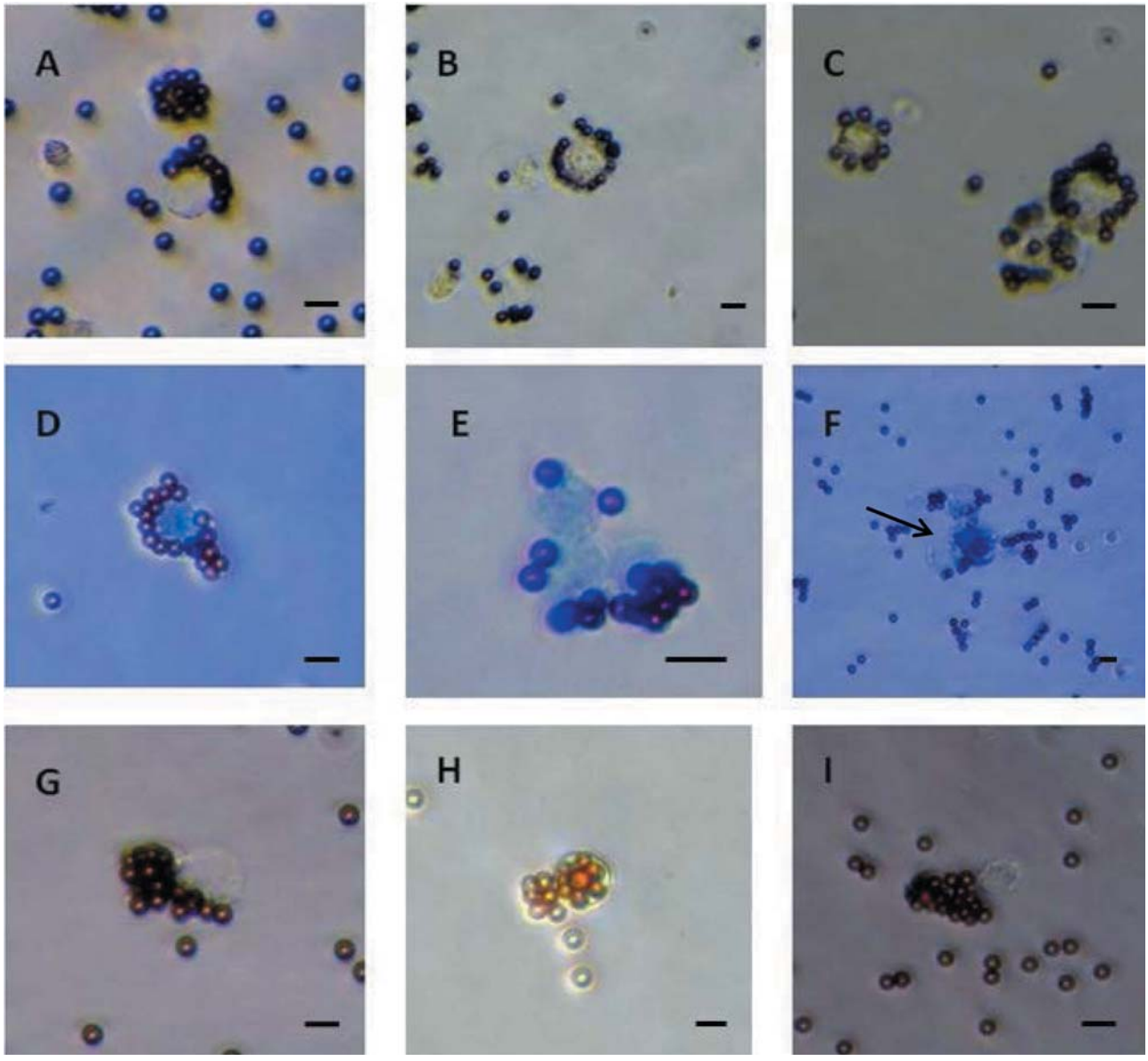

Figure 1. *Circulating tumor cells as observed under light microscopy after immunomagnetic separation from 1 ml of blood. We expect that the cancer cells are enveloped by immunomagnetic beads. The arrows are indicating beads. The cell viability was tested via Trypan blue exclusion method (D-E). In several cases, CTC clusters were detected, see arrow (F). A bar indicates 10 μM.*

changed from negative to positive in 24-50%. There is no difference comparing the frequency of these changes if comparing the frequency in the whole group of patients (Table VI– total conversion %). But if we sum the conversion rates, the probability of change from CTC-positive to CTC-negative is slightly higher if undergoing CHT. In other words, once you are CTC-positive, one has a 50% chance of becoming CTC-negative after CHT. If there are no CTCs before treatment, one's chance of having CTCs after treatment is 25-50%. The probability of becoming CTC-positive in spite of CHT is much higher in the MBC setting (66%), as shown in our small sub.-group (Table VI). Both of these converting groups should be studied more in detail.

Amount of cancer-associated markers in CTCs. The multiplex PCR revealed the presence of the tumor-associated gene transcripts (*EpCAM, MUC1, HER2*) using a cDNA from an enriched CTC-fraction as a template. The presence or absence of the gene products can be expressed quantitatively in ng/ul

Table I. *Clinicopathological features of patients in the study (n=197).*

	Patients	CTC positivity (%)	*p*-Value
Tumor size	N=168		
T1	22/77	28.6	n.s.
T2	23/69	33.3	n.s.
T3	11/21	52.4	n.s.
T4	4/16	25.0	n.s.
Nodal status	n=187		
N0	24/76	31.5	n.s.
N1	30/79	37.97	n.s.
N2	6/18	33.3	n.s.
N3	3/14	21.4	n.s.
ER status			
Positive	34/118	28.8	n.s.
Negative	21/64	32.8	n.s.
PR status			
Positive	24/96	26.0	n.s.
Negative	27/83	32.5	n.s.
HER2 status			
Positive	12/42	28.6	n.s.
Negative	37/134	27.6	n.s.
Triple negative	14/44	31.8	n.s.
Grade	N=153		
High	27/84	32.1	n.s.
Intermediate	16/54	29.6	n.s.
Low	9/15	60	n.s.
Menopausal status	N=186		
Premenopausal	26/79	32	n.s.
Postmenopausal	34/103	33	n.s.
Men	2/4	50	n.s.
Bone marrow status	N=19(30)		
DTC positive	8/19	42	n.s.
No DTCs	11/19	57	n.s.

ER: Estrogen receptor; PR: progesterone receptor; HER2: v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2; CTC: circulating tumor cells.

Table II. *CTC positivity rate in different therapeutical settings.*

	Patients	CTC positivity Before CHT	CTC positivity After CHT
Neoadjuvant treatment	38	35%	5%
Adjuvant treatment	100	26%	13%
Paliative treatment in MBC	42	42%	12%

CHT: Chemotherapy; MBC: metastatic breast cancer .

Table III. *CTC positivity in peripheral blood and DTC positivity in bone marrow in parallel withdrawn samples .*

	N=16	CTC-positive	CTC-negative
DTC-positive	6/16 (37.5%)	4/16 (25%)	2/16 (12.5%)
DTC-negative	10/16 (62.5%)	4/16 (25%)	6/16 (37.5%)

for the detected product by means of capillary electrophoresis. The cut-off levels for positivity and negativity are set by the Adnatest protocol. We compared levels of detected products for primary BC and MBC patient groups.

Based on the results, *HER2* gene product is abundant in the same amount in early BC and MBC patients, but non-significantly higher amount of *MUC1* was detected in the MBC group (p=0.80). Comparing the average amount for all of the three markers in all positive samples (BC+MBC), their amount in MBC group is two to 3-times higher than the average. Higher levels of *HER2* and *MUC-1* were detected in bone marrow samples than in blood (Table VII).

Discussion

In several studies, the presence of tumor cells in the bone marrow and blood places patients with early-stage BC at higher risk for relapse and shows worse survival. However, whether and how this information could be used in the context of clinical care remains uncertain. The 2007 ASCO expert panel concluded that measurement of CTCs should not be used to influence treatment decisions in early or metastastatic BC disease (18). Our study was started with a focus on showing the potential role of CTCs in the management of patients with BC. We hypothesized that CTC testing could further stratify patients into sub-groups and identify patients needing to be 'treated' differently from the current guidelines (19). The first dosing set (*e.g.* six CHT doses according to the standard oncological guidelines) could be extended after the CTC checkpoint if CTCs were found. Due to the RECIST criteria (20), the therapy effect can be evaluated after completing the CHT. But could we not do better? We could save much time if we included CTCs in the therapy efficiency evaluation process.

There is no doubt about the role of CTCs in the dissemination process because many studies have shown their relationship to worse prognosis and shorter disease-free survival deviation for patients with early BC (21). However, there is still a problem in answering the question whether we do indecd whether capture the relevant cells, do the caught cells truly represent the tumor. The methods for CTC examination are still not standardized except for the Food and Drug Agency (FDA)-approved CellSearch® test (22). There is still ongoing discussion whether PCR-based methods are sensitive enough reduced to antibody-based approaches (3). The differences in CTCs detection rates between published clinical studies may then simply arise from using different CTCs enrichment methods. To help answer the question about the relevance of captured CTCs, we need better molecular

Table IV. *Circulating tumor cells positivity rate and HER2-positive by primary tumor status.*

Primary tumor	Patients	CTC positivity	%	HER2+ CTC	%
HER2-positive	42	12/42	28.6%	8/12	68.2%
HER2-negative	134	37/134	27.6%	13/37	35%
Triple-negative	44	12/44	27.0%	4/12	33.3%

Table V. *Circulating tumor cells positivity in peripheral blood and Disseminating tumor cells positivity in bone marrow in parallel withdrawn samples.*

	N=16	CTC positive	CTC Negative
DTC-positive	6/16 (37.5%)	4/6 (66.6)	2/6 (33.3%)
DTC-negative	10/16 (62.5%)	4/10 (40%)	6/10 (60%)

characterization of the captured cells, which is one of the advantage of the Adnatest™ used in our study.

On the other hand, there is need for visualization of CTCs before mRNA analysis to confirm the origin of the cells. The very slight modification of the standard Adnatest™ isolation process enabled us to observe the cells with bound magnetic beads under light microscopy and to implement staining protocols for these cells. Thanks to the approaches used for CTCs testing in mouse experimental models of metastasis, we were able to setup cultivation protocols for human CTCs (20). The culturing methods were further developed to test ability of CTCs to invade and proliferate (21). The visualization of CTCs enabled us to introduce further checkpoints in the CTC detection process in this study.

CTC detection rates in early BC reported by other researchers using different approaches range from 9% to 50% depending on the clinical stages investigated (14, 21, 25-34). Interestingly, in a study comparing CTC abundance before and after surgery in early BC, CTCs were detected in 30% of patients (35). Detection rates in our study (26% for patients in adjuvant therapy after surgery, 35% for neoadjuvant therapy before surgery) do correspond with rates published elsewhere. The patients included in our study were destined to undergo CHT after surgery. We could then expect that these patients represent the high-risk group of BC and CHT prevents disease recurrence. From this point of view, it is interesting that only 26% of the patients were CTC-positive. We should ask the question if the remaining 74% could not be treated differently, probably less aggressively? After completing therapy, 5-12% of these patients remained CTC-positive. What might be the CTC marker enabling us to distinguish between the responders and non-responders? It is clear so far that the presence of CTCs after completion of chemotherapy is statistically associated with reduced disease-free survival and overall survival (36). On the other hand, the presence of CTCs before chemotherapy has not been associated with worse prognosis as shown from the results of the SUCCES trial in 1,500 patients (25). It is important to underline that the presence of CTCs was not associated with any pathological characteristics in our study, apart from vascular invasion (32), unfortunately, vascular invasion was not evaluated in our study.

If we focus on the CTC abundance examined within those undergoing chemotherapy, we may summarize the following: there is a 30-50% probability that CTC status will change after treatment. The change from a CTC-positive to a CTC-negative status may be an advantage for patients (37) and signifies a good prognosis compared to patients remaining positive in all of the testings (38). The results show a great need to focus on patients becoming CTC-negative for several reasons. One of these is the probability of stratification of high-risk patients, for which the CHT has not been effective and another type of treatment could be helpful. This could prevent metastasis formation in the so-called therapeutic window, which occurs after the completing of the first therapy dosing, according to the guidelines (19).

The difference in molecular characteristics between the patients who always tested CTC-positive but became CTC-negative could be used for identification of patients who most probably will respond to CHT and identify so-called markers of resistance or sensitivity. Analyzing these results, we may summarize that if a patient tests as CTC-positive before therapy, which means a worse prognosis, the chance of being CTC-negative after chemotherapy is around 50%. For the patients remaining CTC-positive, we may continue the therapy with different agents. These results suggest that CTCs may contribute to predicting the efficacy of treatment similarly to tumor markers (39).

This fact may be due to the frequency of pluripotent (stem cells) cells expressing both MUC1 and HER2. Based on the analysis of the data obtained after semi-quantitative gene expression testing, we may assume the approximate amount of the gene-specific product. We have observed that the expression of cancer-associated genes is proportionaly higher in the group of patients with MBC if compared to the patients under adjuvant treatment. The difference is obvious for *EpCAM* and *MUC1* genes, on the other hand, the HER2 expression level was comparably equal for patients with early BC and those with MBC.

From the therapeutical point of view, the most important predictive information presented in our study is the changing dynamics of HER2 expression, both in patients with HER2-negative and those with HER2-positive tumors. The detection of HER2-positive CTCs in patients with HER2-negative

Table VI. *Circulating tumor cells (CTC) positivity in peripheral blood of patients with early BC undergoing adjuvant chemotherapy (CHT) and those with metastatic breast cancer under palliative treatment. All reported patients were examined for CTCs at least twice. We focused on the evaluation of the 'conversion rate' for both CTC-positive and CTC-negative groups. From the results shown, it is clear, that half of the CTC-positive patients remai CTC-positive after treatment.*

	Before treatment		After treatment			
	CTC-positive	CTC-negative	CTC-positive	CTC-negative	Total %	Conversion rate
At least 2 CTC tests within CHT (N=78)						
Positive	11		11		14%	
Negative		41		41	53%	
Change to positive		13	13		17%	13/54 (24%)
Change to negative	13			13	17%	13/24 (54%)
	24/78 (31%)	54/78 (69%)	24/78 (31%)	54/78 (69%)		26/78 (33%)
At least 3 CTC tests within CHT (N=25)						
Positive	5		5		20%	
Negative		7		7	28%	
Change to positive		7	7		28%	7/14 (50%)
Change to negative	6			6	24%	6/11 (54%)
	11/25 (44%)	14/25 (56%)	12/25 (48%)	13/25 (52%)		13/25 (52%)
Early BC adjuvant treatment N=15 (3 CTC tests within treatment)						
Positive	3		3		20%	
Negative		5		5	33.3%	
Change to positive		4	4		26%	4/9 (44%)
Change to negative	3			3	20%	3/6 (50%)
	6/15 (40%)	9/15 (60%)	7/15 (46%)	8/15 (54%)		7/15 (46%)
Metastatic BC under palliative treatment N=8 (3 CTC tests within treatment)						
Positive	2		2		25%	
Negative		2		2	25%	
Change to positive		3	3		37%	3/5 (66%)
Change to negative	1			1	12%	1/3 (33%)
	3/8 (37%)	5/8 (62%)	5/8 (62%)	3/8 (37%)		4/8 (50%)

primary tumors has been shown in several published studies (3, 7, 41, 42). It has been already shown Meng *et al*.'s work that patients with HER2-negative primary tumors responded to the HER2-targeted therapy if CTCs were HER2-positive (7). This would support the role of CTCs not only in the risk stratification process, but also directly in therapy guidance. CTCs might be very useful for assessing therapy type, especially in MBC, whose lesions are difficult to test (43). On the other hand, there is a question to be answered: Is the evaluation of HER2 status on CTCs representative of the HER2 status of metastases as already indicated in the study of Pestrin *et al*. (42)?

The CTC count may be useful in patient stratification and therapeutic selection, particularly in those with positive CTCs, for whom various therapeutic choices may procure differential palliative benefit (5). But in connection with the character of CTCs, CTCs were strongly predictive of survival in all BC subtypes except HER2 cases which had been treated with targeted-positive therapy in the metastatic setting (4). This would mean that the presence of HER2-positive CTC cells could completely change the strategy of treatment in HER2-negative patients and by that, their prognosis as well. As presented by Apostolaki *et al*. *HER2* mRNA-positive CTCs also emerged as an independent prognostic factor for DFS and OS in early BC (44).

The results presented in 2009 by Fehm *et al*. showed that the meaning of CTCs and DTCs in patients with early BC is different (3). Only a few studies have compared their value for patients prognosis (26, 34, 45). According to the published data, CTCs are more closely related to the biology of the primary tumor than are DTCs (3). To test the differences between DTCs and CTCs, we tested CTC and DTCs abundance in 16 patients and used the obtained mRNA for further gene expression analysis (data not shown). Although the number of patients is low, these patients were under 40 years of age belonging to the very high-risk group.

Table VII. *Comparison of HER2, MUC1 and EpCAM detected PCR-product levels (ng/μl) in early and metastatic breast cancer. Additionally, the endpoint PCR product levels detected for DTC-associated genes in bone marrow are shown (average for the tested group of early breast cancer patients).*

CTC –associated endpoint PCR-product levels	*Actin*	*HER2*	*MUC-1*	*EpCAM*
Early BC - CTC	8.27	0.42	0.92	0.36
Recidive - CTC	9.53	0.09	0.14	0.26
Metastatic BC - CTC	8.54	0.73	2.54	1.23
Bone marrow - DTC	11.10	2.15	5.10	3.30
Average of CTC+ samples	8.12	0.50	1.46	0.68

**Cut-off levels for CTC-positivity are set by Adnatest™ as >0.15 ng/μl

Our data showing that 25% of them were positive for CTCs and DTCs and almost 40% were negative for both. This will definitely help to stratify patients for additional CTC testing and influence their treatment in the future. Concluding with fact that CTC status also recently identified a subset of patients with significantly poorer outcome among low-risk node-negative patients who did not receive adjuvant systemic therapy (46), this could make a CTC test really very useful in patient stratification.

Conclusion

DTCs and CTCs are likely to play an important role in the development of distant metastases in BC. CTCs in the bloodstream and DTCs abundance in bone marrow can be detected much earlier than progression by other methods (*e.g.* imaging) and is thought to be an early indicator of tumor spread. Our results indicate that CTCs could be used as a marker for the success of therapeutical intervention in adjuvant and neoadjuvant settings. The persistence of CTCs or DTCs positivity could be a reason for use of another therapeutical intervention for maintenance therapy, *e.g.* metronomic chemotherapy, hormonal treatment prolongation or other targeted therapy. The predictive value of CTCs for therapeutic interventions should be evaluated in relation to HER2 more intensively. The molecular profiling of CTCs is one of the crucial points for more efficient personalized treatment in the future instead of rebiopsy of metastases. CTCs may enable clinicians to identify patients with early BC who deserve special attention because of high risk of disease recurrence.

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